Bacterial interactions in the female genital tract

A triangle affair between pathogens, microbiota, and host

Martin Singer

“The further one goes, the less one knows.”

Lao Tzu - Tao Te Ching
Bacterial interactions in the female genital tract –
A triangle affair between pathogens, microbiota, and host

PhD thesis, Vrije Universiteit Amsterdam, the Netherlands

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“I’m being quoted to introduce something, but I have no idea what it is and certainly don’t endorse it.”

Randall Munroe
INTRODUCTION
GENERAL INTRODUCTION

Every person on earth enjoys the presence of millions upon millions of bacteria that are present on, or even in, his or her body. These living creatures not only interact with the person they are found on, but also with each other. This interaction can take the form of infection, symbiotic relationship, or commensal colonization of the host. A shift in environmental characteristics, can equally shift the nature of this relationship from beneficial to pathogenic.

This thesis will focus on the female genital tract and the interactions occurring therein. The first bacteria to come to mind that interact with the host at this anatomical site, are the commonly known sexually transmitted infections (STI) like Chlamydia and Gonorrhoea. However, there are a number of less commonly known STIs that proof to be unique and interesting subjects of study into host-pathogen interaction. A selection of these STIs are subject of study in this thesis. Much more commonly present in the female genital tract are the bacteria comprising the vaginal microbiome. These bacteria are consistently in contact with the host, often living in a state that is mutually beneficial.

The host itself is also a focus in this thesis. The host immune response affects bacteria in the female genital tract most significantly. Because of this, we look into the genetic basis of immune related genes and their effect on infections at this anatomical location. The expression of cytokines is another subject of interest. There are a number of mitigating factors that might come into play between the transcription of genetics and the actual expression of immune related genes, which could result in different expression than is expected.

Providing better insight into the various interactions between the pathogens, the microbiota, and the host will not only lead to a better understanding of how things work on a molecular level, it is also a requirement for the development of new treatments and therapies. Considering the function of the female genital tract, more knowledge on the subject of vaginal microbiota and pathogen interaction with the host may lead to new breakthroughs in treatments for subfertility, and work as a supporting force for the creation of healthy pregnancies.
1. **Inducing inflammation: the innate immune system**

When a pathogenic bacterium enters a healthy human host, a healthy immune system interacts with this invasion in a manner that is both effective and not too harmful to the host body. This interaction consists of two parts: the innate immune system and the adaptive immune system. The first phase of the immune response is the responsibility of the innate immune system, which includes proper recognition of the invading pathogen. This recognition is the function of the four groups of so called Pattern Recognition Receptors (PRR): Toll like receptors (TLR), NOD like receptors (NOD), RIG-I-like receptors, and C-type lectin receptors (1-5), which are present in and on the host cell. All these PRR groups contain a number of PRRs which recognise specific pathogen associated molecular patterns (PAMPs) unique to that specific PRR. The PRRs that are most significant for the detection of bacteria related to the female genital tract and their respective PAMP targets are listed in Table 1. PRRs can also bind to damage associated molecular patterns (DAMP), however, these are not related to the infections covered in this thesis.

The main difference between TLRs and NODs is the location of their expression (6). TLRs are transmembrane proteins, and are generally expressed on the surface of the cell. An exception to this localization is the presence of three TLRs in endosomes of the cell, with TLR9 being the most significant TLR expressed in the endosome membrane for the detection of bacteria studied in this thesis. In contrast, NODs are found in the cytoplasm of the cell. Here the NODs act as a scaffold for the creation of an inflammasome at the time of binding with their respective PAMPs.
Table 1: Pattern recognition receptors most significantly related to bacterial detection and their main ligands.

<table>
<thead>
<tr>
<th>PRR</th>
<th>Main ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Bacterial lipoproteins</td>
</tr>
<tr>
<td>TLR2</td>
<td>Bacterial peptidoglycans</td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellin</td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated CpG DNA</td>
</tr>
<tr>
<td>NOD1</td>
<td>Peptidoglycans</td>
</tr>
<tr>
<td>NOD2</td>
<td>Peptidoglycans</td>
</tr>
</tbody>
</table>

Figure 1: Distribution and localization of pathogen recognition receptors. TLRs and NODs are stimulated by their specific ligands, after which they initiate complex pathways which all end at the transcription factor NF-κB. NF-κB transcribes DNA for production and secretion of pro-inflammatory cytokines. PAMPs: Pathogen associated molecular patterns.

One major similarity between TLRs and NODs is the eventual outcome of pattern recognition. When a receptor is stimulated, a molecular signalling pathway is activated which ends at the transcription factor NF-κB (7). Among other effects this transcription factor enables expression of a number of pro-inflammatory cytokines, leading to the early signs of immune response (inflammation and attraction of innate immune cells). Additionally, it leads to the initial steps of activating the adaptive immune response for further response to the infection.
2. The variety in immune response to vaginal pathogens

There is a wide variety of bacterial STIs that can plague the female reproductive system. Symptoms for these diseases are often directly linked to the host immune response aimed at the pathogen, and tissue damage resulting from the infection. This can vary from light inflammation and itchiness, to infertility and miscarriage. In this thesis we cover a part of the host interaction with common bacterial STIs: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Haemophilus ducreyi*. Although these are all bacterial STIs, the host immune responses to the different pathogens differ strongly.

*C. trachomatis* is the cause of Chlamydia, and is the most common bacterial STI in the world. In 2012 it was shown that every year there are approximately 130 million new cases of infection with *C. trachomatis*, and despite efforts to reduce this number Chlamydia remains highly prevalent (8). *C. trachomatis* is an intracellular bacterium, and the localization of *C. trachomatis* in the endosome of the cell means that for a large part of the infection only a selection of PRRs can make contact with the pathogen. The significant role of TLR9 in chlamydial infections has been shown in previous studies, corroborating this claim (9). Additionally, the surface of *C. trachomatis* cells is largely made up of lipopolysaccharides (LPS) which stimulate the TLR4 receptor. Therefore TLR4 has a central role in the immune response to *C. trachomatis* (10).

Similarly to *C. trachomatis*, *N. gonorrhoeae* is highly prevalent around the world, with approximately 78 million new cases of infection every year (8). Infection with *N. gonorrhoeae* causes gonorrhoea, a well-known sexually transmitted disease. *N. gonorrhoeae* shows an interesting interaction with TLR9, with which it interacts after phagocytosis of the bacteria. The lysis of the bacteria allows binding of the unmethylated CpG DNA to TLR9. Due to the makeup of the unmethylated CpG DNA of *N. gonorrhoeae*, TLR9 is less efficient in the recognition of *N. gonorrhoeae* (9). It is likely that this is part of the defence of the bacteria to suppress the immune response of the host, as *N. gonorrhoeae* is found in the extracellular space, and is therefore vulnerable to immune activity. Indeed, a number of studies have shown that *N. gonorrhoeae* actively suppresses signalling related to inflammatory signalling in the immune response (11, 12).

*T. pallidum* causes syphilis. Although untreated syphilis can lead to severe neurological symptoms and even death, the overall clinical burden caused by syphilis has reduced significantly
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due to a severe drop in cases. Currently, the prevalence in Europe is approximately 0.005\% (13). *T. pallidum* is an interesting pathogen with regards to pathogen detection in the host. The bacterium is Gram-negative, which usually means that there is LPS in the membrane of the bacterial cell for TLR4 to recognise. However, in practice *T. pallidum* does not appear to interact with TLR4. Rather it interacts with TLR2, which is a receptor that mainly targets Gram-positive bacteria through recognition of the associated glycolipids and lipoteichoic acid (14, 15). *T. pallidum* has specific membrane lipoproteins that interact with TLR2, which is uncommon for gram-negative bacteria (16).

*H. ducreyi* causes the lesser known chancroid. This is typically an ulcer on or around the genitals which, if left untreated, can begin to necrotise. Unlike the previous three well studied pathogens, the exact interactions between *H. ducreyi* and host PRRs is not entirely understood. The ulcer-based nature of the symptoms and previous immunological studies downstream from the PRRs do emphasize how impactful the innate immune system is in creating a proper response to infection with *H. ducreyi* (17, 18).

The variety between individuals in immune responses to infections is not only caused by the invading bacteria. Host genetics have a significant impact on the immune response as well. The most notable way host genetics can have this effect is through polymorphisms, which are host specific. Single nucleotide polymorphisms (SNPs) are point mutations that are present in a significant part of the population. A point mutation means that a single base pair in the host DNA is affected by a nucleotide change. For example, where in most of the Dutch population people have a C nucleotide at a given base pair location, a person with a SNP at this location may have an A nucleotide instead. When this genetic data is translated, it may end up affecting the expression, shape, and/or function of the related genes. A schematic overview of this process can be seen in Figure 2. This may in turn affect the ability of the host to respond to infections. As an example of this, a combination of SNPs, called a haplotype, in the gene coding for TLR9 showed effects on susceptibility and development of late complications related to *C. trachomatis* infections (9).
Figure 2: The effect of a Single Nucleotide Polymorphism (SNP) on the functionality of a protein. At the top of this figure two strands of DNA are shown. One with a wildtype DNA composition, and one where a single nucleotide has been altered in the form of a SNP. This SNP is marked as the red C nucleotide. In this case, the SNP causes a difference in the amino acid sequenced derived from the DNA, as the wildtype stop codon is replaced by an arginine amino acid. A difference like this can lead to a vastly different protein structure, influencing the function of the protein greatly. It should be noted that an altered protein structure does not always lead to reduced effectiveness.

Other SNPs can result in differences in, for example, recognition of invading bacteria, cytokine expression, and thus the host's ability to clear infections. Besides currently known genetic factors which are often related to the most relevant PRR pathways of the specific bacteria, there are likely to be more genetic factors to be discovered in the future, especially for a less studied pathogens such as *H. ducreyi*, which is further studied within this thesis. In addition, genetic differences between bacteria can also affect the interaction between PRRs and bacteria. A study of bacterial genetic sequences related to the interaction with TLR9 is therefore also included in this thesis.

3. **Profiling the vaginal microbiome**

Besides individual bacteria contributing to infection, large amounts of bacteria are present on and in the host. These are often not harmful, and even beneficial to the host in some way. The complete collection of bacteria inhabiting an anatomical site is called the microbiome.
The recognition of the microbiome and the way it is characterized has been the subject of much change, especially with the advent of molecular characterization techniques. Initial full microbiome characterization attempts used a mixture of culturing and 16s ribosomal RNA analysis (39;40). This was used mostly to examine the diversity of the bacteria making up the microbiome, and to create a general overview of the phylogenetic characteristics. This technique proved lacking for characterization of vaginal microbiomes when it was found that bacteria making up the microbiomes that have high impact on the vaginal health were often difficult to cultivate, and some even proved entirely uncultivatable (41;42).

The introduction of PCR allowed for the rapid amplification of the 16s gene and, when combined with hybridization techniques, made microbiome characterization attempts faster and easier (43). However, before a PCR could be done the bacteria needed to be cultured, meaning the drawbacks of cultivation were still there. The advent of metagenomics sought to solve this problem by genetically characterizing the vaginal microbiota without first culturing it (44). In this approach, the 16s rRNA of the bacteria is sequenced using next generation sequencing techniques such as pyrosequencing or Illumina (Solexa), sequencing directly on a microbiome sample. More recently, a high throughput method of analyzing human microbiota was developed called the IS-pro. This technique combines differentiation accuracy with taxonomic classification using phylum specific PCR primers and fluorescent labels, making it more suitable for clinical settings than other next-generation sequencing techniques (45). Besides IS-pro, sequencing is also being developed further. So called 3rd generation sequencing techniques, are currently in their infancy, primarily being used for specialized cases (19)4. However, these show enormous potential in increasing the amount that can be sequenced regarding both reads and samples, and lowering the time this takes significantly. A timeline showing the usage of the techniques is shown in Figure 3.
4. Characteristics of the vaginal microbiome

The vaginal microbiome plays an important part in the host immune system. Consisting of commensal bacteria living in a symbiotic fashion with the host, it provides a mechanical barrier shielding the vaginal epithelial layer from potential pathogens. A vaginal microbiome in a healthy state has a single anaerobic *Lactobacillus* as the dominant presence (20). *Lactobacilli* have the capacity to acidify the vaginal environment through production of lactic acid, which works against any opportunistic pathogens seeking to infect the vagina. *Lactobacilli* also generally produce H$_2$O$_2$, and bacteriocin substances which aid in the protection against invading pathogens and make sure that *Lactobacilli* remain the dominant presence (20-23).

The most common kinds of *Lactobacillus* spp. are *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*. The microbiome compositions dominated by these *Lactobacilli* have been labeled by Ravel et al. as Community State Type (CST) I, II, III, and V, respectively (24-27). CST IV is defined as a diverse microbial profile where no *Lactobacillus* has a significantly dominant presence over the other present bacteria. CST IV is also linked to Bacterial Vaginosis (BV) (26). Besides these most commonly found microbiome profiles, there are a number of different *Lactobacilli* that can also be found in the healthy vaginal microbiome, primarily including: *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei*, *L. delbrueckii*, *L. salivarius*, and *L. vaginalis* (26). It is important to note however, that *Lactobacilli* are not the only bacteria that can be found in a healthy vaginal microbiome, as low amounts of anaerobic bacteria such as *Prevotella*, *Megasphaera*, *Gardnerella vaginalis*, and *Atopobium vaginae* can colonize the vagina without leading to an unhealthy state (22, 26).
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During pregnancy the vaginal milieu changes. An increased level of oestrogen during gestation results in an increased production of vaginal glycogen, which is an environment that benefits Lactobacillus spp. Unsurprisingly, this environment leads to an increase in microbiome profiles featuring L. vaginalis, L. crispatus, L. gasseri, L. iners, and L. jensenii in pregnant women (28-31). Any change in the vaginal microbiome that occurs during pregnancy almost exclusively goes from domination by one Lactobacillus species to another (31, 32). Additionally, non-Lactobacillus species hold significantly lower presence in pregnant women (24, 28, 31). The increased abundance of Lactobacillus spp. during pregnancy may be one way the female body attempts to prevent non-indigenous bacteria in the vagina from colonizing and to prevent bacterial vaginosis (BV), infections, or preterm birth (24).

Besides pregnancy, the sexual phase of a woman’s life is also a factor in the composition of the vaginal microbiome. Before a woman is sexually active, the vaginal microbiome is more stable and less prone to infection and overgrowth (33, 34). This is characterized by the low amounts of BV detected in women before they become sexually active (33). Once women are of reproductive age, the menstrual cycle does not appear to cause significant change to the vaginal microbiome, although other studies emphasize that this effect is not entirely certain yet (22, 35). Post-menopausal women do have a significantly changed vaginal microbiome, as dryness and tissue atrophy are cause for a decrease in Lactobacilli (36).

Ethnicity is also a distinctive factor for vaginal microbiome profiles. Figure 4 shows a comparison of multiple studies that used next-generation sequencing to characterize the vaginal microbiome and label CST diversity in hosts of various ethnicities. It clearly shows varying levels of CST abundance, and even CST presence in differing populations. Hispanic, African, and African American women were found to have less abundance of the dominant Lactobacillus spp. and a higher average pH level compared to Asian and Caucasian women (26, 37, 41). Perhaps as a consequence of these vaginal microbiome characteristics, ethnicity is linked to pre-term birth, the prevalence of vaginal inflammation, prevalence of STIs, and fertility problems such as miscarriage in several studies (39, 41, 42). The differences in the vaginal microbiome between ethnicities also exist in pregnant women, raising concern for populations with a relatively large abundance of BV linked microbiome profiles, as this would imply a higher risk for adverse pregnancy outcomes (43, 44).
Figure 4: Comparison of dominant vaginal microbiome species found in studies using Next-generation sequencing to characterize the vaginal microbiome and label CST diversity in hosts of various ethnicities (26, 37-40).

5. The vaginal microbiome in relation to reproductive health

A healthy vaginal microbiome can become unbalanced due to a number of reasons. STIs, smoking, and vaginal hygiene are some reasons for a decrease of *Lactobacillus* spp. (35, 45). This allows opportunistic bacteria that are not normally part of the vaginal flora to colonize the vagina (46, 47). Additionally, bacteria that are part of the normal vaginal flora in low amounts are able to overgrow the healthy vaginal flora, often leading to BV (48).

There are a number of recent studies showing that BV increases risk of pregnancy loss, preterm delivery, and miscarriage, as well as lowering the rate of conception significantly (32, 41, 49-52). The risk for these pregnancy complications goes up significantly. For instance, pregnancy loss was two and a half times as likely to occur in women with BV compared to women with a healthy microbiome (42). When the BV state is persistent in the patient, or if there is a higher than average amount of BV related bacteria present, there is an even higher potential for adverse pregnancy outcomes (42, 53-55). Additionally, women undergoing their first pregnancy suffer a higher risk for pregnancy loss in the second trimester when the vaginal microbiome is shown to contain relatively low amounts of *Lactobacillus* spp. or
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no *Lactobacillus* spp. at all (56). It is likely that the lack of *Lactobacilli* creates an opportunity for other opportunistic bacteria to colonize the vagina, which often leads to BV. It is currently unclear if assisted reproductive therapy (*e.g.* IVF) is affected by the composition of the vaginal microbiota. This thesis aims to clarify the role of the microbiome composition in successful IVF treatments.

6. The vaginal microbiome and bacterial STIs

Even though the healthy vaginal microbiome has a number of mechanical and molecular mechanisms to protect its dominance over the microbiome and protect the host, bacterial pathogens still manage to infect the host. When the microbiome composition is in dysbiosis, this becomes easier.

During dysbiosis of the vaginal microbiome the host is between two and three times as likely to be infected by *C. trachomatis* (57-59). The reduced or even absent mechanical barrier functionality of the microbiome would be a logical reason for the increased infections, as *C. trachomatis* is more likely to reach the targeted epithelial layer. However, there is an additional molecular reason for the increased susceptibility. *C. trachomatis* is reliant on tryptophan for its growth, which it can only obtain from outside sources. During dysbiosis *Prevotella* spp. are often considerably more abundant than in a healthy microbiome. *Prevotella* spp. produces tryptophan which *C. trachomatis* can use, facilitating an infection (60).

A dysbiotic microbiome also increases susceptibility for *N. gonorrhoeae* infections. The most recent studies find an approximate 1.5 to 2 times increase in susceptibility for this infection when the vaginal microbiome is in a dysbiotic state (58, 59). It is currently theorized that the lack of mechanical barrier function provided by the microbiome is the main contributor to the increased susceptibility to *N. gonorrhoeae*. *C. trachomatis* and *N. gonorrhoeae* currently represent the best understood bacteria with regards to their interaction with the microbiome of the host, primarily due to their relatively high prevalence and clinical impact. Unfortunately, for other bacterial STIs a large gap in the knowledge on this subject still exists.

It is well established that BV makes the host more susceptible to common STIs, however evidence shows that this action might go both ways. Studies show that STI infected women are subsequently also more likely to develop dysbiosis as a result (59). However, it is not yet
entirely clear whether this is because of pathogen-host-microbiome interaction, or whether there is a common set of risk factors for STIs and development of dysbiosis.

Regardless of the mechanisms behind it, it is clear that BV poses a clinical threat to those who have developed it through an increased susceptibility to STIs. This raises the question whether treatment of BV should be considered as a preventative measure for STI susceptibility. Certainly, in recent years the attention to modulation of the vaginal microbiome has increased rapidly. Approaches vary between standard antibiotic treatments to lactic acid releasing mechanisms (61-63). However, a conclusive longitudinal study highlighting the effectiveness of this strategy does not yet exist.

7. Aims and outline of this thesis

7.1 Aims
Bacteria in and around the host can have an enormous impact on its health. Within the female genital tract, such impact is often related to reproductive health in particular. Studies in this thesis examine the multifaceted subject of bacterial interactions within the female genital tract. This includes the impact of host and bacterial genetics and expression on the immune response, the commensal microbiota composition and its effect on assisted reproduction, and a description of interactions between known pathogens and microbiota in the host.

7.2 Outline
This thesis is divided into two sections:

- Part 1: Pathogens in the vaginal tract and their interaction with the immune system
- Part 2: The interaction between the vaginal microbiota and host reproductive health

Part 1:
The expression of cytokines is a direct result of the interaction of bacteria with the recognition pathways of the innate immune system. However this expression differs between people based on various factors. What effect the differences in cytokine level expressions have on bacterial infections is discussed in Chapter 1 of this thesis. A meta-analysis of the
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available literature gives a clear image of the impact of host specific differences on infections with common STIs.

Chapter 2 provides an *in silico* examination of bacterial genetic sequences. The aim was to clarify the potential stimulation or inhibition of TLR9 signaling through the unmethylated CpG DNA makeup of various bacteria present in the female genital tract.

*H. ducreyi* is an STI that is less commonly known. Because of this, not much is known about its interaction with the host during infection. Chapter 3 sheds light on the effect of host genetic polymorphisms on the immune response during *H. ducreyi* infection. In turn this increases knowledge of *H. ducreyi* pathogenesis as a whole.

Part 2:
The simultaneous interaction of pathogens, commensal microbiota, and the host immune response is, due to its inherent complexity, lacking in study. In Chapter 4 an effort is made to clarify current knowledge related to this triangular integration for the otherwise well described pathogens of *C. trachomatis* and *M. genitalium*.

Chapter 5 quantifies and qualifies the current knowledge related to the vaginal microbiota and its relation with IVF outcomes through systematic review and meta-analysis of available scientific literature.

The relation between the vaginal microbiome and success of IVF treatment has been hinted at. Chapter 6 details the protocol used for the study performed in Chapter 7, which aims to determine microbiome compositions that provide a positive or negative effect on IVF outcomes. Furthermore, it examines the potential to use this knowledge as a predictive rule for IVF outcome.

Recent literature suggests that a urinary microbiome exists which is distinct from the vaginal microbiome. Chapter 8 examines to what degree urine shows a similarity with vaginal samples in its microbiome profiles on a per person basis. Additionally, a discussion is had on the viability of the distinct urinary microbiome as a concept.
REFERENCES

INTRODUCTION


INTRODUCTION


“Dogma is like tough meat. It is best well-chewed.”

**Kor Phaeron** -
Fabius Bile: Primogenitor (Novel),
Part One, Chapter Eight
CHAPTER 1

Effect of cytokine level variations in individuals on the progression and outcome of bacterial urogenital infections – a meta-analysis

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ABSTRACT

Bacterial urogenital infections like chlamydia, gonorrhoea, and syphilis are widespread inflammatory diseases, which may be accompanied by severe complications. These complications can range from basic inflammation to tubapathology, infertility, and neurological dysfunction, though infections go unnoticed in the majority of cases. Cytokines in the host play a vital role in both the initial and long term immune response and inflammation. However, levels of cytokine expression vary between individuals. A meta-analysis was performed to evaluate the effect of cytokine expression differences on severity of infections with these pathogens. Studies comparing expression of cytokines in humans with inflammation or inflammation based complications were identified using NCBI, Google Scholar, and Cochrane databases. Only studies into human cytokine expressions were included, and 3 articles per subject were required to be suitably analysed during the meta-analysis. 52 articles were included for meta-analysis. It was shown that differences in IL-1, IL-6, IL-8, IL-10, TNFα, and IFNγ affect the clinical outcome of *C. trachomatis* infection significantly. Similarly, IL-1 and IL-8 expression during *N. gonorrhoeae* infection significantly affects the outcome of the disease. For *T. pallidum* infection it was shown that IFNγ variation in hosts could be linked to severity of disease. However, a lack of studies to use in the meta-analysis and fluctuation in the resulting data depending on the adjustments makes adequate evaluation difficult.
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During infection with bacteria the human body can detect pathogen associated molecular patterns needed to initiate its innate immune system. Specifically, activation of the NF-κB pathway leads to production of pro-inflammatory cytokines like Interleukin (IL)-1, IL-2, IL-4, IL-8, and Tumor Necrosis Factor (TNF)α (1, 2). The cytokine functions also include activation of T-helper 1 and 2 cells required for cellular and humoral immune responses, respectively. Th1 cells produce IFNγ and IL-2, while Th2 cells secrete IL-4, TGFβ, and IL-10 (3). These cytokines and their functions are summarised in Table 1.

*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Treponema pallidum* are the most common bacterial sexually transmitted disease worldwide. Resulting complications include inflammation for all, with more severe complications including tubapathology and related fertility problems for *N. gonorrhoeae* and *C. trachomatis*, and cutaneous lesions and neurosyphilis for *T. pallidum*. Pathogen recognition receptors such as Toll-like receptors (TLRs) are responsible for identifying pathogens in the human host and initializing the immune response against them (5-9). One of the results is an influx of immune related cells, like CD4+ T-helper cells, at the sites of infection (10, 11). However what cells are recruited to the site of infection and which cytokines are released can differ depending on the pathogen and the host.

Cytokine expression follows similar pathways in all humans, but has been shown to vary between individuals (12-15). The inflammatory nature of the complications related to *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* means a difference in expressions of inflammatory cytokines can alter the course of the disease and the resulting complications in the host. Especially tubapathology and related fertility problems have been shown to be affected by differences in expressed inflammation (5, 16).

The aim of this literature review was to critically analyse, create insight, and create a standardized measurement of effect for cytokine expression differences related to outcome of various bacterial urogenital infections. This will shed light on the overlap and differences in the effects of differing cytokine expressions between multiple pathogens.
Table 1: Cytokine functions and sources of production (4).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primary Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>Activation of macrophages</td>
<td>Th1 cells</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Immunoregulation</td>
<td>Th1 cells</td>
</tr>
<tr>
<td>IL-2</td>
<td>Promotion of T-cell proliferation and differentiation into Th1</td>
<td>Th1 cells / NF-κB activation</td>
</tr>
<tr>
<td>IL-10</td>
<td>Down regulation of inflammation by blocking NF-κB activation and Th1 cell expression</td>
<td>Th1 and Th2 cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>Promotion of differentiation into Th2 cells</td>
<td>Th2 cells / NF-κB activation</td>
</tr>
<tr>
<td>IL-5</td>
<td>Stimulates B-cell growth and eosinophil activation</td>
<td>Th2 cells</td>
</tr>
<tr>
<td>IL-6</td>
<td>Regulation of temperature during inflammation</td>
<td>Th2 cells / NF-κB activation</td>
</tr>
<tr>
<td>IL-9</td>
<td>Regulation of hematopoietic cells during inflammation</td>
<td>Th2 cells</td>
</tr>
<tr>
<td>IL-13</td>
<td>Regulation of allergic and parasitic inflammation</td>
<td>Th2 cells</td>
</tr>
<tr>
<td>IL-1</td>
<td>Increase of vascular endothelial activity and permeability, and activates lymphocytes</td>
<td>NF-κB activation</td>
</tr>
<tr>
<td>IL-12</td>
<td>Activation NK cells</td>
<td>NF-κB activation</td>
</tr>
<tr>
<td>TNFα</td>
<td>Increase of vascular endothelial activity and permeability</td>
<td>NF-κB activation</td>
</tr>
<tr>
<td>IL-8 / CXCL8</td>
<td>Recruitment of neutro- and basophils to infection site</td>
<td>NF-κB activation</td>
</tr>
</tbody>
</table>

METHODS

Search methods

Pubmed.gov and Google Scholar databases were used to obtain the scientific articles for this study. To limit the number of articles found by searching Google Scholar, only the first 100 articles from every search in this database were analysed. Search terms used are shown in Table 2. Searches were done in the period of 1 October 2014 till 1 December 2014. No age limit was applied when searching for articles.

Table 2: Search strategy in the PubMed and Google Scholar databases. “Pathogen” indicates any bacterial urogenital pathogen. “Cytokine” indicates any cytokine, chemokine, or interleukin.

<table>
<thead>
<tr>
<th>Search terms</th>
</tr>
</thead>
<tbody>
<tr>
<td>“cytokine” expression “pathogen”</td>
</tr>
<tr>
<td>“pathogen” interleukin</td>
</tr>
<tr>
<td>“cytokine” “pathogen”</td>
</tr>
</tbody>
</table>
Table 3: Exclusion and inclusion criteria.

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines not directly related to pathogen</td>
</tr>
<tr>
<td>No cytokine profiling</td>
</tr>
<tr>
<td>Secondary: Data not compatible with other studies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA or mRNA cytokine quantification</td>
</tr>
<tr>
<td>Bacterial urogenital infections (e.g. C. trachomatis, N. gonorrhoeae, and T. pallidum)</td>
</tr>
<tr>
<td>Based on in vivo human studies</td>
</tr>
</tbody>
</table>

Selection of studies

Abstracts were analysed and studies that did not include cytokine profiling were excluded. Both articles showing quantified cytokine profiles and articles showing relative cytokine expression differences were included. Additionally, any articles not linking the cytokine profiles to the pathogen directly were excluded. Full exclusion and inclusion criteria are shown in Table 3. Statistical data of included studies was extracted and reviewed for compatibility with the Comprehensive Meta-analysis (CMA; Biostat inc., Englewood, NJ) tool. Data of studies that was not compatible with other studies for analysis was excluded from the meta-analysis.

Statistical analysis

Dixon’s Q test for outliers was used to assess for heterogeneity between included study results. P values below 0.05 were considered significantly heterogenic results. Targets for the heterogeneity assessment were the standardized values given as output by the CMA tool. When variability between study results was proven we used a random effects model for analysis of the pooled study results with a significance set at P<0.05. Otherwise, a fixed-effect model was used. A one-study-removed analysis was performed to assess for effect of large studies on the pooled study results. Cumulative analysis of the results was performed to assess for trends in study results over time. Publication bias was assessed using funnel plots. When publication bias was suspected, a trim-and-fill method was used to adjust the results of the initial fixed effect or random effect model analyses. All analyses were conducted using the CMA tool.
RESULTS

The search strategy resulted in 2551 initial search results for analyses. After analyses of the articles, 185 articles were considered for the meta-analysis. 23 articles were included into the meta-analyses based on data in the articles. Figure 1 shows the process of selection of the articles, while Table 4 shows the characteristics of the studies.

![Flow diagram showing the process of article selection.](image)

Calculation of the heterogeneity of the included study results showed a significant value in studies into cytokines affecting *C. trachomatis* and *N. gonorrhoeae* infections (Q test, \( P<0.01 \)). This was likely due to the various methods, settings, and sample groups used in analysis in the different studies. Because of this, a random effects analysis model was used for the meta-analyses of this subgroup. The analysis showed that multiple cytokines significantly affect the outcome of the studied diseases; Table 5 shows the odds ratios found for the analysed cytokines per pathogen in the meta-analysis and the included articles per analysed cytokine. Only cytokines that were studied in relation to a pathogen in at least 3 different studies were included for meta-analysis as lower numbers are not compatible with the CMA tool. Analyses of IFN\( \gamma \) and TNF\( \alpha \) for *C. trachomatis*, IL-1 for *N. gonorrhoeae* and IFN\( \gamma \) for *T. pallidum* are highlighted in this review due to the impact of the findings. Other results can be found in Appendix 1.
## Table 4: Characteristics of studies included in the meta-analysis.

<table>
<thead>
<tr>
<th>Year of publication</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2000</td>
<td>4</td>
<td>(17-20)</td>
</tr>
<tr>
<td>2001</td>
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<td>2002</td>
<td>1</td>
<td>(22)</td>
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<tr>
<td>2004</td>
<td>1</td>
<td>(23)</td>
</tr>
<tr>
<td>2007</td>
<td>4</td>
<td>(24-27)</td>
</tr>
<tr>
<td>2008</td>
<td>2</td>
<td>(28, 29)</td>
</tr>
<tr>
<td>2009</td>
<td>5</td>
<td>(30-34)</td>
</tr>
<tr>
<td>2012</td>
<td>1</td>
<td>(35)</td>
</tr>
<tr>
<td>2013</td>
<td>1</td>
<td>(36)</td>
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<tr>
<td>2014</td>
<td>3</td>
<td>(37-39)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>9</td>
<td>(23, 24, 27-33)</td>
</tr>
<tr>
<td>North America</td>
<td>6</td>
<td>(17, 19-21, 25, 37)</td>
</tr>
<tr>
<td>Africa</td>
<td>2</td>
<td>(18, 39)</td>
</tr>
<tr>
<td>Europe</td>
<td>3</td>
<td>(26, 34, 36)</td>
</tr>
<tr>
<td>South America</td>
<td>2</td>
<td>(35, 38)</td>
</tr>
<tr>
<td>Australia</td>
<td>1</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of people included</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>1</td>
<td>(25)</td>
</tr>
<tr>
<td>10-49</td>
<td>6</td>
<td>(20, 23, 34-37)</td>
</tr>
<tr>
<td>50-99</td>
<td>8</td>
<td>(18, 19, 22, 27, 28, 30, 32, 33)</td>
</tr>
<tr>
<td>100-249</td>
<td>4</td>
<td>(17, 21, 26, 29)</td>
</tr>
<tr>
<td>250+</td>
<td>4</td>
<td>(24, 31, 38, 39)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling type</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical swab</td>
<td>12</td>
<td>(17, 19, 23, 24, 27-33, 37)</td>
</tr>
<tr>
<td>Blood</td>
<td>8</td>
<td>(19, 20, 22, 24, 28, 31, 34, 35)</td>
</tr>
<tr>
<td>Semen</td>
<td>1</td>
<td>(26)</td>
</tr>
<tr>
<td>Urine</td>
<td>1</td>
<td>(20)</td>
</tr>
<tr>
<td>Urethra swab</td>
<td>1</td>
<td>(21)</td>
</tr>
<tr>
<td>CSF</td>
<td>1</td>
<td>(36)</td>
</tr>
<tr>
<td>Conjunctiva swab</td>
<td>1</td>
<td>(18)</td>
</tr>
<tr>
<td>Vaginal fluid</td>
<td>3</td>
<td>(25, 38, 39)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>28</strong></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1

Targeted study population

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis positive patients</td>
<td>18</td>
<td>(17-19, 21-24, 26-33, 38, 39)</td>
</tr>
<tr>
<td>N. gonorrhoeae positive patients</td>
<td>4</td>
<td>(19, 20, 25, 39)</td>
</tr>
<tr>
<td>T. pallidum positive patients</td>
<td>3</td>
<td>(34-36)</td>
</tr>
<tr>
<td>Male subjects</td>
<td>7</td>
<td>(18, 20, 21, 26, 34-36)</td>
</tr>
<tr>
<td>Female subjects</td>
<td>22</td>
<td>(17-20, 22-39)</td>
</tr>
<tr>
<td>Patients with fertility problems</td>
<td>7</td>
<td>(22, 23, 28-30, 32, 33)</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>1</td>
<td>(25)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>33</strong></td>
<td></td>
</tr>
</tbody>
</table>

Quantification technique

<table>
<thead>
<tr>
<th>Technique</th>
<th>Number of articles</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>17</td>
<td>(17-21, 23-26, 28-33, 36, 38)</td>
</tr>
<tr>
<td>Cytometric bead array</td>
<td>2</td>
<td>(27, 35)</td>
</tr>
<tr>
<td>Luminex multiplex assay</td>
<td>3</td>
<td>(34, 37, 39)</td>
</tr>
<tr>
<td>Enzyme immune-assay (EIA)</td>
<td>1</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23</strong></td>
<td></td>
</tr>
</tbody>
</table>

1= Articles can be included in multiple categories

Table 5: Meta-analysis odds ratios found for cytokines per pathogen.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Pathogen</th>
<th>Meta-analysis OR</th>
<th>Number of articles</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>C. trachomatis</td>
<td>$&lt;0.001$</td>
<td>2.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>C. trachomatis</td>
<td>0.003</td>
<td>1.5</td>
</tr>
<tr>
<td>IL-8</td>
<td>C. trachomatis</td>
<td>$&lt;0.001$</td>
<td>1.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>C. trachomatis</td>
<td>$&lt;0.001$*</td>
<td>0.5</td>
</tr>
<tr>
<td>IFNγ</td>
<td>C. trachomatis</td>
<td>0.03</td>
<td>2.3</td>
</tr>
<tr>
<td>TNFα</td>
<td>C. trachomatis</td>
<td>0.001</td>
<td>2.4</td>
</tr>
<tr>
<td>IL-1</td>
<td>N. gonorrhoeae</td>
<td>0.009</td>
<td>2.5</td>
</tr>
<tr>
<td>IL-8</td>
<td>N. gonorrhoeae</td>
<td>0.140</td>
<td>2.4</td>
</tr>
<tr>
<td>IFNγ</td>
<td>T. pallidum</td>
<td>0.492</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$&lt;0.001$*</td>
<td>0.2*</td>
</tr>
</tbody>
</table>

*= after trim-and-fill correction

Analysis of articles into IFNγ expression during C. trachomatis consistently showed an increased likelihood of complications such as Pelvic Inflammatory Disease and tubal pathology during or after infection (17, 18, 22-24, 27, 28, 37). After applying the random-effect model due to the heterogeneity of the study results, a statistically significant increase in complications due to C. trachomatis infection was found when IFNγ expression was increased (Figure 2A, P<0.03, OR: 2.3, 95% CI: 1.1 – 4.8). To exclude effects of single large
studies, a one-study-removed analysis was conducted (Figure 2B). No significantly different pooled outcome was obtained this way, indicating that no single study affected the pooled outcome significantly. A cumulative analysis of the study results showed little temporal progression of the pooled outcome over time (Figure 2C). This indicates that no significant deviations from original findings have happened over time. Potential for publication bias was examined using a funnel plot analysis (Figure 2D). It showed that one included study could be considered an outlier with higher standard error and log odds ratio than should be expected.

The analysis of articles looking into TNFα expression during *C. trachomatis* infection showed that many of them found a relation between TNFα expression and infertility, likely due to tubapathology caused by the infection (23, 29, 32). The analysis was done using the fixed-effect model as there was no significant heterogeneity of the study results. A statistically significant effect of TNFα expression was found on the complications resulting from a *C. trachomatis* infection and is shown in Figure 3 (P=0.001, OR: 2.4, 95%CI: 1.5 – 4.1). Like the relation found between *C. trachomatis* infection and IFNγ expression, this analysis also showed that no single study had a significant effect on the outcome of the meta-analysis and that temporal progression was not a significant factor. Additionally, no publication bias was found for the included studies (Appendix 1: Figure A5).

Analysis of articles examining the link between IL-1 expression and inflammation during *N. gonorrhoeae* infections showed the highest effect of cytokine expression on inflammation of all the analyses (19, 25, 39). Using Dixon’s Q test for outliers, we found that there was no statistically significant heterogeneity between the studies, which meant we could use the fixed-effect models for the analysis. Through this we found a statistically significant increased chance for more severe expression of inflammation during *N. gonorrhoeae* infections when IL-1 expression was increased (P=0.009, OR: 2.5, 95% CI: 1.3 – 4.9) as can be seen in Figure 4. As can be seen in Figure A6 of Appendix 1, one-study-removed, cumulative, and publication bias testing showed no significant factors weighing on the results.

Meta-analysis of articles containing research into IFNγ expression during *T. pallidum* infection in humans indicated that increased expression of IFNγ is linked to development of neurosyphilis (34-36). Although in Figure 5 it appears there is an amount of heterogeneity between the study results, no statistically significant heterogeneity between the included studies was found. Therefore, a fixed-effect model was used for the meta-analysis. This
analysis did not result in a statistically significant relation being found between IFNγ expression and the chance of developing neurosyphilis during a *T. pallidum* infection (*P*=0.492, OR: 1.5, 95% CI: 0.5 – 4.2) as can be seen in Figure 5. However, when adjusting for publication bias using a trim-and-fill method an adjusted result of *p*<0.001, OR=0.2 was found. One-study-removed analysis showed a larger pooled outcome, indicating that the study of Knudsen *et al.* (2009) affected the pooled outcome significantly (Appendix 1: Figure A8B). A cumulative analysis of the study results showed increasing temporal progression of the pooled outcome over time (Appendix 1: Figure A8C). Potential for publication bias was examined using a funnel plot analysis. It showed that all included studies were borderline outliers with higher standard error and log odds ratio than should be expected (Appendix 1: Figure A8D).

Meta-analysis of articles into other cytokines also showed a significant effect of expression on the outcome of infections with *C. trachomatis* and *N. gonorrhoeae*. For *C. trachomatis* a significant link was found in the articles between the expression of IL-1, IL-6, and IL-10 and fertility disorders. For IL-8 a link between expression and level of inflammation was found, though no direct complications could be linked to it. For *N. gonorrhoeae* a link was found in the articles between the expression of IL-8 and the onset and level of inflammation in the host. However, no direct complications could be linked to the expression of these cytokines either.

**Figure 2 (A):** Meta-analysis of pooled results for *C. trachomatis* severity linked to IFNγ secretion in human infections using a fixed-effect model. **(B):** One-study-removed analysis to examine single study effects showed no significant effects. **(C):** Cumulative analysis showing some temporal progression of results. **(D):** Funnel plot indicating that there was evidence of statistically significant publication bias among one of the studies.
### Study name | Statistics with study removed | Odds ratio (95% CI) with study removed
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Armo et al. 1990</td>
<td>2.034</td>
<td>0.764</td>
</tr>
<tr>
<td>Holland et al. 1996</td>
<td>2.451</td>
<td>1.062</td>
</tr>
<tr>
<td>Debattista et al. 2002</td>
<td>3.039</td>
<td>2.018</td>
</tr>
<tr>
<td>Reddy et al. 2004</td>
<td>2.085</td>
<td>0.875</td>
</tr>
<tr>
<td>Agrawal et al. 2007</td>
<td>2.099</td>
<td>0.857</td>
</tr>
<tr>
<td>Agrawal et al. 2008</td>
<td>2.097</td>
<td>0.872</td>
</tr>
<tr>
<td>Aliyar et al. 2014</td>
<td>1.902</td>
<td>0.895</td>
</tr>
<tr>
<td></td>
<td>2.273</td>
<td>1.082</td>
</tr>
</tbody>
</table>

### Study name | Cumulative statistics | Cumulative odds ratio (95% CI)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Armo et al. 1990</td>
<td>3.189</td>
<td>1.531</td>
</tr>
<tr>
<td>Holland et al. 1996</td>
<td>2.428</td>
<td>1.097</td>
</tr>
<tr>
<td>Debattista et al. 2002</td>
<td>0.808</td>
<td>0.125</td>
</tr>
<tr>
<td>Reddy et al. 2004</td>
<td>1.257</td>
<td>0.342</td>
</tr>
<tr>
<td>Agrawal et al. 2007</td>
<td>1.645</td>
<td>0.664</td>
</tr>
<tr>
<td>Agrawal et al. 2008</td>
<td>1.902</td>
<td>0.895</td>
</tr>
<tr>
<td>Aliyar et al. 2014</td>
<td>2.273</td>
<td>1.082</td>
</tr>
<tr>
<td></td>
<td>2.273</td>
<td>1.082</td>
</tr>
</tbody>
</table>

### Graphs:

- **B**: Odds ratio (95% CI) with study removed.
- **C**: Cumulative odds ratio (95% CI).
- **D**: Scatter plot of standard error against log odds ratio.
Figure 3: Fixed-effect model analysing the effect of TNFα expression on the outcome and severity of *C. trachomatis* infections.

<table>
<thead>
<tr>
<th>Study name</th>
<th>Statistics for each study</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddy et al. 2004</td>
<td>3.369, 1.010–11.242, 0.048</td>
<td></td>
</tr>
<tr>
<td>Srivastava et al. 2008</td>
<td>2.124, 1.037–4.352, 0.040</td>
<td></td>
</tr>
<tr>
<td>Gupta et al. 2009</td>
<td>2.555, 1.004–6.501, 0.049</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.444, 1.461–4.087, 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4: Fixed effect model analysis of the effect of IL-1 expression on the severity of *N. gonorrhoeae* infections in humans.

<table>
<thead>
<tr>
<th>Study name</th>
<th>Statistics for each study</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodges et al. 1998</td>
<td>1.167, 0.356–3.825, 0.799</td>
<td></td>
</tr>
<tr>
<td>Cauci et al. 2007</td>
<td>1.752, 0.486–6.321, 0.391</td>
<td></td>
</tr>
<tr>
<td>Masson et al. 2014</td>
<td>6.070, 2.044–18.024, 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.481, 1.252–4.918, 0.009</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5: A fixed-effect model analysis of the effect of IFNγ expression on the severity and progression of *T. pallidum* infections in humans.

<table>
<thead>
<tr>
<th>Study name</th>
<th>Statistics for each study</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knudsen et al. 2009</td>
<td>0.176, 0.039–0.006, 0.026</td>
<td></td>
</tr>
<tr>
<td>Druet et al. 2012</td>
<td>14.409, 1.204–175.270, 0.035</td>
<td></td>
</tr>
<tr>
<td>Pastuszak et al. 2013</td>
<td>9.328, 1.453–56.984, 0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.452, 0.501–4.212, 0.492</td>
<td></td>
</tr>
</tbody>
</table>

0.01, 0.1, 1, 10, 100
DISCUSSION

This is the first meta-analysis to show that expression level differences of eight different cytokines during bacterial urogenital infections have a significant quantified effect on the complications and outcome of these diseases. During *C. trachomatis* infection the most notable effect was found for IFNγ and TNFα expression level differences, indicating that higher expression is a risk factor for more severe complications like tubapathology. During *N. gonorrhoeae* infection differences between individuals of especially IL-1 expression were related to clinical outcome of the disease. During *T. pallidum* infections the effect of differences in cytokine expression were mostly linked to the stage of the disease. Differences in expression of IFNγ were most noticeable, though not significant, and were related to progression of the infection to neurosyphilis.

Meta-analysis focussing on cytokines expressed during *C. trachomatis* infection showed the most significant results and included the greatest variety of studied cytokines. Notably, multiple studies specifically linked cytokines to an increase in tubapathology and related complications (18, 22, 24, 28). These results highlight the relevance of the expression of these cytokines. Cytokines found to have an effect on *C. trachomatis* infection outcome were linked to NF-κB and T-helper cell cytokine expression. A strong relation was found for IFNγ, which is primarily secreted by Th-1 cells to elicit a cellular immune response and has been found vital to controlling *C. trachomatis* infections (40, 41). Also highlighted was the relation between TNFα expression and the development of infertility due to the infection. TNFα is secreted after activation of the NF-κB pathway and is responsible for inflammatory cellular characteristics such as vascular permeability. Higher levels of TNFα have previously been related to an increase in scarring during trachoma (42). These results indicate that intensity of infection and immune response are related to severity of complications in patients.

The meta-analysis aimed at cytokines expressed during *N. gonorrhoeae* infection produced multiple significant results. Expression of IL-1 and IL-8 was found to have a significant effect on the development of urethritis during *N. gonorrhoeae* infection. There was an increase in inflammation during the infection when expression of these cytokines was increased, however no direct links with any further symptoms or complications could be made (19, 25, 39). The strongest relation between expression and inflammation was found for IL-1. Mainly responsible for inflammatory cellular characteristics and the activation of T helper cells, IL-1 was also
CHAPTER 1

found in the analyses focussing on *C. trachomatis*. IL-8 was found to have a weaker effect that also increases inflammation and likelihood of inflammation related complications during increased expression. This indicates that primarily NF-κB pathway related cytokines affect the outcome of *N. gonorrhoeae* infections, and that the innate immune response is the most important factor in formation of complications.

Meta-analysis focussing on cytokines expressed during *T. pallidum* infections and the effect this had on the severity of the disease was only possible on IFNγ. This was due to the relatively low amount of articles found for the *T. pallidum* meta-analyses. For example, IFNγ was covered in only three articles and lead to a statistically insignificant relation between disease progression and expression of IFNγ. A trim-and-fill procedure focussed on removing publication bias from this article pool adjusted the data in such a way that it did become statistically significant, however the OR found in the meta-analysis went from 1.5 to 0.2. This indicates that the trim-and-fill method had such an effect on the data of this analysis that the results should be taken as an indication rather than statistically significant proof of a relation between IFNγ expression and outcome of *T. pallidum* infections. The fact that IFNγ expression was also linked to tubapathology in *C. trachomatis* infections would support the idea that it also has an effect on the inflammation based complications of *T. pallidum* infections.

The results found in this meta-analysis are largely supported by studies that could not be included. An interesting point brought up by another article showed that cytokine expression levels also differed depending on the presence of bacterial vaginosis or lactobacillus dominated vaginal microbiomes (38). As bacterial vaginosis is often difficult to detect, some included study results may be influenced by its presence. Besides this, non-included articles also showed that the expression of a number of cytokines not included in this study have a significant effect on the outcome of STDs. For instance, IL-17 expression was shown to be linked to development of asymptomatic and symptomatic neurosyphilis (41). Additionally, the level of expression of CXCL13 and CXCR5 was linked to upper genital tract pathology during *C. trachomatis* infection (43). Unfortunately, due to lack of sufficient data these cytokines could not be analysed in the meta-analyses.

Comparing the results from the different pathogens similarities can be seen. IL-1 expression was found to affect the severity of both *C. trachomatis* and *N. gonorrhoeae* infections, and IFNγ was found to affect the severity of *C. trachomatis* infections and was possibly linked to
T. pallidum infections. The link between immune responses and the severity of C. trachomatis and N. gonorrhoeae infections was previously suggested by studies into the genetics of components of the NF-κB pathway (44, 45). Unfortunately, there are currently no studies linking genetic targets from the immune response to severity of both T. pallidum and C. trachomatis. Targets that affect the severity of multiple pathogens can potentially lead to therapy solutions that also affect multiple pathogens.

Looking beyond the scope of the included pathogens some additional predictions can be made. The inflammatory nature and expression of complications during or after C. trachomatis or N. gonorrhoeae infections shows remarkable similarities with Mycoplasma genitalium. This poorly studied pathogen commonly stays asymptomatic or presents as inflammation in the urogenital tract. However it has also been related to Pelvic Inflammatory Disease as well as tubapathology leading to infertility (46, 47). Additionally, cytokines expressed during infection with M. genitalium are similar to those expressed during infection with C. trachomatis or N. gonorrhoeae, though no analysis of the effect of different expression levels has been done for this yet (48). We suggest that the apparent similar disease progression and complications linked to M. genitalium mean that expression of cytokines shown to have a significant effect on the severity of C. trachomatis or N. gonorrhoeae infections could also affect the outcome of M. genitalium in a similar way.

Whereas an M. genitalium infection shows similarities to infection with C. trachomatis or N. gonorrhoeae, an infection with Haemophilus ducreyi shows some similarities with the skin-based complications arising during infection with T. pallidum. These similarities are mainly found in the ulcers or lesions on the skin where immune-cells such as macrophages and CD4+ T-cells converge. For both of these pathogens IFNγ appears to be one of the most, if not the most, important cytokine in combatting the infection (49). It is therefore likely that any difference in expression of IFNγ has similar effects on the progression and outcome of H. ducreyi infections as they have been shown to do for infections with T. pallidum.

Higher expression of cytokines and the link with complications during infection with STDs has also been explored from a genetics perspective. Effects similar to the ones found in this article were found in analysis of TNFα and IL-10 genes in people infected with C. trachomatis (50-52). Additionally, mutations in related genes have been shown to affect the expression of cytokines. Unfortunately, studies involving genetic analyses of cytokines are not as numerous...
as studies into cytokine expression, and have included fewer different cytokine genes. This means that many of the results found for cytokines included in this study cannot be compared to a comparable genetics study. This rings especially true for studies into \textit{N. gonorrhoeae} and \textit{T. pallidum}.

A limitation of this study was the use of a funnel plot to check for publication bias. Although this method suggested multiple studies were affected by publication bias, an outlying result from the funnel plot should not be seen as conclusively biased. Besides publication bias, an outlying study may also have genuinely found varying results. Seeing as the affected studies only showed stronger effects that were also in line with findings found in other studies, publication bias is debatable. Another limitation was the lack of access to data from articles. This lead to a large number of excluded articles with data that could not be processed in the meta-analysis yet did show significant results on the topic.

In conclusion this meta-analysis has shown that there is a statistically significant link between IL-1, IL-6, IL-8, IL-10, IFNγ, and TNFα expression and severity of \textit{C. trachomatis} infections, and a link between IL-1 and IL-8 expression and severity of \textit{N. gonorrhoeae} infections. Analysis could not be run on pathogens besides these and \textit{T. pallidum} due to the low number of studies into them.

In the future these results can be used to assess the severity of infections with these pathogens based on the genetic disposition and expression of inflammatory cytokines. The overlapping results between pathogens also point towards new candidate gene study targets that could link genetic markers to severity of infections. Additionally these results may hint at potential targets for future interventions and therapies that can be used for a broader scope of STDs. The effects found in this meta-analysis that do not show a strong link between expression of cytokines and complications, such as IL-6 for \textit{C. trachomatis} and IL-8 for \textit{N. gonorrhoeae}, suggest that the impact of these cytokines on \textit{C. trachomatis} and \textit{N. gonorrhoeae} infections is limited, although this impact may increase in combined analyses. On the other hand, for example the chemokine MIP shows an effect on the immune-response to \textit{N. gonorrhoeae} in multiple studies, and more cytokines like this exist. However these did not reach the requirements for inclusion in this meta-analysis. More research into these cytokines will make another meta-analysis in the future more inclusive and might add additional insights into the interplay between cytokine expression and disease complications.
DECLARATIONS

Supplementary data
Supplementary data are available at FEMSPD online.

Conflict of interest
None declared.
REFERENCES


“The chances of finding out what's really going on in the universe are so remote, the only thing to do is hang the sense of it and keep yourself occupied.”

Douglas Adams
The Hitchiker’s Guide to the Galaxy
CHAPTER 2
CpG DNA analysis of bacterial STDs

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ABSTRACT

Background: Bacterial infections in the genital tract frequently result in morbidity through a variety of inflammation based symptoms. One component of the bacteria that may trigger host inflammatory response is their DNA. CpG motifs in this DNA are known targets for Toll-like receptor 9 (TLR9), which is a pathogen-recognition receptors focusing on CpG DNA. The activation of TLR9 induces the NF-κB inflammatory pathway. This study aims to provide a broad view of the inflammatory potential of CpG DNA motifs in bacteria related to genital diseases: *C. trachomatis*, *E. coli*, *N. gonorrhoeae*, *G. vaginalis*, *H. ducreyi*, *L. crispatus*, *L. gasseri*, *M. hominis*, *M. genitalium*, *T. pallidum*, and *U. urealyticum*.

Methods: Publicly available genomic sequences of the bacterial species and strains have been analyzed in silico to produce a CpG index number. This CpG index number shows the relative inflammatory potential of the genome and has previously been used in a study by Lundberg et al. (2003). Higher CpG index values suggest a strong CpG induced inflammation potential during infection and vice versa.

Results: The highest observed CpG index belongs to *G. vaginalis* with a value of 26.2, suggesting a strong pro-inflammatory potential when in contact with TLR9. The lowest index belongs to *N. gonorrhoeae* with a value of -79.5, suggesting a strong immunoinhibitory effect on TLR9 contact. Interestingly, *Lactobacilli* showed a mean CpG index value of 4.2, suggesting a weak inflammatory potential.

Discussion: Our results show varying CpG index values between bacterial species. Comparison of CpG indices with the clinical course of several pathogens shows the CpG index helps clarify the clinical course of infection. However, we found no links between CpG index values and either obligate pathogenicity or facultative pathogenicity through bacterial vaginosis. *Lactobacilli* showed relatively low CpG indices which do suggest a lower inflammatory potential from these bacteria.
INTRODUCTION

Bacterial Sexually Transmitted Diseases (STD) and Genital Tract Infections (GTI) can cause high levels of morbidity, are often accompanied by social stigma, and are frequently widespread (1). Symptoms can range from slight inflammation and discharge to infertility and death. In these diseases, inflammatory responses may not always have the positive effect of initiating immune responses to clear the infection (2). Tissue scarring and an inability to clear bacteria often occur in these infections. Others possess ways of reducing inflammatory response to allow for better survival in the host. The scale of inflammatory response relies on the ability of the host to detect the pathogen and initiate key inflammatory pathways.

One way of initiating inflammation is through the detection of bacterial DNA (3). Bacterial DNA has unmethylated Cytosine-phosphor-Guanine (CpG) motifs, while mammals generally have methylated CpG motifs which are close to anti-inflammatory DNA sites (4-6). Unmethylated CpG motifs are targets for the intracellular Toll-like receptor 9 (TLR9) (7). This receptor is minimally, but consistently expressed in epithelial cells of the genital tract (8, 9). When TLR9 binds to specific unmethylated CpG motifs it activates the NF-κB pathway, which is a major pathway related to immune response. Activating this pathway initiates a chain reaction resulting in the release of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, IL-12, and type 1 interferons (4, 10). These cytokines directly affect the cellular and humoral immune response as well as regulate the inflammation at the site of infection.

As inflammation is a main cause for symptoms in bacterial STDs, we look into how the CpG properties of these pathogens can explain differences in symptoms and outcomes of bacterial STDs, including: Haemophilus ducreyi, Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum, and Mycoplasma genitalium.

The first group of pathogens; C. trachomatis, N. gonorrhoeae, and M. genitalium are STDs with similar symptoms and course of infection. These diseases are often asymptomatic, but can also show similar inflammation based symptoms during infection. These can range from mild cervicitis to pelvic inflammatory disease, ectopic pregnancy, and tubopathology associated infertility. It has been shown that activation of the NF-κB pathway through various TLRs is a vital part of the initial immune response to all of these diseases (11-14). Previous study into the CpG properties of these pathogens showed that C. trachomatis serovars C and D have an
immunostimulatory effect on the immune system while CpG properties of *N. gonorrhoeae* demonstrates a strong inhibitory potential towards TLR9 binding (12). *C. trachomatis* serovars E and the highly inflammatory L2b, as well as various strains of *M. genitalium* will be analyzed for the first time in this study.

The second group of pathogens; *T. pallidum* and *H. ducreyi* are STDs that are characterized by ulcers and lesions on the genitals and skin during infection. It has been shown that NF-κB pathway activation through TLR stimulation is vital for initiating an immune response against *T. pallidum* (15, 16). However, this has not yet been shown for *H. ducreyi* infections. The cellular response to *H. ducreyi*, including macrophages and polymorphonuclear leukocytes, does suggests that cytokine signaling originating from NF-κB pathway activation plays a significant role in the infection (17, 18). CpG properties indicating a potentially strong activation potential of TLR9 could indicate the primary immune response during infection with these diseases.

Bacterial vaginosis is a disease of the genital tract commonly described as abnormal vaginal discharge, often accompanied with a foul smell, in women of childbearing age. There is no single causative agent of bacterial vaginosis. Instead it is caused by an imbalance in the natural vaginal microflora. One or more commensal bacteria overgrow the naturally dominant Lactobacilli. Some of the bacteria associated with bacterial vaginosis are *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*. An immune response against bacterial vaginosis appears to be lacking. There are no polymorphonuclear leukocytes in the vaginal fluids of women with bacterial vaginosis, however it has been shown that inflammatory cytokines such as IL1 and TNF-α are present (19). This suggests the imbalance of bacteria is recognized by the immune system, but an effective immune response is inhibited. Host response mechanisms to bacterial vaginosis appear to largely revolve around the activation of the NF-κB pathway (20-22).

Unlike these pathogens, commensal bacteria are naturally found in the host and generally cause no adverse effects. In this study we include the commensal bacteria *Lactobacillus crispatus*, *Lactobacillus gasseri*, and an *Escherichia coli* strain linked to asymptomatic growth in the urinary tract. *L. crispatus* is a beneficial vaginal bacterium whose decrease is characteristic of bacterial vaginosis. The vaginal bacterium *L. gasseri* is also found to protect the vagina from infections. Lactobacilli acidify the vagina and produce hydrogen peroxide which reacts with myeloperoxidase to form reactive molecules toxic to pathogens. Women without vaginal
lactobacilli have an increased risk of HIV and gonorrhoeae (23, 24). It has been shown that Lactobacilli may or may not induce an immune response through the NF-κB pathway on a species dependent basis (25). E. coli is a bacterium generally linked to intestinal inflammation and urinal tract infections. However, E. coli can also occur asymptomatically in both the intestines and the urinal tract (26). The immunopathogenesis of E. coli has been clearly linked to the NF-κB pathway, primarily through activation of TLR4 (27, 28). However this has only been shown for pathogenic strains.

In this study we aim to provide a broader view of the inflammatory properties of bacterial genomes in diseases related to the vaginal or genital tract. These genomes are analyzed in silico, to assess the inflammatory potential of CpG motifs in these pathogens, and to predict the role TLR9 plays in the respective host-bacterium interactions and whether strain differences affect this role.

MATERIALS AND METHODS

Publicly available bacterial genome data has been used for all analyses in this study. NCBI genome databases have been used to obtain the genomes required for analysis. Genomes most focused on by the scientific community that did not have specific uncommon characteristics were chosen for the analysis. Genomes were chosen based on frequency of inclusion in research and lack of traits differentiating them from the usual organism. CpG analysis per genome has been done using previously described genome analysis methods (29). These methods allowed determination of the amount and build of CpG motifs in a genome, predicted number of CpG motifs when looking at the genomes size, and GC content. The analyzed strains in this study comprise strains of the bacteria: C. trachomatis, E. coli, G. vaginalis, H. ducreyi, L. crispatus, L. gasseri, M. genitalium, M. hominis, N. gonorrhoeae, T. pallidum, and U. urealyticum as shown in Table 1.

In silico analyses

Size and GC content of the analyzed genomes were gathered from the NCBI genome databases. The average amount of CpG hexameres (NNCGNN) per kb of genome was calculated from the total amount of CpG hexameres per genome. CpG hexameres found per genome were compared to the amount of CpG hexameres expected based on the size and
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the GC content of the genome. We determined the frequency of inflammation stimulatory or inhibitory CpG DNA motifs in their respective genomes (12, 29, 30). As definition for stimulatory or inhibitory motifs we used published consensus motifs derived from E. coli sequences (30). These comprise inhibitory hexamere motifs NCCGNN and NNCGRN, and stimulatory hexamere motifs RRCGY. From the difference between these frequencies we produced CpG indices showing the CpG-based immunostimulatory or immunoinhibitory potential of the disease as has previously been described (31, 32).

Table 1: Micro-organism names, strains and relevant NCBI references to sequences.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Disease</th>
<th>Strain</th>
<th>NCBI reference sequence</th>
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</thead>
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Ethics statement

The authors declare no human material was used during this study.
RESULTS

Table 2 shows the CpG indices for the examined micro-organisms. An index above 0 predicts immunostimulatory properties of the DNA and an index below 0 predicts immunoinhibitory properties. The indices do not predict a set amount of inflammation. Larger indices indicate a more potent inflammatory or inhibitory potential. Amount of inflammation belonging to index values can be predicted by comparing scores and *in vitro* or *in vivo* responses.

*G. vaginalis* has the highest index with one strain reaching a value of 26.2, and a mean value of 23.9. Both the included *E. coli* strain and *T. pallidum* also appear to have larger than average mean CpG values, with mean values of 21.1 and 17.7, respectively. The lowest index belongs to *N. gonorrhoeae* with one strain having a CpG value of -79.5 and a mean CpG value of -77.1. *N. gonorrhoeae* was the only bacteria showing a negative CpG value in the analysis.

A large cluster of genomes were found to have relatively low mean CpG values of <10. The mean CpG value of *C. trachomatis* strains that were not L2b was 3.1, with the included L2b strain showing a slightly lower CpG value of 2.9. *H. ducreyi* showed a CpG value of 6.6. The two included *Mycoplasma* species, *genitalium* and *hominis*, were found to have mean CpG values of 1.5 and 3.8, respectively. The single strain of *U. urealyticum* was found to have a CpG value of 8.4. Lastly, the Lactobacilli were found to have index values of 3.7 and 4.6. Figure 1 shows the mean CpG index values for every pathogen on a CpG axis.

![Figure 1: Scale bar showing the position of the mean CpG values per pathogen.](image-url)
Table 2: Results of \textit{In silico} CpG analyses.

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<tr>
<th>Genome</th>
<th>Strain</th>
<th>Size (Mb)</th>
<th>G+C%</th>
<th>CpG per kb(^b)</th>
<th>Total CpG(^c)</th>
<th>Stimulatory(^d)</th>
<th>Inhibitory(^e)</th>
<th>CpG index(^f)</th>
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<td>31.7</td>
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<td>39.1</td>
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<td>48.3</td>
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<td>138.7</td>
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<tr>
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<td>41.2</td>
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\(a\): Deviations in amounts of CpG hexameres compared to the expected amount based on GC content.
\(b\): CpG hexameres occurring per 1kb of DNA
\(c\): Total number of CpG hexameres compared to the expected amount
\(d\): Number of stimulatory CpG hexameres (RRCGYY) compared to expected amount
\(e\): Number of inhibitory CpG hexameres (NCCGNN and NNCGRN) compared to the expected amount
\(f\): Index calculated from the difference between stimulatory deviation and inhibitory deviation indices, multiplied by the total CpG index, normalized by multiplying with the amount of CpG hexameres per 1 kb.
DISCUSSION

The immune response to bacterial STDs is primarily initiated through activation of TLRs. TLR9 is likely to be a big factor due to activation of the receptor by bacterial DNA CpG motifs. This study has produced an overview of potential TLR9 activation through inflammation stimulating or inhibiting CpG motifs related to a variety of bacterial STDs, bacteria linked to bacterial vaginosis, and commensal bacteria found in the genital tract.

The group of bacterial STDs with symptoms largely related to symptoms induced by inflammation in the host was a likely target to show high potential TLR9 activation through the CpG indices. Surprisingly, *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* do not show any indices higher than 3.1. Although there is a relatively low CpG index for both *C. trachomatis* serovars E and 2b, cervical and colonic epithelial cells infected with *C. trachomatis* do secrete pro-inflammatory cytokines in relatively large amounts (33). Additionally, IL-1α secreted by endocervical epithelial cells was previously found to amplify the inflammatory response by stimulating additional cytokine production without activating more TLRs (34). A study by Ouburg *et al.* shows that TLR9 does not influence the susceptibility to acute *Chlamydia* infection (12). This information plus the relatively low stimulatory CpG index of 3.1 of *C. trachomatis* may indicate that it elicits an immune response via another route than TLR9. TLR4 is a likely alternative candidate, as it recognizes chlamydial LPS via its coreceptor CD14 (35). Similarly, the highly inflammatory strain L2b/UHC-1 shows a comparable CpG index also suggesting that TLR9 and CpG DNA are not vital factors in inflammation during *C. trachomatis* infection.

Similarly to *C. trachomatis*, symptoms during infection with *N. gonorrhoeae* are also largely based on inflammation. However it was found to have an inhibitory CpG index of -73.1, similar to findings in a study by Ouburg *et al.* (12). It has been described that *N. gonorrhoeae* uses several strategies to avoid the immune system. The CpG index of -73.1 of *N. gonorrhoeae* may explain that this pathogen suppresses Th1 and Th2 responses by reducing binding with TLR9 and activation of NF-κB (36). This reduces the secretion of IL-4 and IL-12 that are needed to activate the Th1 and Th2 responses. Reports show that shortly after infection, CD4+ T cell and CD8+ T cell levels declined (37). This may also explain why *N. gonorrhoeae* demonstrates an asymptomatic clinical course in most cases (38). Based on these studies,
it is likely that when inflammatory symptoms arise during *N. gonorrhoeae* infection, it is likely through activation of the immune response without activation of TLR9.

*M. genitalium* was found to have a minimal CpG index of only 1.5. Comparing this with the minor CpG index for *M. hominis* of 3.8 and a higher CpG index of 8.4 for the closely related *U. urealyticum* some similarities can be seen. The values suggest minor inflammatory properties of the micro-organisms’ DNA and significance of TLR9 in the inflammatory response to these organisms. No research has been done on the roles of either CpG or the TLR9 pathway in the bacteria. However, a previous study did indicate TLR1 and TLR2 to induce the NF-κB pathway in *M. genitalium* (14). Therefore, we suggest that activation of the immune response is largely initiated through these pathways instead.

The bacterial STDs *H. ducreyi* and *T. pallidum*, both characterized by the formation of lesions or ulcers as symptoms, showed CpG indices of 6.6 and 17.7, respectively. During *H. ducreyi* infections, increased secretion of TLR9 related pro-inflammatory cytokines including IL-12 and IFNy would activate and increase differentiation of Th1 cells. A Th1 cellular immune response is needed for clearance of *H. ducreyi* (39). The effect of some point mutations in TLR9 on activation of the cellular immune response was shown by Sanders *et al.*, showing a protective effect of TLR9 +2848 in a study targeting bacterial meningitis (31). Unpublished data from our group showed a protective association for TLR9 +2848*G* and a significant risk enhancing effect for TLR9 -1237*T* plus TLR9 +2848*A* during *H. ducreyi* infections (manuscript in preparation). This indicates TLR9 activation through CpG motifs in *H. ducreyi* DNA is vital for a proper immune response to this infection.

Similarly during *T. pallidum* infections a cellular immune response is vital for clearance of the infection (40). With pro-inflammatory cytokines being found inside lesions, indicating activation of the NF-κB pathway plays an important role in the initial immune response as well as activation of the cellular response. The relatively high CpG index of 17.7 found for *T. pallidum* indicates that TLR9 can be the primary inducer of the NF-κB pathway during infection with *T. pallidum*.

*G. vaginalis* was found to have the highest mean CpG index of 25.7, suggesting that it has DNA with significant inflammatory properties. However, bacterial vaginosis because of *G. vaginalis* overgrowth only produces mild inflammatory signs. *G. vaginalis* has previously
been found to create a biofilm and from there induce controlled inflammation, using the host’s immune response to further its infection (41). Additionally, Ghione et al. has found that a Th2 response activating B-cells produces antibodies specific to G. vaginalis influencing the infection but not clearing it (42). We suggest that the high CpG index found in this study can be explained as part of the way G. vaginalis gains advantage from the inflammation while inside a biofilm.

The commensal bacteria L. gasseri and L. crispatus show a stimulatory effect on the immune system. In contrast to our findings, a study by Ghadimi et al. describes that the binding of the commensal bacteria L. rhamnosus to TLR9 elicits an intracellular signaling cascade in a manner that reduces the expression of IL-8. TNF-α is being attenuated by reducing IkBa and p38 phosphorylation, which are downstream signaling proteins in the NF-κB pathway (43). Additionally, recent findings suggest that there is a species specific effect on the inflammatory response of the host to Lactobacillus spp. (25). For example L. iners was found to induce pathogen recognition receptor activity and expression of pro-inflammatory cytokines. Conversely, L. crispatus was found to not exhibit these effects. This suggests a potential disparity between different Lactobaccillus species that may explain the different findings. Indeed, one study found that cytokine production differed (slightly) between Lactobacillus species, and that this cytokine response is primarily due to activation of TLR9 (44). This may indicate that the relatively small difference between the two species examined here is a fluctuation that apparently has an in vivo effect on the production of TLR9 related cytokines.

In contrast to the relatively low CpG indices of the examined Lactobacilli, the examined E. coli strain showed a high immunostimulatory CpG index of 21.1. Although studies into commensal E. coli strains have primarily focused on TLR4 and TLR5, one study has shown cytokine expression profiles during stimulation of TLR9 with commensal E. coli DNA linked to NF-κB activation (45-47). It is strange then, that the presence of the E. coli strain does not lead to symptoms that normally occur during E. coli pathogenic infections. Previous analysis of the E. coli ABU 83972 genome found that the innate immune response of the host is modified during infection with this bacterium (26). Specifically the IL-1 and IL-6 signaling pathways are affected. The authors suggest that the bacteria uses this modified immune response to adapt on a host-specific basis, to a point where both host and bacterium can benefit from
CHAPTER 2

the commensal growth. Therefore, in this specific strain of *E. coli* the immunostimulatory potential of the high CpG index is successfully circumvented.

Comparing our results to previous studies into CpG indices of microbial organisms allows us to put the CpG indices into context (31, 32). Lundberg et al. examined viral DNA to find CpG indices up to 148.7 for Bovine Herpesvirus-1 and a low of -9.4 for Epstein Barr virus. They suggest that viral DNA characteristics make it hard to compare CpG indices of these viruses, and mention that the results may have been affected by the CpG motifs used for analysis, as they were determined from bacterial DNA. Nevertheless, they showed a predictive value in the CpG index as the negative results relate to low inflammation in clinical infections and relatively high results relate to strong inflammatory responses *in vivo* (32). The study of Sanders et al. focused on bacterial meningitis and can be better related to this study. Interestingly, their analyses of *N. meningitidis* resulted in a CpG index of -106.8, suggesting a very strong immunoinhibitory relation similar to the one found in this study for *N. gonorrhoeae*. *H. ducreyi* has a CpG index of 6.6, only 0.6 points removed from *H. influenzae* with an index of 7.2. Sanders *et al.* relate even the weak CpG indices to clinical inflammation during their respective diseases (31).

Looking at the clinical pictures of pathogens included in this study, the bacteria *H. ducreyi* and *T. pallidum* cause visible soars or ulcers during their clinical course while *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* have the shared characteristic of causing tubal pathology, which in all cases can lead to infertility and ectopic pregnancy. The ulcer and lesion producing group has CpG indices that are overall higher than the group of pathogens related to tubapathology, even though the clinical course of the last group of diseases shows clear inflammation in the host. However, previous studies have shown that pathogens related to tubapathology are detected more accurately through other pathways. The difference in CpG index values in this group may be explained by the fact that *C. trachomatis* is intracellular, and *N. gonorrhoeae* extracellular, thus the two are exposed to different immunological factors. This has already been shown for *M. genitalium*, which is detected through TLR1 and TLR2 instead (14). There are also two non-pathogenic groups of bacteria studied here. The first is the commensal group including *L. crispatus*, *L. gasseri*, and an asymptomatic *E. coli* strain. The second is the bacterial vaginosis group consisting of *G. vaginalis*, *M. hominis*, and *U. urealyticum*. These may show symptoms like increased vaginal discharge, change of smell, and itchiness (48). These two groups both show widely varying positive CpG indices.
depending on the examined organism. This indicates that TLR9 initiation potential is likely highly specific to an organism, and related to multiple factor such as interaction with the immune system. Additionally, it suggests that CpG / TLR9 interaction cannot account for specific inflammatory symptoms. Previous study has shown that bacterial CpG specifically induces the proinflammatory cytokines IL-6, IL-12, and Interferon γ (49). However, the symptoms created during infection with the included organisms are formed by a complex system including both host and bacterial factors for which the CpG index value reflects the intensity of the initial inflammation.

Including all the studied bacteria into one biological model is difficult, as many of these bacteria have different ways of avoiding or interacting with the immune system. However, the comparison of CpG indices with clinical outcomes of the diseases showed that there are similar characteristics between some bacteria. As was previously mentioned, positive CpG indices result in stimulation of TLR9, which activates the TLR9 related NF-κB pathway. At the end of this pathway, upregulated transcription of NF-κB targeted genes causes more inflammatory cytokines such as IL-1 and TNF-α to be released. We suggest that a relatively low or negative CpG index still allows the DNA of the bacteria to bind. However, this DNA then does not stimulate TLR9, or does not stimulate TLR9 as strongly into activating the NF-κB pathway. Conversely, a positive CpG index means the DNA binds to TLR9 more easily or activates the NF-κB pathway in a stronger manner.

Reflecting back on this study some strengths become clear. The methods used in this study have previously been shown to have significant predictive value. This study is also the first to look at CpG DNA and its effect on inflammation for such a large group of relevant bacteria in the genital tract. However, some limitations do apply. Though the predictions have previously been shown to have significant value, in vitro study is needed for verification. Also this study has only looked at sequenced strains. Therefore some results may not be in line with what can be seen in infections with current wild type strains in in vivo infections. Additionally, the used CpG sequences were all derived from studies on E. coli. There is no study into whether these sequences act like stimulatory and inhibitory motifs for all the bacteria studied here or if there are any additional relevant sequences.

This study has indicated inflammatory potential in bacterial STDs through analysis of the bacterial genomes. If this result can be corroborated in vitro it can clarify the
immunopathogenesis for the bacteria studied here. In the future this data can be used to specifically focus research into inflammation during infections with the studied bacteria. Additionally, results found in this study can be used to compare indices of other microorganisms studied using the same methods.

**Conclusion**
In conclusion our results show varying CpG index values between bacterial species. Comparison of CpG indices with the clinical course of several pathogens shows the CpG index helps clarify the clinical course of infection. However, we found no links between CpG index values and either obligate pathogenicity or facultative pathogenicity through bacterial vaginosis. *Lactobacilli* showed relatively low CpG indices which do suggest a lower inflammatory potential from these bacteria.

**Competing Interests**
The authors declare that they have no competing interests.

**Authors’ Contributions**
MS and DdW participated in the design of the study, carried out the genome analyses and drafted the manuscript. SM and SO conceived and participated in the design of the study. They also coordinated the study and helped finalize the manuscript. All authors read and approved the final manuscript.
REFERENCES


“That without experimentation, a willingness to ask questions and try new things, we shall surely become static, repetitive, moribund.”

Anthony Bourdain
CHAPTER 3

Host polymorphisms in TLR9 and IL10 are associated with the outcomes of experimental Haemophilus ducreyi infection in human volunteers

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ABSTRACT

Background. In humans inoculated with Haemophilus ducreyi, there are host effects on the possible clinical outcomes - pustule formation vs. spontaneous resolution of infection. However, the immunogenetic factors that influence these outcomes are unknown. Here we examined the role of 14 single nucleotide polymorphisms (SNPs) in seven selected pathogen recognition pathways and cytokine genes on the gradated outcomes of experimental infection.

Methods. DNAs from 105 volunteers infected with H. ducreyi at 3 sites were genotyped for SNPs using real-time PCR. The participants were classified into 2 cohorts by race and four groups based on whether they formed 0, 1, 2, or 3 pustules. $X^2$ tests for trend and logistic regression analyses were performed on the data.

Results. In European Americans, the most significant findings were a protective association of the $\text{TLR9} +2848 \text{ GG genotype}$ and a risk enhancing association of the $\text{TLR9} \text{ TA haplotype}$ with pustule formation; logistic regression showed a trend towards protection for the $\text{TLR9} +2848 \text{ GG genotype}$. In African Americans, logistic regression showed a protective effect for the $\text{IL10} -2849 \text{ AA genotype}$ and a risk enhancing effect for the $\text{IL10} \text{ AAC haplotype}$.

Conclusions. Variations in $\text{TLR9}$ and $\text{IL10}$ are associated with the outcome of $H. ducreyi$ infection.
BACKGROUND

**Haemophilus ducreyi** causes chancroid, a sexually transmitted disease that presents as painful genital ulcers and facilitates the transmission and acquisition of the human immunodeficiency virus (HIV) type 1 (1). Due to syndromic management of genital ulcers, the global prevalence of chancroid is currently undefined but has declined in many former areas of high endemicity (2, 3). Recently, *H. ducreyi* was found to be the leading cause of cutaneous ulcers in children in yaws-endemic communities of the South Pacific islands and equatorial Africa (3-7). Thus, *H. ducreyi* is an important threat to global health.

To study the biology of *H. ducreyi*, we developed a model in which healthy adult volunteers are inoculated at 3 sites on an upper arm with identical doses of the genital ulcer isolate, 35000HP (HP, human passaged) (8, 9). Papules develop at infected sites within 24 h and either spontaneously resolve or progress into pustules within 2 to 5 days. Within a person, the outcomes (resolution vs. pustule formation) of infected sites tend to be similar, suggesting a host effect on disease progression (10, 11). When reinfected, volunteers initially classified as "resolvers" or "pustule formers" segregate towards their initial outcomes, confirming a host effect on susceptibility (10).

Experimental pustules and natural ulcers represent a failed immune response. These lesions resemble suppurative granulomas in that they consist of polymorphonuclear leukocytes (PMN) that form an epidermal abscess, a collar of macrophages admixed with regulatory T cells below the abscess, and a deep dermal infiltrate of memory CD4, CD8, and NK cells (12-15). Unlike most bacteria that cause granulomas, *H. ducreyi* is surrounded by PMN and macrophages and is extracellular (16, 17). Thus, evasion of phagocytosis underlies disease progression (18-21). The mechanism of bacterial clearance in resolvers is unknown but likely involves enhanced phagocytic clearance, which may be shaped by the microenvironment at the infected site (10, 22). Comparative transcriptional analysis of skin biopsies obtained after a repeat infection shows that relative to resolvers, the lesional microenvironment of pustule formers is marked by a hyperinflammatory, dysregulated state (22). When infected with *H. ducreyi*, monocyte-derived myeloid dendritic cells (DC) obtained from resolvers have a transcriptional response typical of type 1 DC, while those derived from pustule formers have a mixed response with features of type 1 DC and regulatory DC, marked by upregulation of IL-10 (22). In addition, the preinfection microbiome of resolvers share a similar community structure
that significantly differs from the preinfection microbiome of pustule formers, which is more diverse (23). This finding may reflect biases in innate immunity between the two groups that drive different compositions of the microbiome (23). These data led us to hypothesize that there may be an immunogenetic basis for differential innate immune responses to H. ducreyi that ultimately determine disease outcome.

Host immunogenetic factors are associated with the outcome of other bacterial sexually transmitted infections (24-27). For instance, single nucleotide polymorphisms (SNP) in the Toll-like receptor (TLR) 4 and TLR9 genes affect the susceptibility to and severity of Chlamydia trachomatis infections (24, 25). These polymorphisms affect the ability of the TLRs to detect pathogen associated molecular patterns, impeding the host immune response to infection.

In this study, we examined whether SNPs in genes that encode pathogen recognition receptors (PRR), control innate immune responses, or encode cytokines correlated with the outcomes of experimental infection in two cohorts of experimentally infected European Americans (EA) and African Americans (AA). As innate immune responses appear to be important in determining outcome, we analyzed SNPs in TLRs, nucleotide oligomerization domain (NOD)-like receptors, single immunoglobulin interleukin (IL) 1 receptor (SIGIRR) and IL-10.

METHODS

Sample collection
Between March 2000 and June 2014, we collected blood from 144 healthy adult volunteers, who had no history of previous H. ducreyi infection (Figure 1). Each volunteer was inoculated with strain 35000HP on one arm at 3 sites vertically spaced 3 cm apart on the skin overlying the upper deltoid via 1.9 mm puncture wounds made with an allergy testing device, which delivers the bacteria to the epidermis and dermis. Each site received identical doses of 35000HP, which was prepared from dedicated freezer lots according to US Food and Drug Administration guidelines. Most participants were enrolled in mutant versus parent comparison trials and were also infected on the opposite arm with isogenic mutants derived from 35000HP, which can be attenuated or fully virulent for pustule formation (9). Resolvers who formed pustules at sites inoculated with virulent mutants were considered capable of pustule formation; 3 such participants were excluded from the analysis.
In the model, we attempt to deliver a standard dose of ~ 90 colony forming units (CFU) of 35000HP. However, *H. ducreyi* has a tendency to clump, which causes variation in actual dose. Data based on infection of 299 participants show a significant effect of dose on pustule formation, which increases by 0.7% per CFU ($P = 0.001$). To adjust for potential differences in doses between the resolvers and pustule formers, we excluded 15 participants who had been inoculated with 35000HP doses < 34 CFU and ≥ 130 CFU.

From the remaining 126 persons, 19 samples were lost and 2 samples were not amplifiable; thus, we recovered amplifiable DNA from 105 participants. The participants were divided into EA and AA cohorts by self-report. Each cohort was divided into 4 groups, who formed 0 (resolvers), or 1, 2, or 3 pustules (pustule formers) at 35000HP-inoculated sites. The participants included 59 EA (33 males and 26 females; age range 21 – 59 years; mean age ± standard deviation (SD), 36.3 ± 11.8 years) and 46 AA (29 males and 17 females; age range 21 – 64 years; mean age ± SD, 42.3 ± 10.6 years) (Figure 1).
CHAPTER 3

Ethics Statement
Study protocols and informed consent statements were approved by the Division of Microbiology and Infectious Diseases of the National Institutes of Allergy and Infectious Diseases and by the Institutional Review Board of Indiana University.

DNA isolation
Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using the Accuspin™ System–Histopaque®-1077 kit (Sigma-Aldrich). DNA was isolated from PBMC using the High Pure PCR Template Preparation Kit (Roche Applied Science).

SNP determination
The isolated DNA was genotyped for 14 SNPs in seven genes (Table I) using Real Time PCR assays on the LightCycler 480 (Roche Molecular Diagnostics, Almere, The Netherlands). The PCR conditions were: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 60 s, and elongation at 72 °C for 1s. For the SNP /IL10 -1082 A>G, annealing was done at 55 °C for 1 minute each cycle, using primer sequences as described in Supplementary file 1, which can be found in the online version of this article.

Table 1: Genes, SNPs, alleles, rs numbers, and haplotype configurations analyzed in this study. Abbreviations: SNP, single nucleotide polymorphisms; rs number, reference SNP identification number.

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Statistical Analyses
Statistical analyses were performed using Graphpad Instat 3. Results between sample groups were examined for Hardy Weinberg equilibrium. X² tests for trends were performed where appropriate to assess differences in genotype distributions between the groups (0, 1, 2, or 3 pustules). Haplotype distribution (Table 1) was inferred using PHASE software and analysed using X² tests for trends. Carrier trait analyses were performed to examine synergy in protective or risk enhancing associations of different SNPs and haplotypes. To reduce data complexity, binary logistic regression was performed using SPSS v20.

Analysis of H. ducreyi CpG motifs
To determine the potential immunostimulatory activity of 35000HP DNA, we calculated the CpG index for H. ducreyi exactly as described previously (28, 29). The results for H. ducreyi were compared to those calculated previously for Streptococcus pneumoniae, Neisseria meningitidis, and Haemophilus influenzae.

RESULTS

Genotyping results
Amplifiable DNA was recovered from 105 persons who were infected with H. ducreyi and met inclusion criteria. The participants included 59 EA and 46 AA who formed 0, 1, 2, or 3 pustules (Figure 1). In each cohort, there were no significant differences in the doses of H. ducreyi among the four outcome groups (data not shown). Table 2 shows the overall frequency of the genotypes in each cohort.

We assessed potential links between SNPs and haplotypes and the outcome of infection by using X² tests. Within each ethnicity, X² tests for trend on the SNPs and haplotypes showed multiple significant results (Figure 2 and Figure 3). There were significant protective associations against pustule formation for the TLR9 +2848 GG (P = 0.004) and *G genotypes (P = 0.041) and for the IL10 AGC haplotype (P = 0.009) in the EA cohort. A significant risk enhancing association for pustule formation was found for the haplotype TLR9 TA in the EA cohort (P = 0.005); a borderline risk enhancing association was found for the haplotype IL10 AAC (P = 0.058) in the AA cohort. No significant results were found for the other analysed SNPs or haplotypes.
Table 2: Genotype frequencies and percentages of the SNPs in the cohorts. Data represent the number of persons and (their percentage) in each cohort. Abbreviations: SNP, single nucleotide polymorphism; EA, European American; AA, African American; WT, wild type; HZ, Heterozygous; MT, Mutant allele

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>EA cohort</th>
<th></th>
<th>AA cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>HZ</td>
<td>MT</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
<td>--------</td>
<td>-----------</td>
<td>---</td>
<td>-----------</td>
</tr>
<tr>
<td>TLR2</td>
<td>-16934</td>
<td>T&gt;A</td>
<td>6 (10)</td>
<td>27 (48)</td>
<td>26 (44)</td>
</tr>
<tr>
<td>TLR2</td>
<td>+2477</td>
<td>A&gt;G</td>
<td>54 (92)</td>
<td>5 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TLR4</td>
<td>+896</td>
<td>A&gt;G</td>
<td>52 (88)</td>
<td>7 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TLR9</td>
<td>-1237</td>
<td>T&gt;C</td>
<td>46 (78)</td>
<td>11 (19)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>TLR9</td>
<td>+2848</td>
<td>A&gt;G</td>
<td>19 (32)</td>
<td>28 (47)</td>
<td>12 (20)</td>
</tr>
<tr>
<td>NOD1</td>
<td>+32656</td>
<td>T&gt;GG</td>
<td>35 (59)</td>
<td>22 (37)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>NOD2</td>
<td>+2104</td>
<td>C&gt;T</td>
<td>52 (88)</td>
<td>7 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NOD2</td>
<td>+3020</td>
<td>C insertion</td>
<td>54 (92)</td>
<td>5 (8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>-146</td>
<td>G&gt;T</td>
<td>47 (80)</td>
<td>10 (17)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>+53</td>
<td>C&gt;T</td>
<td>47 (80)</td>
<td>10 (17)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>+935</td>
<td>G&gt;A</td>
<td>27 (46)</td>
<td>25 (42)</td>
<td>7 (12)</td>
</tr>
<tr>
<td>IL10</td>
<td>-2849</td>
<td>A&gt;G</td>
<td>36 (61)</td>
<td>20 (34)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>IL10</td>
<td>-1082</td>
<td>A&gt;G</td>
<td>20 (34)</td>
<td>25 (42)</td>
<td>14 (24)</td>
</tr>
<tr>
<td>IL10</td>
<td>-819</td>
<td>C&gt;T</td>
<td>27 (48)</td>
<td>26 (44)</td>
<td>6 (9)</td>
</tr>
</tbody>
</table>
Figure 2: Bar plots and trend lines for SNPs and haplotypes found to have significant effects on the outcome of experimental infection in European American using the $X^2$ tests for trends. The data shows the % of volunteers who carried a particular SNP or haplotype in the four outcome groups. Analyses are shown for A, TLR9 +2848 *G genotype; B, TLR9 +2848 GG genotype; C, TLR9 haplotype TA; and D, IL-10 haplotype AGC. The data in panels A, B and D show protective effects against pustule formation, while the data in panel C shows a risk enhancing effect.
Figure 3: Bar plot and trend line for the *IL10* AAC haplotype, which had a significant risk enhancing effect on the outcome of experimental infection in African Americans using the $X^2$ tests for trends. The data shows the % of volunteers who carried this haplotype in the four outcome groups.

**Carrier trait analyses**

We assessed the synergy in protective or risk enhancing associations between combined SNPs or haplotypes and the outcome of infection by $X^2$ tests for trends. Two combinations of variables showed a significant association with the severity of *H. ducreyi* infection. In the EA cohort, only the *TLR9* +2848 *G* genotype combined with the *IL10* AGC haplotype had an increased significance of a protective effect compared to any of the single SNPs ($P = 0.012$). In the AA cohort, the *IL10* -2849 *G* genotype combined with the *SIGIRR* TTG haplotype had an increased significance of a protective effect compared to single SNPs or haplotypes ($P = 0.02$).
Logistic regression

We used forward stepwise binary logistic regression with dichotomized groups of the formed pustules as the dependent variable to produce models for each cohort. Only variables with $P < 0.2$ in the $X^2$ tests for trend were included in the models. In the EA cohort, the model included SNPs at TLR2 -16934, TLR9 +2848, and SIGIRR +935, the TLR9 haplotype TG and the IL10 haplotypes AGC and GGC. In the AA cohort, the model included SNPs at IL10 -819 and IL10 -2849, the SIGIRR haplotype TTG, and the IL10 haplotype AAC. The major results are shown in Table 3. In the EA cohort, there was a trend towards a protective association with the TLR9 +2848 GG genotype ($P = 0.052$); in the AA cohort, the IL10 -2849 AA genotype showed a significant protective association ($P = 0.032$) and the IL10 AAC haplotype a significant risk enhancing association ($P = 0.024$). In general, these results were consistent with the trends analysis shown in Figure 2 and Figure 3. No significant results were found for the other analysed SNPs and haplotypes.

Table 3: Results of logistic regression on probable association models. Abbreviations: EA, European American; AA, African American; OR, odds ratio; CI, confidence interval.

<table>
<thead>
<tr>
<th>Genotype / Haplotype</th>
<th>Outcome</th>
<th>Cohort</th>
<th>$P$ value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR9 +2848 GG</td>
<td>1,2,3 vs. 0</td>
<td>EA</td>
<td>0.052</td>
<td>0.42</td>
<td>0.17-1.01</td>
</tr>
<tr>
<td>IL10 -2849 AA</td>
<td>1,2,3 vs. 0</td>
<td>AA</td>
<td>0.032</td>
<td>0.18</td>
<td>0.04-0.86</td>
</tr>
<tr>
<td>IL10 AAC Haplotype</td>
<td>1,2,3 vs. 0</td>
<td>AA</td>
<td>0.024</td>
<td>3.08</td>
<td>1.16-8.13</td>
</tr>
</tbody>
</table>

* Group dichotomization by outcome (0, 1, 2 or 3 pustules).

Calculated CpG index

Since TLR9 is activated by CpG motifs in bacterial DNA, we calculated a CpG index for 35000HP DNA and compared it to results previously described for several other bacterial pathogens (28). While a CpG index < 1 is considered immunoinhibitory, a CpG index >1 is regarded as immunostimulatory. The calculated CpG index for H. ducreyi was 6.6, which was similar to the indices calculated for S. pneumoniae and H. influenzae (Table 4).
Here we sought to find contributions of host immunogenetic factors on the outcome of experimental *H. ducreyi* infection. Because the Hardy-Weinberg equilibrium showed differences in the genotypes of the AA and EA, these cohorts were analysed separately. Our cohorts were unique in that the participants had clearly distinguishable phenotypes and could be placed into defined groups (0, 1, 2 or 3 pustules), which allowed us to do a trend analysis. The fact that all our participants were infected with *H. ducreyi* likely permitted us to find significant genetic associations with disease outcomes despite our small sample size.

Table 4: Calculated CpG indices.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Size (Mb)</th>
<th>G+C (%)</th>
<th>CpG/ kb&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total CpG&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Stimulatory&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Inhibitory&lt;sup&gt;f&lt;/sup&gt;</th>
<th>CpG index&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemophilus ducreyi</em></td>
<td>1.7</td>
<td>38.2</td>
<td>40.9</td>
<td>112.2</td>
<td>124.8</td>
<td>110.4</td>
<td>6.6</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em>&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.91</td>
<td>38.2</td>
<td>72.8</td>
<td>109.1</td>
<td>105.5</td>
<td>96.4</td>
<td>7.2</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em>&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.22</td>
<td>39.5</td>
<td>78.0</td>
<td>69.5</td>
<td>82.4</td>
<td>66.5</td>
<td>8.6</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em>&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.27</td>
<td>51.5</td>
<td>132.7</td>
<td>130.6</td>
<td>78.4</td>
<td>140.0</td>
<td>-106.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Deviations in specified motif occurrences compared to those expected based on genomic G+C content.

<sup>b</sup>Consensus stimulatory and inhibitory CpG hexamer motifs

<sup>c</sup>Number of CpG hexamer motifs (NNCGNN) in each genome normalized to 100 kb of DNA

<sup>d</sup>Total frequency of CpG hexamer motifs (NNCGNN)

<sup>e</sup>Frequency of stimulatory CpG hexamers (RRCGYY)

<sup>f</sup>Frequency of inhibitory CpG hexamers (NCCGNN and NNCGRN)

<sup>g</sup>Index calculated from the difference between stimulatory and inhibitory hexamers, multiplied by the total CpG hexamers, normalized to 1 kb.

<sup>h</sup>Data are taken from Table 3 of reference (28).

In the EA cohort, we found that the tendency to resolve experimental infection was associated with the TLR9 +2848 *G* and GG genotypes, but the TA haplotype of this gene showed a risk enhancing effect for pustule formation. In contrast, Sanders *et al.* showed a protective association for TLR9 +2848 GA or AA alleles in control children vs. those with bacterial meningitis in the Netherlands; the protective effect is against *N. meningitidis* but not against *S. pneumoniae* or *H. influenzae* (28). The TLR9 +2848 AA genotype is also associated with
a decreased incidence of positive blood cultures in children who have meningococcal meningitis, again suggesting that some degree of protection against *Neisseria meningitidis* is conferred by this genotype (30).

One explanation of the different effects of these *TLR9* alleles on susceptibility to bacterial infection could be that the activation of TLR9 is triggered by binding of unmethylated bacterial CpG DNA motifs, which lead to the production of inflammatory cytokines (31). The amount and structure of CpG motifs in bacterial DNA affect its ability to activate TLR9; calculated CpG indices > 1 are pro-inflammatory while those < 1 are anti-inflammatory (28, 29). The calculated CpG index for *N. meningitidis* is very low (-106.8) relative to *S. pneumoniae* (8.6) and *H. influenzae* (7.2). This data led to the hypothesis that the *TLR9* +2848 GA or AA alleles might compensate for the anti-inflammatory potential of meningococcal DNA and protect the host against disease (28). The CpG index of *H. ducreyi* 35000HP DNA calculated by the same methodology (28, 29) was 6.6. Since pustule formation is marked by hyperinflammatory responses in tissue and dendritic cells (22), perhaps the *TLR9* +2848 *G and GG alleles counter hyperinflammatory responses to *H. ducreyi* that lead to tissue damage. Similarly, in Ghanaian children with malaria, the *TLR9* +2848 GG genotype is not associated with protection against parasitemia (i.e., infection) but is associated with protection from symptomatic disease (i.e., inflammation) (32). In our cohort, the contrasting result found for the *TLR9* TA haplotype may be due to the fact that this haplotype lacks the protective *TLR9* +2848 *G and GG genotypes. Since *TLR9* +2848 G is a synonymous coding SNP, how this SNP affects *TLR9* expression and subsequent activity is unclear.

Variation in *IL10* polymorphisms and IL-10 production are linked to various immunosuppressive or inflammatory conditions. In our study, we found the *IL10* -2849 AA genotype in the AA cohort had a statistically significant protective effect against pustule formation. Two studies reported an association between *IL10* -2849 AA and low IL-10 production by endotoxin-stimulated whole blood (33, 34). The finding that *IL10* -2849 AA is associated with resolution is consistent with our previous report showing that DC cells derived from resolvers have less IL-10 transcription and secretion than pustule formers in response to H. *ducreyi* (22). IL-10 is an anti-inflammatory cytokine that inhibits the activation and function of T cells, NK cells and macrophages (35). Production of high levels of IL-10 by DC during *H. ducreyi* infection could promote Th2 as well as regulatory T cell responses and inhibit the activation of Th1 cells and macrophages, leading to impaired clearance of *H. ducreyi* (22).
CHAPTER 3

The *IL10* AGC haplotype had a protective effect on *H. ducreyi* infection in the EA cohort, while the *IL10* AAC haplotype showed a risk enhancing effect in the AA cohort. Several studies suggest that protection against infection is linked to low IL-10 producing haplotypes while risk enhancement is linked to high IL-10 producing haplotypes (36, 37). The AAC haplotype has been shown and the ACG haplotype is assumed to be low IL-10 producers due to the inclusion of the genotype *IL10* -2849 A (36). If this is the case, one would expect both haplotypes to be protective against *H. ducreyi*. However, the levels of IL-10 expression could be influenced by *IL10* -1082 genotypes; PBMC from European cohorts with the *IL10* -1082 GG genotype secrete more IL-10 than those with the *IL10* -1082AA genotype in response to *C. trachomatis* (38). Similarly, *Helicobacter pylori* infected patients with the *IL10* -1082 GG genotype express more IL-10 in mucosal biopsies than those with the AA genotype (37). Additionally, the general genetic background of the EA and AA cohorts might affect IL-10 expression. As no plasma or peripheral blood samples were available from the *H. ducreyi*-infected cohorts, we were unable to correlate their IL-10 secretion capacity with the two IL-10 haplotypes.

In the EA cohort, the *TLR9* +2848 *G* genotype combined with the *IL10* AGC haplotype had an increased significance of a protective effect compared to the single SNPs, which may be due to the potential anti-inflammatory effects of both SNPs discussed above. In the AA cohort, the *IL10* -2849 *G* genotype combined with the *SIGIRR* TTG haplotype also had an increased significance of a protective effect. The *IL-10* -2849 *G* genotype is associated with high production of IL-10 (33, 34). *SIGIRR* is a negative regulator of the TLR pathways and SIGIRR deficiency in mice leads to hyperinflammatory response and tissue damage in microbial infections (39). Currently, there are no other reports on associations of the *SIGIRR* TTG haplotype with any inflammatory conditions. The *SIGIRR* -146TT genotype, which is contained in the TTG haplotype, is significantly associated with the susceptibility to systemic lupus erythematosus (40). Perhaps hyperinflammatory responses potentially conferred by the *SIGIRR* TTG haplotype are offset by potentially higher levels of IL-10 induced by the *IL-10* -2849 *G* genotype, leading to a balanced inflammatory response against *H. ducreyi* and effective clearance of the pathogen.

In the human challenge model, there are no effects of race or age on pustule formation, but men form pustules at rates approximately 1.7 fold higher than women, consistent with the high male to female ratio seen in natural chancroid (1). Men and women were included in this study. Analysis for potential gender influences on the results using Mantel-Haenszel
tests in conjunction with Tarone’s tests showed no significant differences between results related to gender.

Since differences in innate immune responses are associated with the outcome of *H. ducreyi* infection, we chose to include genes only from innate immune pathways in this study. One effect of this targeted approach was a reduced need for corrections for multiple comparisons. In addition, the statistical tests utilized in this study provide a clear picture through both univariate and multivariate testing, while the logistic regression model already accounts for multiple comparisons in its design.

Although we found associations between *TLR9* and *IL-10* SNPs with outcome, no significant links were found for other SNPs in several other genes encoding PRRs. Compared to most immunogenetic studies, which usually compare large groups of infected patients to healthy controls, our cohorts were small; it is possible that the lack of finding other associations was due to our small sample size.

In summary, this is the first study to shed light on the immunogenetic factors affecting the outcome of *H. ducreyi* infection. Our results could be used to predict the risk of susceptibility to *H. ducreyi* infection in future studies. Studies on the effects of the *TLR9, IL10, and SIGIRR* SNPs on immune responses to *H. ducreyi* are also needed to gain better understanding of differential host susceptibility to the pathogen.

**ACKNOWLEDGMENTS**

We thank the volunteers who participated in the study and Diane Janowicz, Kate Fortney, Sheila Ellinger, and Beth Zwickl who were involved in the infection experiments. We also thank Barry Katz and Susan Ofner for their help in designing the cohorts, Jolein Pleijster and James Williams for technical support, Tatiana Foroud for her advice, and Byron Batteiger and Margaret Bauer for their thoughtful criticism of the manuscript. This work was supported by the National Institutes of Health U19 AI31494, AI27863S1, and AI059384 to S.M.S. The human challenge trials were also supported by the Indiana Clinical and Translational Sciences Institute and the Indiana Clinical Research Center [UL RR052761].
REFERENCES


“We cannot fathom the marvelous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm—a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven.”

Charles Darwin
CHAPTER 4

The two-sided role of the vaginal microbiome in Chlamydia trachomatis and Mycoplasma genitalium pathogenesis

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ABSTRACT

Sexually transmitted infections (STI) can have major consequences for the reproductive health of women. *Mycoplasma genitalium* is a STI that is not as well studied but causes pelvic inflammatory disease (PID) among other complications. Another well-known STI is *Chlamydia trachomatis*, notorious for its capability to cause infertility. Both *C. trachomatis* and *M. genitalium* share some of the same clinical aspects. Parts of the pathogenesis of *C. trachomatis* and *M. genitalium* infections are unclear but potential factors are the microbiome and other STIs. The healthy vaginal microbiome is dominated by *Lactobacillus* spp; these bacteria protect the host against invading bacteria like *C. trachomatis* and *M. genitalium* by producing antibacterial compounds and providing a mechanical barrier. A dysbiosis of the vaginal microbiome is characterized by a non-*Lactobacillus* spp dominated microbiome, also known as bacterial vaginosis (BV). BV and BV associated bacteria play a role in the pathogenesis of STIs such as *C. trachomatis* and *M. genitalium*. The different species of BV associated bacteria have distinct characteristics that could play a role in *C. trachomatis* and *M. genitalium* infections. Host factors should also be considered when analysing the interaction of *C. trachomatis* and *M. genitalium* and the microbiome. One important factor is the hormonal homeostasis. Oral hormonal contraception influences the vaginal milieu and could influence the infection process of STIs. Overall, this review attempts to give an overview of the pathogenesis of *C. trachomatis* and *M. genitalium* infections and the relationship between *M. genitalium*, *C. trachomatis*, and the vaginal microbiome.
INTRODUCTION

Sexually transmitted infections such as *Mycoplasma genitalium* and *Chlamydia trachomatis* are infections that are transmitted through sexual contact. When these bacteria try to invade the genital tract it will encounter a microbiome, which in women is the vaginal microbiome. The vaginal microbiome can be seen as a complementary part to our immune system that consists of bacteria. Humans and their microbiome live in a mutualistic relationship: the bacteria profit of the resources the host provides and in turn protect the host against invading pathogens(1). The typical healthy vaginal microbiome is dominated by *Lactobacillus spp.* (1-7). Different studies show that certain strains of the *Lactobacillus spp.* protect the host better against the colonisation of exogenous microorganisms than other strains *Lactobacillus* (1, 3, 8, 9). The *Lactobacilli* defend the host by producing a wide variety of antibacterial produces, such as creation of an acidic environment by producing lactic acid(1, 3). These characteristics play a role in the barrier function of the microbiome.

The microbiome has a close interaction with the host. Not only is the host immune system essential for fighting off external threats, the barrier function of the microbiome is also essential. The first barrier in the host immune system is the epithelial barrier. This barrier is not only in constant contact with the microbiome but also under influence of the host hormonal cycle(10). The female sex hormones estradiol and progesterone influence the immune system indirectly through the epithelial layer. For example, the female sex hormones stimulate the immune system by triggering the epithelial cells to produce cytokines such as IL-8(10). This also applies to the production of antibacterial products and even the transport of immunoglobulins into the lumen(10). In short, the epithelial layer and the hormonal cycle strongly contribute to the host immune response, tying them to the susceptibility to diseases.

Susceptibility to various vaginal pathogens that invade the vagina forms a potential health risk. An important risk factor for infection by pathogens is the weakened barrier function of the vaginal microbiome. An example of this weakened barrier is when there is a dysbiosis as is the case with bacterial vaginosis (BV)(8). In BV the composition of the vaginal microbiome shifts from the dominant *Lactobacillus spp.* to a more diverse microbiome. Characteristic for BV is the rise in pH resulting in a more neutral pH environment(4). Studies showed that women that have BV are more prone to STIs such as *C. trachomatis* and *M. genitalium* infections(11-13). Pathogens like *C. trachomatis* and *M. genitalium* may utilise the failing defences
to invade(3). There are various factors that play a role in the development of BV. Douching (extensive washing and rinsing of the vagina) for example has a big impact on the microbiome and could lead to BV in women with an already altered flora(14). Whether this impact is also seen in women with a normal Lactobacillus spp. dominated microbiome is currently unclear. Continuous condom use on the other hand protects against BV and other invading pathogens such as Mycoplasma species, however no lower incidence was seen in urogenital C. trachomatis infections(15). Overall this suggest that there may be an overlap in prevention of STIs and prevention of BV development.

C. trachomatis is a STI notorious for late complications like infertility(16). Other clinical consequences of C. trachomatis infections are PID and cervicitis(17). C. trachomatis has a characteristic infection process that consists of an infective form and a non-infective form, the latter of which is obligatory intracellular. The infectious form is known as elementary body (EB) that binds to the host cell and invades the cell(18). The EB resides in an inclusion in the cell, wherein the EB further differentiates. These inclusions are membrane bound vacuoles that help the bacteria to escape phago-lysosomal fusion. The EB differentiate into reticulate bodies(RB) that multiply various times, after which the RB can differentiate back to EBs. These EBs are released through lysis of the host cell and the cycle starts again(1, 8, 19). Important to note is that C. trachomatis is dependent on tryptophan for its growth. However, C. trachomatis is unable to synthesise tryptophan and utilises the environment for its tryptophan metabolism. If C. trachomatis resides in an environment that is tryptophan poor, it will differentiate into a persistent aberrant form which is not infectious(19).

A lesser known STI is M. genitalium, which poses a serious health risk because of clinical consequences like PID, cervicitis, and possible infertility(4, 6, 16). M. genitalium can also cause pregnancy complications such as preterm delivery and premature rupture of membranes(6, 20). Characteristic of M. genitalium is that it is a facultative anaerobe bacterium and lacks a cell wall. Interestingly, some studies associate M. genitalium with healthy individuals while other studies associate M. genitalium with BV or preterm delivery (2, 21). This leads to the hypothesis that the pathogenesis of Mycoplasma spp. is dependent on co-infection and/or the composition of the microbiome. Other species of Mycoplasma have been studied more extensively, certain characteristics also apply to the pathogenesis of M. genitalium(22). Because of the limited information there is on M. genitalium, we will use data that is available from other species Mycoplasma in this review.
Recently, Tamerelle et al. (2018) published a meta-analysis investigating the association between the vaginal microbiota and a number of STIs among which C. trachomatis and M. genitalium (23). This study concluded that for M. genitalium not enough studies were published that could be used for a meta-analyses. The aim of this study is to reveal the gaps of knowledge to provide guidance to future researchers with an interest in this topic. Interestingly, Tamerelle et al. (2018) showed that there is an association between vaginal microbiota with low Lactobacillus spp. and susceptibility for C. trachomatis infection (23). In the current study we outline the possible pathogenesis for this phenomena.

Few studies have addressed the exact relationship between C. trachomatis and M. genitalium. A London study found an unexpectedly high co-infection rate of C. trachomatis and M. genitalium of 0.5% under participants of a screening program and STI clinic patients (24). As infection rates expected through the even division of M. genitalium infections in all patient groups would be roughly 0.16%, this may suggest that these pathogens may occur more frequently in co-infections. Other studies have investigated co-infection in high risk groups that resulted in a rate of up to 39% (24-29). A possible explanation for the differences in prevalence is the composition of the microbiome, which could influence the susceptibility for STIs.

The aim of this paper is to evaluate two STIs i.e. C. trachomatis and M. genitalium and the interaction with the microbiome of the vagina based on available literature. The focus will be on the female genital tract because of the late complications these particular infections can have on women.

**MATERIALS AND METHODS**

The search terms used for this review are summarized in Table 1. The search was conducted in Web of Science, Cochrane Library, PubMed/MEDLINE, and Embase in January 2018. The results were limited to publications in English only; no publication date limitations were applied.

The articles were screened based on their relevance for answering the research question at hand. This means that the articles contained information about Chlamydia trachomatis and/or Mycoplasma genitalium and the interactions between these bacteria and the microbiome of the female reproductive tract. Additionally, any article found to be relevant by the authors was added.
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Table 1: Search terms used for this review.

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<th>Search term</th>
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</thead>
<tbody>
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<td>Chlamydia</td>
</tr>
<tr>
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</tr>
<tr>
<td>Microbiome</td>
</tr>
<tr>
<td>Microflora</td>
</tr>
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<td>Microbiota</td>
</tr>
<tr>
<td>Mycoplasma</td>
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<tr>
<td>Vaginal microbiome</td>
</tr>
</tbody>
</table>

The inclusion and exclusion criteria used are stated in Table 2. The articles did not have to meet all the inclusion criteria to be included. Adult women and the vaginal microbiome are required for inclusion. Furthermore, the articles were manually curated to fall in the scope of this paper.

Not much is known about the pathogenesis of *M. genitalium*. To broaden the search results *in vivo* and/or *in vitro* studies concerning *M. genitalium* were included. To further widen the perspective of Mycoplasma pathogenesis, other Mycoplasma spp. were included such as *Mycoplasma hominis* and *Candidatus Mycoplasma giererdii*. In contrast, *C. trachomatis* is a well-known and extensively studied bacterium. Therefore, to narrow the results down and concentrate on the microbiome aspect we excluded the *in vitro* studies related to *C. trachomatis* unless no similar *in vivo* study was available.

Table 2: Inclusion and exclusion criteria used for the inclusion and exclusions of articles.

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult woman (of reproductive age)</td>
<td>Animal studies</td>
</tr>
<tr>
<td><em>In vitro M. genitalium</em></td>
<td>Assessments of diagnostic methods</td>
</tr>
<tr>
<td><em>In vivo C. trachomatis</em></td>
<td>Genetic test analyses</td>
</tr>
<tr>
<td><em>In vivo M. genitalium</em></td>
<td><em>In vitro C. trachomatis</em></td>
</tr>
<tr>
<td>Vaginal microbiome</td>
<td>Reviews</td>
</tr>
</tbody>
</table>
RESULTS

The vaginal microbiome composition and its differences

Every woman is unique and so is every vaginal microbiome. There are a number of factors that cause differences between vaginal microbiomes. There are big differences between microbiomes in women of different ethnicities(30). One example is the difference between African and European women(31). Healthy African women are more likely to have a \textit{L. iners} dominated microbiome and healthy European women are more likely to have a \textit{L. crispatus} dominated microbiome(31, 32). This discrepancy could be attributed to the many factors that influence the vaginal microbiome. For example hygiene practises such as douching(33). Whether menses influences the microbiome composition is still unclear. There have been a number of studies that investigated the vaginal microbiome composition during menses, but the results contradict each other. One states that the microbiome stays stable(34) while the other reports small fluctuations(35). Lastly, Gajer \textit{et al.}(2012) states that menses does influence the microbiome in some instances and the author suggest that this may be due to genomic heterogeneity in the dominating \textit{Lactobacillus} \textit{spp.}(36). Antibiotics are also known to influence the vaginal microbiome. When used, the microbiome shifts to a microbiome not dominated with \textit{Lactobacilli}(34). Finally, it is clear that pregnancy impacts the microbiome(37). The microbiome does not undergo major composition changes during pregnancy compared to non-pregnant women. The microbiome of pregnant women is more stable and more often \textit{Lactobacillus} \textit{spp.} dominant than that of non-pregnant women(38).

In general it seems that the microbiome is not static, but rather a dynamic system that is under the influence of menstrual cycle, antibiotics, ethnicity, pregnancy, and potentially many more factors.

The microbiome can fluctuate between different healthy bacterial compositions but it can also get out of balance and form a more permanent dysbiosis such as BV. BV is characterised by a diverse non-\textit{Lactobacillus} dominated microbiome that is colonised by specific types of bacteria such as \textit{G. vaginalis}(4). There are noticeable differences in vaginal microbiome composition between women with BV. \textit{M. hominis} was associated with BV in European women in general(31), Belgian(2), and Greenlandish(39) women, while there was no clear correlation found in African women(31). \textit{L. iners} shows a greater presence than \textit{L. crispatus} in BV but neither were dominant. This was also seen in African sex workers(9).
Interestingly, *M. hominis* is one of the bacteria that are associated with BV but that also can occur in seemingly healthy women(31, 35). This shows that bacteria that are BV associated can be present in healthy women as well. BV is not static, but rather a dynamic process.

There is evidence that the microbiome can fluctuate between composition and in some instances it can fluctuate between BV and healthy(40). Taken together, these findings emphasize that there is a wide range of possible healthy compositions of the vaginal microbiome, because bacteria such as *M. hominis* could be both BV associated in some women and associated with a healthy microbiome in others. Most important is that the dominance of *Lactobacillus spp.* is vital for the vaginal health.

### The role of Lactobacillus in Mycoplasma genitalium and Chlamydia trachomatis pathogeneses

A homogeneous *Lactobacillus spp.* dominated vaginal microbiome is associated with healthy women(30). A distinct characteristic for *Lactobacillus spp.* is the production of H$_2$O$_2$ and lactic acid that are antimicrobial and inhibit invading bacteria(41). Lactic acid has been shown to be a competent inhibitor of *C. trachomatis*(3), while H$_2$O$_2$ inhibits BV associated bacteria such as *M. genitalium*(42). Some strains of *Lactobacillus spp.* are more capable of inhibiting pathogens due to the capacity of producing antibacterial components(43).

Multiple studies have been conducted to explore why *L. crispatus* is associated with the healthy microbiome, with the ability to produce high concentrations of lactic acid as most notable outcome. Due to the increased lactic acid production the pH of the vaginal tract is lowered, this lactic acid rich environment inhibits the spread of *C. trachomatis*(1, 3). Gong et al.(2014) suggested three possible mechanisms that are responsible for the effect lactic acid has on Chlamydia EBs: destruction of the surface molecule(s), destruction of the membrane, and disruption of the internal metabolism(3). Further research is needed to determine what the exact mechanisms are. Not all *Lactobacillus spp.* are competent in warding off potential invading bacteria, for example *L. iners* is frequently seen with BV associated bacteria(2, 13, 44). It has also been demonstrated that a vaginal microbiome dominated by *L. iners* does not protect against *C. trachomatis* infection(45). An explanation for this phenomenon is that *L. iners* produces less H$_2$O$_2$ and is associated with lower lactic acid production compared to other *Lactobacilli spp.*(46, 47). Genomic based identification showed that 9% of the *L. iners* were capable of H$_2$O$_2$ production compared to *L. crispatus* where 95% were capable of H$_2$O$_2$ production(46). These data show that a low pH, lactic acid, and high concentration
of H₂O₂ in the vaginal tract are important as protection against invading bacteria such as *C. trachomatis* and *M. genitalium*.

The epithelial layer of the vagina is an important factor in the interplay between *Lactobacilli* and invading bacteria, as it is in close contact with the microbiome. The glycogen produced by epithelial cells can be an energy source for *Lactobacillus* spp. (48). The epithelial layer also has a barrier function, and immune cells interact with the vaginal microbiota. The vaginal immune system does not attack *Lactobacillus* spp. but inhibits potential harmful bacteria (49). Furthermore, the available free glycogen that is produced by the epithelial layer plays a role in the abundance of *Lactobacillus* spp. especially *L. jensenii* and *L. crispatus* (50). The glycogen is released by the shedding of the epithelial cells and is then utilized by the *Lactobacillus* spp. (50). An important factor that influences the glycogen production is estrogen (41, 51). Estrogen stimulates the production of glycogen in the epithelial cells. Overall, glycogen production is beneficial for the host because more lactic acid producing *Lactobacillus* spp. means a lower pH which protects against invading bacteria. This organization of naturally occurring system is one example of how *Lactobacilli* live in symbiosis with the host and helps protect against invading pathogens such as *C. trachomatis* and *M. genitalium*.

A factor that can disturb the relationship between the host and *Lactobacilli* is the use of hormonal contraceptives like the estradiol-progestin combined oral contraceptive pill (COCP) (52). The use of the COCP may have a beneficial effect on the microbiome by stimulating the *Lactobacillus* spp. because of the higher glycogen production. This is demonstrated in women who use COCP. They are found to be colonised more often with *L. crispatus* and *L. jensenii*, which are more beneficial to the host than most other *Lactobacilli* (52). This suggest that there is a benefit for women to take hormonal anticonception. There is a conflicting report that shows a higher *C. trachomatis* prevalence in risk groups that used contraception (53). However, this concerns a high risk group and the association could be caused by the risk behaviour of these women. In general it appears that the hormonal contraception is beneficial for women by promoting the beneficial strains of *Lactobacillus*.

**Immunological reaction to Chlamydia trachomatis and the interaction with the microbiome**

By bypassing the barriers such as those established by *Lactobacillus* spp. *C. trachomatis* can infect the human cells. Upon recognition of a *C. trachomatis* infection the immune system produces more IL-12, among other inflammatory cytokines (54). IL-12 is an inflammatory
cytokine that stimulates the production of interferon-γ (IFN-γ). This is produced by macrophages, stimulates inflammation, and induces indoleamine-2,3-dioxygenase 1 (IDO1). IDO1 inhibits *C. trachomatis* by limiting the available tryptophan that is necessary for *C. trachomatis* growth. Another source of tryptophan is the BV associated bacterium *Prevotella* spp. It has been shown that tryptophan produced by *Prevotella* can be used by *Chlamydia* for survival when other sources of tryptophan are low or depleted. Prevention of large quantities of *Prevotella* aiding *C. trachomatis* is another positive aspect of healthy *Lactobacilli* dominated microbiota.

The relation between BV and *C. trachomatis* infection has been the subject of a number of studies. Dutch patients with *C. trachomatis* infection more often have a diverse vaginal microbiome, composed of species other than *Lactobacillus* (44). Some reports do show a higher incidence of *C. trachomatis* infection during BV (12), while another study shows no increased *C. trachomatis* incidence (55). The most notable difference between these studies is the study population. The study that showed lower *C. trachomatis* incidences during BV consisted of African women and the study that showed a higher *C. trachomatis* incidences during BV consisted of a mix of African American women and white American women. This may suggest that ethnicity may play a role in the infection process. This suggest that ethnicity may play a role in the infection process. As has been discussed previously, ethnicity plays a major role in vaginal microbiome composition. Healthy microbiomes but also BV compositions can have different composition for African women compared to European women (31) Not only BV-associated bacteria play a role in the susceptibility for *C. trachomatis* infection. *L. iners* dominated microbiomes increase the risk for *C. trachomatis* in infection as well (45). *L. iners* produces significantly less lactic acid which is an important inhibitor of *C. trachomatis* (3, 47). Overall, this suggests that the composition of the microbiome plays an important role in the infection process of *C. trachomatis*.

An important factor to consider in the interaction between host and microbiota is the polymorphisms in immune genes, as these may influence the immune response. For example, polymorphisms in the TNF-α gene (*TNFA*-208G>A) can influence the quantity of expression and thus the inflammatory response of the host. This is mainly seen in a disturbed microbiome, Nugent score >7, and could cause preterm birth (56). This is relevant for *C. trachomatis* infection because TNF-α plays a role in the innate immune response to *C. trachomatis* infections (57). The exact role of BV in *C. trachomatis* infection and its interaction
with the immune system is still unclear but it is interesting to further investigate how this influences the inflammatory reaction(56).

As explained earlier the relationship between the sex hormone estrogen and the vaginal microbiome is beneficial for the production of glycogen and thus beneficial for *Lactobacilli*. Also mentioned was the influence oral contraception could have on the microbiome. As these hormones influence the *Lactobacillus spp*. so does it also influence other bacteria. When looking at COCP use during a *C. trachomatis* infection there is a significant increase in inflammatory cytokines(55). It is clear that the hormones in COCP influence the microbiome and the inflammatory cytokines. Fichorova *et al.* (2015) suggest that this could be due to the differential regulation of inflammatory of the different combinations of hormones. Another explanation could be that the hormones that are used in the hormonal contraception are synthetic and not the natural occurring hormones and therefore could trigger a reaction.

**Immunological reaction to Mycoplasma genitalium and the interaction with the microbiome**

*M. genitalium* is, like *C. trachomatis*, an intracellular bacterium. Important for the survival of *M. genitalium* is the ability to adhere to the host cell and to invade the cell. *M. genitalium* uses MgPa adhesins for this process(58, 59). Before invading the host cell the *Mycoplasma spp.* need to survive in the H₂O₂ and lactic acid rich environment created by the *Lactobacillus spp.* that functions as a line of defence. It is evident that in a *Lactobacillus spp.* rich environment there is less *Mycoplasma spp.* infection such as *M. hominis*(60). Especially the H₂O₂ producing *Lactobacillus spp.* such as *L. jensenii* have this effect(61). Not much research has been conducted on how *Lactobacillus spp.* inhibits *Mycoplasma spp.* growth. Further research is needed to determine whether H₂O₂ or low pH by lactic acid is responsible for the effect against *Mycoplasma spp.*

The relationship between the vaginal composition and the pathogenesis of *Mycoplasma spp.* is double sided. *Mycoplasma spp.* does not only occur as a BV-associated bacterium but can also be part of a diverse microbiome(62). *Mycoplasma spp.* can be found in some healthy African women with a diverse microbiome, while there is a strong association with BV in European women(31, 60). *M. genitalium* is more frequently seen in patients with recent BV(63). The relationship between BV and *M. genitalium* can be explained by the fact that in BV there are lower quantities of lactic acid and thus higher pH which is beneficial for
the survival of this bacteria. This is hypothetical and future studies have to investigate the relationship between BV and *M. genitalium*.

The immune system does not only play a part in the fight against infections, it will also limit the inflammatory reaction to reduce potential damage. An example is the 70-kDa heat shock protein (hsp70). This is an antagonist for the inflammatory IL-1. Hsp70 acts in stressful situations to inhibit the damage to the host cells. Hsp70 expression is increased during BV when *Mycoplasma spp.* is present(64). This is in response to an increased inflammatory reaction of the host during BV with *Mycoplasma spp.* This inflammatory reaction can further unbalance the vaginal microbiota and thus cause the host to be more susceptible to invading pathogens. We suggest that the reaction of the immune system to BV with *Mycoplasma spp.* may make the host susceptible for infections with *M. genitalium*.

Besides it’s relation to dysbiosis, there is also a relationship between *Mycoplasma spp.* and other pathogens. One of these relationships is that with *Trichomonas vaginalis*(65). *T. vaginalis* is a protozoan parasite that causes an STI, called Trichomoniasis(16). Studies have shown a significant association between *M. genitalium* and *T. vaginalis*(66, 67). Other *Mycoplasma spp.* such as *Ca. M. giererdii* is also significant associated(65). *Ca. M. giererdii* is a newly found *Mycoplasma* species that is associated with higher pH and is more prevalent in African-American women than European women with a strong correlation with *T. vaginalis*(31). The exact nature of the relationship between *Mycoplasma spp.* with *T. vaginalis* is yet unclear. It is likely that *Mycoplasma spp.* and *T. vaginalis* have an overlap in favourable environment, especially microbiome compositions such as seen in BV. This could also mean that these pathogens do not interact with each other and only exist in the same environment. However, there is still much unclear about if *T. vaginalis* and *M. genitalium* interact with each other.

*M. genitalium* on its own has not been extensively researched. However, it is clear that there is a link between this pathogen and the vaginal microbiome. Especially in a microbiome with low amounts of *Lactobacillus spp.* and high amounts of BV associated bacteria. Not only BV associated bacteria are associated with *M. genitalium* but also *T. vaginalis*. However, no interactions between *M. genitalium* and *T. vaginalis* have been shown to occur. There may be other bacteria that reside in the vaginal microbiome that are associated with *M. genitalium* and could play a role in its pathogenesis. Further research should clarify these interactions.
DISCUSSION AND CONCLUSION

There are many factors that play a role in the interaction between the vaginal microbiome, the host immune system, and the STIs C. trachomatis and M. genitalium. It is a complex relationship with many aspects still to be uncovered. This review attempted to shed a light on the relationship between the microbiome and these pathogens. We established that there is an interaction between lactic acid levels and C. trachomatis, and between H₂O₂ levels and M. genitalium. We also discussed the association between C. trachomatis and Prevotella, in which C. trachomatis benefits of the tryptophan producing Prevotella. The association between Mycoplasma spp. and T. vaginalis and therefore possibly with M. genitalium suggest that an overlap in microbiome may be favourable for the pathogenesis of T. vaginalis and Mycoplasma. Taking this into account, there could be other pathogens that occur in the microbiome that are associated with C. trachomatis and/or M. genitalium. This could also mean that there is overlap in microbiome compositions that are beneficial for C. trachomatis and M. genitalium, which would be one explanation for the previously described co-infections(24).

The vaginal microbiome is the first line of defence of the female genital tract and thus plays an important role in the infection process of bacteria. We found a common consensus that both C. trachomatis and M. genitalium thrive in a microbiome with less Lactobacillus spp. Especially less H₂O₂ and/or lactic acid producing Lactobacillus spp. C. trachomatis is also shown to more frequently infect hosts with a L. iners dominated microbiome. Due to other similarities this may also be the case for M. genitalium. Hormones have an effect on the microbiome. As has been discussed, estradiol stimulate the Lactobacillus spp, which is beneficial for the host. However, contraception use with synthetic estradiol-progestin results in higher inflammation during C. trachomatis infection, suggesting that hormones influence the immune response and possibly impact symptoms and complications. A schematic depiction of the processes affecting C. trachomatis and M. genitalium can be seen in figure 1.

The immune response in reaction to C. trachomatis is largely mapped out, highlighting the role IFN-γ plays in the inhibition of C. trachomatis spread. However, the immune reaction in response to the M. genitalium infection is largely unknown. Hsp70 activity during BV is a clear sign the immune response to M. genitalium before there is an infection is notably different.
from *C. trachomatis* infection. Overlapping in the pathogenesis is BV, this may increase susceptibility for both pathogens possibly through a higher immune reaction.

![Diagram](image)

**Figure 1:** Depiction of the processes affecting *C. trachomatis* and *M. genitalium* in *Lactobacillus* dominated or diverse vaginal microbiota profiles.

The interaction between *C. trachomatis* and *M. genitalium* and the vaginal microbiome is not fully understood. Further studies that investigate the co-infection between *C. trachomatis* and *M. genitalium* may help us better understand how these pathogens cause infections and what role the vaginal microbiome plays in this infection process.

**DECLARATIONS**

**Conflicts of interests**
The authors declare no conflicts of interest
REFERENCES


“The journey is part of the experience – an expression of the seriousness of one's intent. One doesn't take the A train to Mecca.”

Anthony Bourdain
CHAPTER 5
The relation of the vaginal microbiota to early pregnancy development during In Vitro Fertilization treatment – A meta-analysis

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Submitted
ABSTRACT

Study question: What is the aggregated effect found by studies investigating the influence of the vaginal microbiota composition on early pregnancy rates after IVF treatment?

Summary answer: Women with an abnormal vaginal microbiota are roughly 1.4 times less likely to have a successful early pregnancy development after IVF treatment when compared to women with normal microbiota.

What is known already: An abnormal vaginal microbiota composition has been shown to lead to pre-term births, miscarriage, and problems with conceiving. Studies have suggested that dysbiosis reduces successful early pregnancy development during IVF. However, conflicting reports exist.

Study design, size, duration: A systematic review was performed using the Medline and EMBASE databases, using search terms for healthy vaginal microbiota, abnormal vaginal microbiota, fertility and pregnancy.

Participants/materials, setting, methods: The search resulted in six included articles. Of these, all six were used for further meta-analysis.

Main results and the role of chance: We found a correlation between abnormal vaginal microbiota and lower rates of early pregnancy development after IVF treatment (OR = 0.70, 95% CI = 0.49 - 0.99). One study showed the reverse correlation.

Limitations, reasons for caution: Heterogeneity between study methodologies in various forms was found. Heterogeneity in primary outcomes as well as IVF methodology characteristics and sampling times is likely to affect study outcomes.

Wider implications of the findings: We press for caution and ask researchers and readers of the literature to pay special attention to the possible confounding factors that can effect study outcomes, as they appear prevalent.

Study funding/competing interest(s): N/A.

Trial registration number: PROSPERO number: CRD42018093149
INTRODUCTION

Over the past decades, bacterial microbiota in humans has received increasing attention. Through technological advances in molecular typing it has become possible to more easily and accurately characterize an individual’s microbiota. This has led to links between host microbiotas and inflammatory diseases such as in Crohn’s disease, as well as links with susceptibility to infections (1-4). The vaginal commensal microbiota controls pH levels and provide a physical barrier to opportunistic pathogens. These characteristics have been shown to influence the vaginal environment and possibly influence conception and development of the child during pregnancy.

A healthy vaginal microbiota is currently defined by a composition dominated by one of the multiple anaerobic Lactobacilli (5). Through the production of lactic acid, Lactobacilli lower the pH level of the vaginal environment, which protects the vagina from invasion and infection by opportunistic pathogens. Many Lactobacilli also produce hydrogen peroxide ($\text{H}_2\text{O}_2$), bacteriocins, glycogen, and glycerol, which aid in the defense against pathogens and in return secure the dominant habituation of Lactobacilli (5-8). The most commonly found phylotypes of Lactobacillus spp. are L. crispatus, L. gasseri, L. iners, and L. jensenii (9-12). Another common phylotype is a diverse microbial profile, without dominance of Lactobacilli, and is often related to Bacterial Vaginosis (BV) (11). BV is the most common vaginal disorder in women and occurs in up to 20% of pregnant women (5). BV is a disruption of the ecological vaginal balance by overgrowth of a typically non-Lactobacillus anaerobic bacterium (13). This results in an alteration of the milieu and composition of the vaginal microbiota. Notable BV related bacteria are Gardnerella vaginalis, Mobiluncus spp., and Atopobium vaginae (14-17). Symptoms of BV include watery discharge with a fishy malodour. However, roughly 50% of women who have BV are asymptomatic or have less obvious symptoms (18). Clinically, BV is determined based on the widely accepted Nugent criteria, which take into account the presence and abundance of various vaginal bacteria (13).

Recent evidence corroborates that BV increases risk of preterm delivery and pregnancy loss (18-23). This risk is potentially twice as high when compared to women with a healthy vaginal microbiota (24). Persistency and the relative amount of BV related bacteria in the vagina increase the potential for negative pregnancy outcomes (24-27). Additionally, women without previous pregnancy are at higher risk of second trimester pregnancy loss when the vaginal...
CHAPTER 5

microbiota contains low amounts of *Lactobacillus* spp. or no *Lactobacillus* spp. at all (28). Although BV has received the most attention, abnormal vaginal microbiota is not always BV and other conditions have separate effects on pregnancy outcome (14). For instance, loss of *Lactobacillus* without BV-related bacterial growth is more strongly associated with preterm birth than BV (28). All of these studies indicate a significant role of the vaginal microbiota in the development of the child during pregnancy, but primarily focus on later stage negative outcomes of pregnancy, as these appear more clearly expressed.

During IVF transfer a transfer catheter is inserted through the vaginal cavity into the uterus. During this transfer it is not uncommon for the catheter tip to become contaminated with mucus originating in the vagina (29, 30). This mucus is commonly filled with large amounts of bacteria from the vaginal microbiota. We hypothesize that the effect that vaginal microbiota can have on uterine implantation and early development is more expressed during IVF due to its larger presence during transfer.

In this study we aim to systematically review and perform a meta-analysis on the effect of vaginal microbiota composition on the early pregnancy development rate of IVF treatments, defined as the absence of first trimester pregnancy. Through this we hope to shed light on the effect of abnormal vaginal microbiota on the earlier stages of pregnancy.

**METHODS**

Here we have systematically reviewed the effect of human vaginal microbiota on early IVF outcomes. This review was written in compliance with the PRISMA-statement for reporting systematic reviews.

**Information sources and search**

The Medline and EMBASE scientific databases were used to conduct the searches. All included scientific articles were written in English between 1980 and the 6th of April 2018. Articles were evaluated by two of the authors, and discrepant articles were judged by a third author.
The full search strategy and terms can be found in Table 1. We applied the search methodology described in the PRISMA-statement: disease or disorder, outcome measure, methodological terms, patient characteristics, and prognostic factors. Additional sources for articles included references from already included articles.

**Table 1: Search strategy.**

<table>
<thead>
<tr>
<th>Categories</th>
<th>[MeSH] term</th>
<th>Free PubMed terms</th>
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<tbody>
<tr>
<td>Disease</td>
<td>Infertility, Female</td>
<td>Infertility</td>
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<td></td>
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<td>Subfertility</td>
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<td>vaginal microflora</td>
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<tr>
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<td>Bacterial dysbiosis</td>
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<td></td>
<td></td>
<td>Bacterial vaginosis</td>
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</table>

**Study selection**

Articles were selected for further screening when at least one of the following terms was found in the title or abstract: vaginal microbiota composition, vaginal microbiota, bacterial vaginosis, or abnormal vaginal microbiota associated with IVF outcome. The primary outcome measures were the implantation and/or early pregnancy development rate. Inclusion and exclusion criteria can be found in Table 2. Nugent scores above 7 were considered indicative of abnormal microbiota.
Table 2: Inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
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<tbody>
<tr>
<td>Human participants</td>
<td>Diagnosis of abnormal microbiota without Nugent criteria or qPCR</td>
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<tr>
<td>Women of reproductive age</td>
<td>Reviews</td>
</tr>
<tr>
<td>Subfertility or infertility (otherwise healthy)</td>
<td></td>
</tr>
<tr>
<td>In-vitro fertilization</td>
<td>Ultrasound proven fetal heartbeat and/or hCG results before 10 weeks gestation</td>
</tr>
</tbody>
</table>

Statistical meta-analysis

Analyses of the overall effect of the vaginal microbiota on IVF outcome were carried out by integrating the quantitative findings in a random or fixed effect model.

With the aid of the statistical MedCalc Software (Ostend, Belgium), weights were assigned to the different studies for more insight of the pooled effect. When studies shared a common true effect the fixed effects model had been chosen. When the true effect of the studies was assumed to vary extensively, the random effects model was used for estimation of the weighted average of the effect reported in the studies (31). A p-value < 0.10 in the test for heterogeneity indicated the random effects model due to significant heterogeneity. The inconsistency ($I^2$) represented the percentage of observed variation across studies. Values larger than 0% indicate increasing heterogeneity and are presented in a forest plot.

RESULTS

Our database searches initially yielded twenty articles, with an additional 5 articles included from other sources. None of these were duplicate findings. Nineteen articles were then removed based on the title and abstract, leaving six articles for further screening. Full-text assessment of these six articles led to no additional exclusions. All six articles were deemed fit for inclusion in the meta-analysis on the basis of the study outputs. A schematic overview of this procedure is shown in Figure 1.

Synthesis of results

The search produced six studies addressing the relationship between vaginal microbiota composition and outcome of IVF treatment (Figure 1). The selected six studies provided
a total cohort for meta-analysis of 1095 participants wherein 893 women were classified with a normal vaginal microbiota and 202 women with abnormal vaginal microbiota (32-37). The total OR was significant for the distribution of normal vaginal microbiota composition versus abnormal vaginal microbiota of women with or without early pregnancy development after IVF treatment (CI 95% 0.49 - 0.99). The OR of 0.70 represents a negative correlation between abnormal vaginal microbiota and early pregnancy development (Table 3, Figure 2).

For the total OR we chose the random effect model due to the heterogeneity of the different studies included. However, when tested, we found no significant heterogeneity across the studies ($p = 0.15$).

Figure 1: Flowchart of the article selection process.
Table 3: Meta-analysis data of the included IVF articles. Data are represented as n (percent of patients per group). Heterogeneity testing resulted in a Cochran’s Q score of 8.09 with 5 degrees of freedom and a P value of 0.15. I² for inconsistency was 38.23% with a 95% confidence interval of 0.00 to 75.44. VF = Vaginal flora.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Analysis method</th>
<th>Abnormal VF</th>
<th>Normal VF</th>
<th>Total OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haahr et al. (2016)</td>
<td>qPCR</td>
<td>2 (9)</td>
<td>27 (44)</td>
<td>0.13 (0.03 - 0.60)</td>
</tr>
<tr>
<td>Mangit-Bertrand et al. (2013)</td>
<td>qPCR + Nugent score</td>
<td>8 (28)</td>
<td>92 (33)</td>
<td>0.77 (0.33 - 1.81)</td>
</tr>
<tr>
<td>Selim et al. (2011)</td>
<td>Nugent score</td>
<td>9 (35)</td>
<td>21 (47)</td>
<td>0.61 (0.22 - 1.64)</td>
</tr>
<tr>
<td>Eckert et al. (2003)</td>
<td>Nugent score</td>
<td>3 (30)</td>
<td>38 (47)</td>
<td>0.49 (0.12 - 2.01)</td>
</tr>
<tr>
<td>Liversedge et al. (1999)</td>
<td>Nugent score</td>
<td>24 (32)</td>
<td>64 (30)</td>
<td>1.15 (0.66 - 2.03)</td>
</tr>
<tr>
<td>Gaudoin et al. (1999)</td>
<td>Nugent score</td>
<td>7 (18)</td>
<td>53 (26)</td>
<td>0.61 (0.26 - 1.47)</td>
</tr>
<tr>
<td>Total (fixed effects)</td>
<td></td>
<td>53 (26)</td>
<td>295 (33)</td>
<td>0.70 (0.49 - 0.99)</td>
</tr>
<tr>
<td>Total (random effects)</td>
<td></td>
<td>53 (26)</td>
<td>295 (33)</td>
<td>0.66 (0.40 - 1.08)</td>
</tr>
</tbody>
</table>

Figure 2: Forest plot of clinical pregnancy rates following IVF procedure in patients with abnormal vaginal microbiota (BV) compared with patients with normal vaginal microbiota. The figure represents the individual raw OR’s with 95% Confidence intervals and the combined OR of the fixed effect model and the random effect model. The data of these models can be seen in Table 3. The size of the squares for the individual studies was proportional to the weight of the study.
Results of individual studies

The recent study of Haahr et al. investigated the diagnostic performance of qPCR assays and conventional Nugent scoring in predicting IVF outcome for an infertile population from Denmark (32). Haahr et al. conducted this study to distinguish between normal vaginal microbiota and abnormal vaginal microbiota, and to elucidate the difference of the predictive capacity of these methods on IVF outcome. The prevalence of BV assessed by the Nugent score was 21%, compared to the prevalence of 28% of abnormal vaginal microbiota assessed by qPCR. Abnormal vaginal microbiota was defined by high levels of Gardnerella vaginalis and/or Atopobium vaginae. There was no significant difference in abnormal vaginal microbiota between qPCR- and Nugent-determined BV in predicting the IVF failure. However, the pregnancy rate was significantly lower in the abnormal vaginal microbiota group, with an OR of 0.13 (95% CI 0.03-0.60).

Mangot-Betrand et al. revealed a 9.45% BV prevalence in a population of 307 infertile patients (33). Consequently, they assessed the impact of BV on the pregnancy rate after women underwent IVF. Participants with BV- showed higher embryo implantation rates compared to BV+. Nonetheless, the difference was not significant (36.3 % vs. 27.6 %, p=0.418).

Liversedge et al. found no significant difference in conception rate in patients with abnormal vaginal microbiota compared to patient with normal microbiota (OR 1.15, CI 95% 0.66 - 2.03) (36). In this study population, BV was much more prevalent in the group of patients with tubal disease ($p = 0.02$), while a normal microbiota was much more frequent in patients without tubal disease ($p = 0.004$). Selim et al. found significant differences in pregnancy rates to be correlated with single isolated bacteria (34). Specifically, decreased pregnancy rates were found in patients who tested positive for Staphylococcus aureus and Streptococcus viridians compared with patients tested negative. However, they found no significant differences in pregnancy rates between participants with abnormal vaginal microbiota and those with normal microbiota (OR 0.61, CI 95% 0.22-1.64). Eckert et al. investigated the impact of the vaginal microbiota and vaginal inflammation on conception within IVF trajectories and found no significant differences of conception rates between participants with abnormal vaginal microbiota and women with normal vaginal microbiota composition. These findings were confirmed by the study of Selim et al. (57). Besides the quantifications of the vaginal culture they also cultured the embryo transfer catheter-tip. The rate of conception in participants with Streptococcus viridans-positive catheter tips compared with catheter tips with no bacteria was 18% and 39% respectively ($p<0.001$).

In table 4 we describe a number of characteristics that may have affected the IVF outcome of patients in the included studies, and therefore affected the outcome of the meta-analysis.
Table 4: Clinical and participant characteristics of study populations.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>31 (median)</td>
<td>33.5 (mean)</td>
<td>21-44 (range)</td>
<td>21-45 (range)</td>
<td>33 (median)</td>
<td>No info</td>
</tr>
<tr>
<td>Indication for IVF</td>
<td>Male factor /</td>
<td>Male factor / Tubal factor /</td>
<td>No info</td>
<td>No info</td>
<td>Tubal factor (with and</td>
<td>Tubal factor /</td>
</tr>
<tr>
<td></td>
<td>Tubal factor /</td>
<td>Endometriosis / Unknown / Ovarian factor / Single or lesbian</td>
<td>No info</td>
<td>No info</td>
<td>without hydrosalpinx) / Sperm dysfunction (with and without sperm antibodies) / Endometriosis / Ovulatory disorder / Unexplained</td>
<td></td>
</tr>
<tr>
<td>Pituitary Down-</td>
<td>No info</td>
<td>No info</td>
<td>No info</td>
<td>No info</td>
<td>Long agonist protocol</td>
<td>Long agonist protocol</td>
</tr>
<tr>
<td>regulation</td>
<td></td>
<td></td>
<td>No info</td>
<td>No info</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td>No</td>
<td>No</td>
<td>Yes, beginning at ovum pick up (Metronidazol, Once daily for 5 days)</td>
<td>Yes, before ovum pick up (Doxycycline 100mg, Twice daily for 5 days)</td>
<td>Yes, after positive C. trachomatis immunofluoresence test (Ofloxacin), Timing unknown</td>
<td>No info</td>
</tr>
<tr>
<td>Timing of sampling</td>
<td>95% of samples</td>
<td>During ovum pick up</td>
<td>During ovum pick up</td>
<td>During embryo transfer</td>
<td>During ovum pick up</td>
<td>During ovum pick up</td>
</tr>
<tr>
<td></td>
<td>2-4 weeks before start of IVF. Max. 2 months before transfer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Country / Ethnicity</td>
<td>Denmark / 90%</td>
<td>France / no info</td>
<td>Egypt / no info</td>
<td>USA (Washington) /</td>
<td>United kingdom / no info</td>
<td>United kingdom / no info</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td></td>
<td></td>
<td>no info</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>8/130 (6.2)</td>
<td>72/307 (23.5)</td>
<td>No info</td>
<td>No info</td>
<td>No info</td>
<td>No info</td>
</tr>
<tr>
<td>IVF cycle of sampling</td>
<td>No info</td>
<td>Ranked cycles (1,2,3, &gt;= 4)</td>
<td>First cycle</td>
<td>First cycle</td>
<td>One cycle, no info on the cycle number, multiple cycles were possible</td>
<td>No info</td>
</tr>
<tr>
<td>Transfer stage</td>
<td>No info</td>
<td>No info</td>
<td>4-8 cell stage</td>
<td>4-8 cell stage</td>
<td>No info</td>
<td>No info</td>
</tr>
</tbody>
</table>
DISCUSSION

In this review we have used meta-analysis to show the importance of the microbiota composition during the conception and initial gestation through IVF. The positive effect that healthy vaginal microbiota was found to have on the outcome of IVF treatment underlines the importance of microbiota, and microbiota-focused studies investigating the early pregnancy development during IVF.

In another systematic review by van Oostrum et al. (2013) the primary focus was on the relation between the later stages of IVF-induced pregnancy and microbiota (38). However, part of their study was also focused on the effect of BV on conception rates. At the time, van Oostrum et al. found no impingement of BV on clinical conception rates. By including more up-to-date literature and specifically focusing on the early pregnancy development phase, we have indicated that there is an important effect. This can primarily be attributed to the increased size of the aggregated cases and control groups in the meta-analysis of this review. More recently, Haahr et al. reviewed the relation between dysbiotic vaginal microbiota and IVF outcome, with clinical and biochemical pregnancies as secondary outcomes (39). Due to less stringent criteria, such as using the life birth rate instead of clinical pregnancy, they were able to analyse more studies. Notably, Amsel or Nugent criteria were not specifically required for definition of BV. Additionally, definitions of clinical and biochemical pregnancies were not specifically defined, even though our review noted these as subjects of high variability between studies. In line with our findings, the quality of the extracted evidence was scored as very low using the GRADE tool. Once more, our data highlight the need for an increase in study sizes and repetition of studies relating to urogenital microbiota and reproductive health, and importantly give an overview of the research characteristics most likely to affect the outcome of the studies.

The results from the meta-analysis shows a relatively large variation in the effects found in individual studies. Our findings show that the methodology of the studies is likely the reason for this variation, as it differed on multiple points. Firstly, all included studies had slightly different definitions of conception or early pregnancy. Our definition of an ultrasound proven fetal heartbeat and/or hCG results before 10 weeks gestation captured all included articles. However, this still left room for definitions of clinical pregnancy, including only hCG results in Selim et al. and fetal heartbeat proven by ultrasound at 4 weeks of gestation by
Liversedge et al. (34, 36). We find these definitions unsatisfactory, as they skirt the edges of proper diagnosis of clinical pregnancies.

Secondly, the included studies had a number of variations in the IVF protocols. Table 4 shows the varying IVF characteristics as they were used in the respective studies. Especially notable is the varying time points of sampling for the studies. Sampling during the follicular puncture means that the increased estrogen levels are likely to affect the microbiota composition (40, 41). Sampling should ideally be done 2-4 weeks before the start of the IVF procedure, as hormonal levels will be lowest at that point. Additionally, some studies where antibiotics were given had sampling take place shortly after or during the antibiotic therapy. These antibiotics likely affected the compositions of the microbiota in the patients. For ideal sampling, antibiotic use prior to the sampling should be taken as an exclusion criterion for possible patients willing to participate in these studies. Other varying characteristics in Table 4 show the need for uniformity in sampling during IVF studies to produce a study that can be properly compared to previously performed studies. Furthermore, it shows the need for care on the side of the reader of these articles, as these varying characteristics mean study results are not always immediately translatable to other settings.

Another factor that can influence the outcomes of this study is microbiota on the endometrium, which was previously thought of as sterile. A number of studies found that an abnormal endometrial microbiota is associated with implantation failure in reproductive trajectories of subfertile women (42, 43). A study conducted by Moreno et al. (2016) compared the vaginal microbiota with the endometrial microbiota in a cohort of healthy and fertile women (43). All of the endometrial samples revealed bacterial colonization consisting of *Lactobacillus* and less abundant anaerobic bacteria such as *Gardnerella*, *Prevotella* and *Atopobium*. About 20% of the women showed bacterial colonization in their endometrial samples that differed significantly from their vaginal samples, which suggests that the endometrial microbiota may be an independent effector of conception. Even then, a link between the vaginal microbiota composition and the endometrial microbiota could further increase the value of knowing the vaginal microbiota composition before IVF treatment begins. In that sense, knowledge of the interactions between the vaginal microbiota, the endometrial microbiota, and the reproductive tract is crucial to improve the fertility trajectory of subfertile women (44). Notable, a recent study by Benner et al. corroborates this by linking the uterine microbiota to the receptivity and fertility of the endometrium (45). One aspect of
special interest is chronic endometritis, which occurs in roughly 45% of all subfertile women (46). The chronic inflammation is suggested to prevent embryo implantation. Antibiotic intervention of chronic endometritis after bacterial culture revealed improved reproductive results in women with recurrent implantation failure, showing a clear interaction between the host and colonizing bacteria (47). Whether the vaginal microbiota can be linked to the development or severity of chronic endometritis still needs to be studied. A clear link would make diagnosis and treatment of chronic endometritis easier, and potentially lead to improvement of early pregnancy development rates in subfertile women.

There are limitations to consider in this study. The size of the effect found during the meta-analysis is notable. An odds ratio of 0.7 in favor of a healthy vaginal microbiota on the outcome of IVF treatment is much less than that suggested by most of the included studies. Although this result is still significant, it suggests the contribution to IVF failure of other factors that may act together with the resident microbiota.

Additionally, this study has some inherent limitations, including the length of time between included studies. This may mean that the methods applied in earlier studies do not represent their findings as accurately as more recent studies. We believe that the size of the meta-analyses partially corrected for this.

**CONCLUSIONS**

In this review and related meta-analysis we show in a large aggregated cohort that abnormal vaginal microbiota has a strong correlation with the failure of IVF through the absence of first trimester pregnancy. In addition to this, we conclude that there is currently too much heterogeneity in the methodology of studies into the vaginal microbiota during IVF, leading to poor comparability. We suggest researchers and readers of the literature to pay special attention to the possible confounding factors (e.g. hormone levels) that can effect study outcomes, and to make a concerted effort to have a uniform methodology with the current literature, unless deviation is strictly necessary.
CHAPTER 5

DECLARATIONS

Author’s roles
M. Borg and M. Singer performed the meta-analysis and wrote Introduction, Materials and Methods, Results, and Discussion sections. S. Ouburg and S.A. Morré contributed by evaluating the review and ensuring the quality of the manuscript.

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6. Antonio MA, Petrina MA, Meyn LA, Hillier SL. Women colonised by lactobacillus crispatus have a lower risk of acquisition of bacterial vaginosis (BV) than women colonised by other Lactobacilli. Sexually Transmitted Infections. 2013;89.


“We demand rigidly defined areas of doubt and uncertainty!”

Douglas Adams

The Hitchhiker’s Guide to the Galaxy
CHAPTER 6
The ReceptIVFity cohort study protocol to validate the urogenital microbiome as predictor for IVF or IVF/ICSI outcome

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Submitted
ABSTRACT

Background
During the last decade, research has shown that besides the known predictive factors, such as duration of subfertility, a woman’s age, the body mass index, also the microbiome might affect fertility. Micro-organisms together with their genetic information and the milieu in which they interact are called the microbiome. Studies have shown that the presence of certain microbiota during assisted reproductive technology (ART) has a positive impact on the outcome. However, the potential role of using the microbiome as a predictor for outcome of ART has not yet been investigated.

Methods
In a prospective study, 300 women of reproductive age and with an indication for in-vitro Fertilization (IVF) with or without Intra Cytoplasmic Sperm Injection (ICSI) treatment will be included. Prior to the IVF or IVF-ICSI treatment, these women provided a midstream urine sample and a vaginal swab. The composition of the urinary and vaginal microbiome will be analysed with both Next Generation Sequencing and the IS-pro technique. The endpoints of the study are pregnancy achieved after fresh embryo transfer (ET) and within the subsequent year after inclusion.

Discussion
In the proposed study, the predictive accuracy of the composition of the urinary and vaginal microbiome for IVF or IVF-ICSI outcome will be only validated for fresh ET. Follow-up has to show whether the predictive accuracy will be similar during the consecutive frozen ET’s as part of the IVF or IVF-ICSI treatment or for subsequent stimulated or natural cycles. Predictive knowledge of the microbiome profile may enable couples to make a more substantiated decision on whether to continue treatment or not. Hence, the unnecessary physical and emotional burden of a failed IVF or IVF-ICSI treatment can be avoided.

Trial registration
ISRCTN83157250 Retrospectively registered 17 August 2018.
**BACKGROUND**

Assisted Reproductive Technologies (ART) such as *in-vitro* Fertilization (IVF) (1) with or without Intra Cytoplasmic Sperm Injection (ICSI) (2) are used to assist the 10-15% of couples affected by subfertility (3). At present the pregnancy rate is around 25% for the first attempt (cycle) and cumulative ongoing pregnancy rates after additional cycles vary between 40% and 50% depending on the number of treatment cycles (4, 5). Because of the psychosocial, physical and financial burden of ART, prediction of accurate outcome is needed. Current prediction models perform moderately well and are based upon pure clinical parameters such as age, number of previous failed IVF treatments, and the cause of subfertility (6).

However, at the department of Urology and the division of Reproductive Endocrinology and Infertility of the department of Obstetrics & Gynaecology both in the Erasmus University Medical Centre, a new predictive algorithm for the outcome of IVF or IVF-ICSI based on the microbial composition (population of different bacteria in a given sample) has been developed. The collection of microorganisms that live on or in the human body is known as our microbiota and its complete genetic profile as the microbiome. For this study, the urinary microbiome was determined in mid-stream urine sample. All urine samples were collected prior to the start of the actual IVF or IVF-ICSI treatment (7).

The predictive algorithm was developed in a pilot study comprising 42 women who were expected to start with either IVF or IVF-ICSI treatment and uses the percentages of the species *Lactobacillus*, *Staphylococcus* and *Escherichia coli* within the total microbiome of the sample. In the pilot study, the composition of the urinary microbiome prior to the start of the IVF or IVF-ICSI treatment was linked to the outcome of the ART treatment defined as ongoing pregnancy after one treatment cycle and pregnancy rates within one year after the initiation of the treatment. Cluster analysis and principal component analysis revealed that based on the microbiome composition it is possible to separate (by means of clustering) the women into two clusters: pregnant and non-pregnant women. After logistic regression, the bacterial species that dominated this prediction were identified and those were used to construct a predictive algorithm. In the pilot study, the test panel had a specificity of 96% and a sensitivity of 81%. Importantly, by adding the species *Bacillus RG4* the specificity improved up to 100%. The species found to be predictive has still to be confirmed in an independent, separate study. The crucial outcome of this predictive test is its specificity or
better said, the prediction that the treatment will not result in pregnancy, since it could be used to select those women who should not be subjected to treatment because they will have a high chance not getting pregnant.

Hypotheses that might explain these finding is that the microbiome, acts as a sensor for the immunological tolerance that exist in secretory epithelia of a particular woman. Hence, it constitutes a proxy for endometrial receptivity which in turn depends on a similar immune response towards the developing embryo which is trying to implant. Another hypothesis is that the bacterial species included in the predictive algorithm possibly thrive the nutritional environment within epithelial secretions, which might be essential for initial survival of the embryo after transferral into the uterine cavity (8, 9).

In line with our finding that the genus \textit{Lactobacillus} has an important role in reproductive health/outcome, are similar results of several published studies (10-20). Recent publications show that women with infertility problems have a reduced number of \textit{Lactobacillus} compared to healthy women (10-18). Moreover, the presence of non-\textit{Lactobacillus} dominated microbiota (\textit{e.g.} presence of the genera \textit{Gardnerella} and \textit{Streptococcus}) seems to be associated with significant decreases in implantation, ongoing pregnancy and live birth rates (17) and clinical pregnancy rates (19, 20).

The large benefit of such a predictive test might have for patients undergoing IVF or IVF-ICSI treatment necessitates validation of these findings in a larger study with several independent ART clinics. Furthermore, to allow future clinical application of the algorithm, the Sanger sequencing technique used in the pilot study needs to be replaced by a quick and high-throughput method. Therefore, all collected microbiome samples will concomitantly be investigated with a Next Generation Sequencing (NGS) method as well as with the polymerase chain reaction (PCR) based IS-pro technique. These techniques will be used for both urine samples and vaginal samples, to investigate whether the urinary microbiome is affiliated from the vaginal microbiome. Here we describe the design of the ReceptIVFity cohort study.
METHODS

Study aim
With ReceptIVFity, we aim to validate the algorithm in a larger cohort, and to develop a predictive test suitable for the use in daily practice. In this cohort study, the following aims will be addressed:

Primary Objective:
– To assess the specificity and sensitivity of the urinary and vaginal microbiome composition for the prediction of embryo implantation failure of a consecutive IVF or IVF-ICSI procedure.

Secondary Objective:
– To assess the specificity and sensitivity of the urinary and vaginal microbiome composition for the prediction of the cumulative outcome of one year of subsequently performed IVF or IVF-ICSI procedures.

Study design and setting
The prospective study of the urogenital microbiome of subfertile women of reproductive age will be carried out in eight IVF centres in the Netherlands. The participating centres are: Erasmus University Medical Centre (UMC) (Rotterdam), Radboud UMC (Nijmegen), UMC Utrecht (Utrecht), VU University Medical Centre (Amsterdam), Isala Fertility Centre (Zwolle), Sint Elisabeth Hospital (Tilburg), VivaNeo Medical Centre Kinderwens (Leiderdorp), and Maastricht UMC+ (Maastricht). Inclusions will take place over the period from the 1st of June 2015 until the 31st March 2016.

Study population
Women who will visit the infertility outpatient clinics of participating hospitals and who are expected to undergo their first IVF or IVF-ICSI cycle within the next two months will be approached to participate in this study. Inclusion criteria to be fulfilled are: women aged between 20 and 44 years and a male partner. Those excluded from the study are: women with an indication for emergency IVF because of cancer or other reasons, endometriosis American Fertility Score (AFS) III/IV and pre-treatment with a Gonadotrophin-releasing hormone (GnRH) analogue or those who use hormonal contraceptives within three months.
prior to the start of their IVF or IVF-ICSI intake. Those women who are using the oral contraceptice pill for the purpose of cycle timing prior to their treatment cycle are eligible for this study. Women who have had a previous pregnancy or miscarriage in their medical history will also be excluded from participation. Pregnancy outcomes after the first fresh ET will be used as endpoint for this study. Ongoing pregnancy is defined as an intrauterine embryo/foetus with detection of cardiac activity on transvaginal ultrasound between 7-9 weeks of gestation.

**Study materials**

A vaginal swab and a midstream urine sample before the start of the IVF or IVF-ICSI procedure will be self-collected. The swab and the urine sample have to be taken within the two months prior to the ET. A self-collecting method was chosen, because it is minimally invasive and therefore suitable for use in daily practice. Vaginal samples will be taken with FLOQSwabs™ (Copan Italia SpA, Brescia, Italy). The participants will be instructed to insert the swab 3-5 centimetres beyond the vaginal orifice, and move the swab around along the vaginal wall for 10-15 seconds. After this procedure the swabs will be immediately placed in Eppendorf tubes filled with reduced transport fluid (RTF) buffer (Microbiome Ltd., Amsterdam, the Netherlands). Until further processing, samples will be stored in a freezer at -20 °C degrees.

The urine sample collection will be obtained according to a standard ‘clean catch’ protocol, including washing hands thoroughly, cleaning the urinary opening with towelettes and collecting a midstream specimen in a sterile container. The urine sample will be stored at room temperature or in the refrigerator at 2-8 °C for a maximum of two hours until further processing. Further processing will consist of vortexing the urine sample and centrifuging 10 ml of the urine at 1500 relative centrifugal force (RCF) followed by resuspension in 1 ml of urine, which will be stored at -20°C degrees until transport.

Next, vaginal swabs and urine samples will be transported on dry ice by courier from the eight clinics to the microbiological laboratory.

**DNA isolation**

DNA extraction of the vaginal swabs and urine samples will be performed with the Chemagen machine (Chemagen, Baesweiler, Germany) using the buccal swab extraction kit according to the manufacturer’s instructions. First, collected swabs and urine samples will be thawed
and vortexed. 200 µl of sample will be incubated with 200 µl of Chemagen lysis buffer and 10 µl Proteinase K (Qiagen, Hilden, Germany) at 56°C while shaking at 500 rpm. DNA will be extracted using the protocol buccal Swab Prefilling. Finally, DNA will be eluted in 100µl of Chemagen Elution buffer.

**Sequencing of the 16S ribosomal RNA**

Picogreen dsDNA assay (Thermofisher, MA, USA) will be used for sample DNA concentration measurement. For sequencing, the V3-V4 region of the 16S rRNA gene region will be amplified using the individually distinguishable dual index primer sets. The rDNA amplification primers will be the universal primer set 319F/806R and they will be altered to also encode the Illumina sequencing primer and barcode labelling sequences. PCR will include 30 seconds at 98°C, 30 cycles of 10 seconds at 98°C, 15 seconds at 58°C, and 15 seconds at 72°C and three minutes at 72°C. The AMPure XP magnetic bead assay (Beckman Coulter Genomics, Danvers, MA, USA) will be used for purification of the amplified DNA. The following formula will be used for recalculation into nM and equalized to 12 nM:

\[
[nM \text{ DNA}] = \frac{(\text{DNA concentration (ng/µl)} \times 1000)}{(\text{sample fragment size in bp} \times 656,4 (g/mole))}
\]

If DNA concentrations fell below 12 nM, pooled DNA will be concentrated by vacuum evaporation.

**Next generation sequencing**

NGS will be performed using a Miseq tabletop sequencer (Illumina, San Diego, CA, USA) by the Tumor Genome Analysis Core group of the Department of Pathology at the VU University Medical Centre in Amsterdam, The Netherlands. QIIME will be utilized to remove primer and index sequences, while paired end reads with a minimum overlap of six nucleotides and a minimum combined length of 400 nucleotides are assembled to produce identifiable sequences. The Usearch method will be utilized to produce operational taxonomic units (OTU) clusters. During this process, the sequences will be sorted on length and abundance of identical reads, will be checked for chimeric sequences and the sequence similarity threshold is set to 0.97 to denoise the data. The database described by Srinivasan et al. (21) will be used to assign sequences on a genus to species level by using the PyNAST method for sequence alignment and subsequently assignment using the RDP classifier method. The remaining sequences will be BLASTed, and will be included if the sequence can be identified at a genus or species level.
CHAPTER 6

Interspace profiling
Amplification of the intergenic spaces (IS) regions will be performed with the IS-pro assay, according to the protocol provided by the manufacturer (IS-Diagnostics, Amsterdam, the Netherlands). IS-pro is a eubacterial technique based on the detection and categorisation of the length of the 16S-23S rRNA gene IS region. The length of this IS region is specific for each bacterial species. Phylum-specific fluorescently labelled PCR primers will be used for taxonomic classification (22).

Briefly, the procedure consists of two separate standard PCRs: the first PCR mixture contains two different fluorescently labelled forward primers targeting different bacterial groups and three reverse primers providing universal coverage for those groups. The first forward primer is specific for the phyla Firmicutes, Actinobacteria, Fusobacteria, and Verrucomicrobia (FAFV), and the second labelled forward primer is specific for the phylum Bacteroidetes. A separate PCR mixture includes a labelled forward primer combined with seven reverse primers and is specific for the phylum Proteobacteria.

A GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA) will be used to perform the amplifications. After PCR, 5 μl of PCR product will be mixed with 20 μl of formamide and 0.2 μl of custom size marker (IS-Diagnostics, Amsterdam, The Netherlands). DNA fragment analysis will be performed on an ABI Prism 3500 genetic analyser (Applied Biosystems, Foster City, CA, USA). Data will be analysed with the IS-pro proprietary software suite (IS-Diagnostics, Amsterdam, The Netherlands), and the results will be presented as bacterial profiles. Automated species identification of IS-pro peaks will be done with the dedicated IS-pro software suite (IS-Diagnostics, Amsterdam, The Netherlands), in which peaks are linked to a database containing IS-profile information of >500 microbial species. Peaks of <500 relative fluorescence units (RFU) will be regarded as background noise and will be discarded from further analysis (22, 23).

Microbiome analysis and algorithm building
The vaginal microbiome profile of each participant will be assigned to one of five community state types (CST) based on the dominant microbial species, as described by Ravel et al.10 Microbial communities in group I are dominated by L. crispatus, whereas group II, III, and V are dominated by respectively L. gasseri, L. iners, and L. jensenii. Group IV contains a heterogeneous group including species such as Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and Mobiluncus.
The prediction model will be composed by analysis of the microbiological data will be performed in Spotfire 7.6 (TIBCO Spotfire Inc., Somerville, MA, USA). First, a distance matrix will be created on cosine distances of all possible sample pairs. Cosine distances are calculated with the following formula:

\[
dissimilarity = 1 - \cos(\theta) = 1 - \frac{\sum_{i=1}^{n} A_i \times B_i}{\sqrt{\sum_{i=1}^{n} (A_i)^2} \times \sqrt{\sum_{i=1}^{n} (B_i)^2}}
\]

The resulting data will be clustered with the unweighted pair-group method with arithmetic mean (UPGMA) and plotted as a heatmap.

To answer the questions of this study, an algorithm will be built based on the composition of the vaginal microbiome profile. First, the algorithm will be built by exploration of the pregnancy outcomes per CST. In a subsequent step, the data will be analysed for species content and microbial diversity per phylum. The formula will be validated for prediction of failure to become pregnant after (the first) fresh embryo transfer during the IVF or an IVF-ICSI treatment. Sensitivity will be calculated as true positive (TP) / (TP + false negative (FN)) and specificity as true negative (TN) / (TN + false positive (FP)). Predictive accuracy will be determined as the correlation between the predicted outcome and the actual outcome.

The vaginal samples that meet the prediction model for failure to become pregnant will be defined as ‘unfavourable microbiome profiles’. The vaginal samples that will not comply with the prediction model for failure to become pregnant will be defined as a ‘favourable microbiome profile’. Additionally, we will explore whether we can stratify these samples with a favourable microbiome profile into an average and a high chance to become pregnant based on additional bacterial species.

**Statistical analysis**

Statistical analyses of the clinical data will be performed by using SPSS statistics version 24 (IBM corp, Amonk, NY, USA). We will examine two different prediction models. In the prediction model for failure to become pregnant, the primary outcome will be defined as ‘not pregnant’ after the first fresh ET during IVF or IVF-ICSI treatment. In the prediction model for success to become pregnant, the primary outcome will be defined as ‘pregnant’. Normality of data will be determined by using the Shapiro-Wilks normality test. Continuous, normally distributed variables will be presented as mean with standard deviation, and variables with
a skewed distribution as median with the range. Categorical variables will be presented as count and proportions.

Differences between the groups of women who will become pregnant and those who will not will be compared using Chi-square test or Fisher’s exact test for the categorical data, as appropriate. The Independent Samples t-test and Mann-Whitney U test will be used for continuous data. Two-sided P values less than 0.05 will be considered statistically significant. Applying the same method, differences between women with a favourable microbiome profile and those with an unfavourable profile will be compared.

Multivariate analysis will be performed using logistic regression with a selection of covariates that are known predictors for pregnancy outcome; age, duration of infertility and body mass index (BMI).

**DISCUSSION**

In the ReceptIVFity cohort study, the specificity and sensitivity of the urogenital microbiome composition for the prediction of IVF or IVF-ICSI outcome will be determined in a cohort of women of reproductive age who will be expected to undergo their first IVF or IVF-ICSI cycle on short term. ReceptIVFity will investigate the role of a broad range of bacterial species and the influence of other clinical parameters in the success rate of IVF or an IVF-ICSI treatment. Insights in the microbial profile and their impact on the success rate may allow for a new strategy to decide whether to continue with treatment or not. The ultimate goal will be to develop a predictive algorithm that enables identification of the group of women with a low chance to become pregnant prior to the start of the IVF or IVF-ICSI treatment. Women with a low a priori chance to become pregnant might prefer to avoid the unnecessary physical and emotional burden of a IVF or IVF-ICSI treatment with a high change of failure.

**Strengths**

The ReceptIVFity study will be the first cohort study in which the microbial composition will be used as predictor for IVF or IVF-ICSI outcome prior to the start of the treatment. As mentioned in the background section, several studies have found associations between the presence of microorganisms and reproductive outcomes, such as decreased implantation,
pregnancy, ongoing pregnancy, and live birth rates. However, the clinical applicability of these findings has not yet been integrated into daily practice.

The ReceptIVFity cohort study consists of a large patient group/sample size and well-defined patients. A large sample is indicated, anticipating the fact patients can drop out for further treatment/follow up due to several reasons, such as poor response or total fertilisation failure. The included patients with embryo transfers will be used to investigate an association between microbial composition and IVF or IVF-ICSI outcome.

A prospective study design blinded for the test result, avoids bias to continue or not continue the treatment and allows for unbiased follow-up of the treatment outcomes.

The one year follow-up is needed to examine the sustainability of the predicted outcome and will provide insight into the possibilities of becoming pregnant with subsequently performed IVF or IVF-ICSI procedures.

Different techniques can be used to assess the microbiome and a high throughput technique is desirable for future application in daily practice. The prospective study validates the findings from the pilot study by the use of two different techniques. NGS that has become a gold standard for categorising bacteria or characterising microbial communities and the IS-pro technique which has the benefit of presenting results within five hours will be compared in this study. In addition, two sample sites (urine samples and vaginal swabs) will be compared with each other, both sites are easy to obtain by patients themselves.

Developing a predictive test based on the urogenital microbiome composition will contribute to a personalised medicine approach in the future.

Limitations

Because our study uses a well-defined study population, the results will be limited to the IVF or IVF-ICSI population. Whether these results also apply to a general population trying to establish a pregnancy and without ART cannot be extrapolated from these data.

In our study, we collect the samples only once and prior to the start of the first treatment cycle.
CHAPTER 6

The success rate of IVF or IVF-ICSI treatment depends on multiple clinical parameters. Account must be taken for possible confounders in order to develop an independent test for prediction of IVF or IVF-ICSI outcome. Nevertheless, we will collect these clinical data (e.g. age, BMI, duration of infertility) and will correct for these possible confounders.

In summary
In the future, microbiome profiling can be a routine diagnostic test prior to IVF or IVF-ICSI treatment if the negative predictive value is high enough to prevent patients a treatment with emotional and physical burden, that has low chance of success. With this cohort, we aim to contribute to better insight and validation of the urogenital microbiome as predictor for IVF or IVF-ICSI outcome. Finally, our ultimate goal is development of a diagnostic test that enables couples to make a more substantiated decision on whether to continue with treatment or not, based on their personal and individual microbiome profile.

DECLARATIONS

Ethics approval and consent to participate
The protocol was approved by the Institutional Medical Ethical Review Board of all participating centres (MEC-2014-455). Written informed consent was obtained from all participants.

Consent for publication
Not applicable.

Availability of data and materials
The data that support the findings of this study are available from ARTPred B.V. but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of ARTPred B.V..

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

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

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

AEB reports that he is a co-owner of IS-Diagnostics Ltd. In addition, Dr. Budding has a patent 392EPP0 pending.

JSEL reports grants from Dutch Heart Foundation, Ferring, Metagenics Inc.. He received personal consultancy fees from ARTPred B.V., Danone, Euroscreen, Roche, during the conduct of the study. In addition, JSEL is a co applicant on a Erasmus MC patent, that predicts IVF outcome based on the urinary microbiome. This particular patent is licensed to ARTPred B.V.

The other authors declare that they have no competing interests.

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Authors’ contributions
RK, SS, AB, PS, SM, DB and JL coordinate the cohort and were responsible for writing the manuscript. MS, PS, SM and AB coordinate laboratory testing and analysis. SM, JL designed the study. All authors critically reviewed and approved the final manuscript.

Authors’ information
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REFERENCES


It has been said that everything everywhere affects everything else. This may be true. Or perhaps the world is just full of patterns.

Terry Pratchet
CHAPTER 7

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

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Submitted
ABSTRACT

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

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

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

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

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INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

RESULTS

Characteristics of the participants
In total, 303 women were included with the intention to provide a vaginal swab for bacterial profile analysis. One woman did not provide a vaginal swab and was excluded, two women were excluded by not completing the questionnaire, and three samples of women were discarded due to sampling error. Another six women were excluded by not fulfilling the inclusion and exclusion criteria: the use of more than three weeks hormonal contraceptives prior to the start of the IVF or IVF-ICSI treatment (n=5), and miscarriage in medical history (n=1). Retrospectively, 14 women collected the sample more than two months prior to the ET and for this reason these women were excluded as well from further analysis. Hence, 26 women in total were not included due to either protocol violations or selection errors.
Figure 1: Heatmap of all clustered vaginal swab samples. Top section shows the clustering of all analysed microbiome profiles. The clusters that coincide with the five known community state types (CST) are shown (I-V). The columns represent the profile per individual. The rows represent the found species and the length of the interspace region in units of nucleotides (nt). This can be clustered into 3 groups at phyla level: Bacteroidetes, FAFV, Proteobacteria.
Of the remaining 277 women, 85 women dropped out due to the fact that there were no transferable fresh embryos after their first IVF or IVF-ICSI attempt. The various reasons for this are summarised in Supplementary table 1. Finally, data of IVF or IVF-ICSI outcome after the fresh ET was available for 192 women. There were no differences in baseline characteristics between women that were excluded compared to those that were included, except ethnicity subcategory negroid (Supplementary table 2). For the final analysis, we divided the population in two groups, 67 women that became pregnant and 125 who did not. Table 1 summarizes the baseline characteristics of these two groups.

**Univariate analysis**

Univariate analyses showed that there were no statistical differences between the two groups of pregnant women versus non-pregnant women (Table 1), including commonly used predictors for pregnancy outcome (e.g. age, BMI, duration of infertility). Women in the non-pregnant group had similar ages (mean ± SD age 31.92 ± 4.17 years) compared to the women in the pregnant group (age 31.16 ± 3.67 years). The median BMI in the two groups was similar (23.0 kg/m²). The duration of infertility was the same in both groups (median (range) 2.43 (0.43-13.80) vs 2.21 (0.55-12.82) years; p=0.333). Finally, when comparing the remaining clinical characteristics of the IVF and IVF-ICSI treatments, there were no significant differences between the two groups (Supplementary table 3).
Figure 2: The community state types and the accompanying pregnancy outcome. The composition of the vaginal microbiome per community state type (CST) and the accompanying distribution of pregnancy results (not pregnant or pregnant) per CST.
Analysis of the vaginal microbiome profile

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

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

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

<table>
<thead>
<tr>
<th></th>
<th>Pregnant Yes (n=67)</th>
<th>Pregnant No (n=125)</th>
<th>p-value**</th>
</tr>
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<tr>
<td>Age</td>
<td>31.16 (3.67)</td>
<td>31.92 (4.17)</td>
<td>0.214§</td>
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<tr>
<td>Ethnicity</td>
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<tr>
<td>Caucasian</td>
<td>53 (79.1%)</td>
<td>113 (90.4%)</td>
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<td>Mediterranean</td>
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<td>3 (2.4%)</td>
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<td>Hindu</td>
<td>3 (4.5%)</td>
<td>2 (1.6%)</td>
<td>0.339‡</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (3.0%)</td>
<td>1 (0.8%)</td>
<td>0.268‡</td>
</tr>
<tr>
<td>African</td>
<td>0 (0%)</td>
<td>3 (2.4%)</td>
<td>0.552‡</td>
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<tr>
<td>Missing*</td>
<td>4 (6.0%)</td>
<td>3 (2.4%)</td>
<td>-</td>
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<tr>
<td>BMI</td>
<td>23.00 (18.00-32.04)</td>
<td>23.00 (18.00-42.00)</td>
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<td>Menarche</td>
<td>13.00 (9.00-17.00)</td>
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<td>Menstrual cycle</td>
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<tr>
<td>Regular</td>
<td>53 (79.1%)</td>
<td>91 (72.8%)</td>
<td>0.331†</td>
</tr>
<tr>
<td>Mostly regular</td>
<td>6 (9.0%)</td>
<td>18 (14.4%)</td>
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</tr>
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<td>80 (64.0%)</td>
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<td>Idiopathic</td>
<td>7 (10.4%)</td>
<td>22 (17.6%)</td>
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<td>Tube factor</td>
<td>2 (3.0%)</td>
<td>5 (4.0%)</td>
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<td>3 (4.5%)</td>
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<td>3 (2.4%)</td>
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<td>8 (6.4%)</td>
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<td>2 (3.0%)</td>
<td>2 (1.6%)</td>
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<td>Use of medication</td>
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<tr>
<td>Yes</td>
<td>37 (55.2%)</td>
<td>82 (65.6%)</td>
<td>0.122†</td>
</tr>
<tr>
<td>No</td>
<td>28 (41.8%)</td>
<td>38 (30.4%)</td>
<td>-</td>
</tr>
<tr>
<td>Missing*</td>
<td>2 (3.0%)</td>
<td>5 (4.0%)</td>
<td>-</td>
</tr>
<tr>
<td>Urinary tract infection in prior 3 months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (4.5%)</td>
<td>4 (3.2%)</td>
<td>0.697‡</td>
</tr>
<tr>
<td>No</td>
<td>64 (95.5%)</td>
<td>121 (96.8%)</td>
<td>-</td>
</tr>
</tbody>
</table>
Values are given as mean ± SD years, number (%), and median (range). BMI= Body Mass Index (kg/m²).
† By Chi-square test.
‡ By Fisher’s exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
* these subcategories are excluded from analysis.
** comparisons are made between analysed category and the other categories combined.

Figure 3: The role of *Lactobacillus crispatus* and *Lactobacillus iners* in predicting pregnancy outcome. Relative rates of *L. crispatus* versus *L. iners* for each patient. Each circle represents a patient, the colour of the dot shows pregnancy outcome (green – pregnant, red – not pregnant).
A: Patients with an unfavourable profile (34 of 192 women) are depicted as black crosses, they are removed from the dataset in figures B-D. B: Of the remaining 158 women, 63 have a relative abundance of *L. crispatus* ≥ 60%. The pregnancy rate in this group is 15/63=24%. C: Women with a relative abundance of *L. iners* ≥ 60% show a pregnancy rate of 19/38=50%. D: When taking into account *L. crispatus* abundance alone, the group of women with a *L. crispatus* abundance ≤ 60% have a pregnancy rate of 50/96=53%.
Table 2: Univariate analysis of baseline characteristics in women with a favourable or unfavourable microbiome profile.

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

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

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

Unfavourable microbiome profile

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

Favourable microbiome profiles

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

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

Values are given as mean ± SD, number (%), and median (range). IVF= in-vitro Fertilisation; IVF-ICSI= in-vitro Fertilisation with Intracytoplasmic Sperm Injection; mm= millimetre; ET= embryo transfer; SET= single embryo transfer; DET= double embryo transfer.
† By Chi-square test.
‡ By Fisher’s exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
*these subcategories are excluded from analysis.
**comparisons are made between analysed category and the other categories combined.

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

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

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfavourable profile</td>
<td>12.057</td>
<td>2.739-53.078</td>
</tr>
<tr>
<td>Age</td>
<td>1.024</td>
<td>0.937-1.119</td>
</tr>
<tr>
<td>BMI</td>
<td>1.071</td>
<td>0.982-1.167</td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>1.072</td>
<td>0.895-1.285</td>
</tr>
</tbody>
</table>

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

**DISCUSSION**

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

The overall pregnancy rate per cycle of the cohort was 35%, which is similar to that reported by most clinics in the Netherlands in 2016 (average 34.2%).

(15) Women with a low *L. crispatus* abundance profile, four (31%) became pregnant (three ongoing pregnancies (75%)) and four did not finish the IVF procedure. Finally, of the 23 women with a low *L. crispatus* abundance profile, 12 (52%) became pregnant (11 ongoing pregnancies (92%)) and three (13%) did not finish the IVF procedure.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfavourable profile</td>
<td>12.057</td>
<td>2.739-53.078</td>
</tr>
<tr>
<td>Age</td>
<td>1.024</td>
<td>0.937-1.119</td>
</tr>
<tr>
<td>BMI</td>
<td>1.071</td>
<td>0.982-1.167</td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>1.072</td>
<td>0.895-1.285</td>
</tr>
</tbody>
</table>

OR= odds ratio; 95% CI= 95% confidence interval; BMI= Body Mass Index.
abundance had a one and a half times higher chance to become pregnant after the first fresh ET, while women with a high \textit{L. crispatus} profile had a third times lower chance of becoming pregnant compared to the overall pregnancy rate.

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

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

Our findings show that a high abundance of \textit{Lactobacillus} appears to be advantageous for IVF and IVF-ICSI outcome, but a high abundance (> 60%) of \textit{L. crispatus} is not advantageous. Studies that generalize on the possible beneficial effects of \textit{Lactobacilli} on fertility will have to be interpreted with caution. They have to take into account that different \textit{Lactobacillus} species also have different characteristics; e.g. the ability to produce lactic acid from degradation of glycogen conversion. The acidity achieved by \textit{L. crispatus} is the highest compared
to *L. iners*, *L. jensenii* and *L. gasseri*.\(^{(10)}\) Witkin *et al.* suggests that lactic acid production may facilitate the release of innate immune system components that inhibit growth of bacteria other than *Lactobacilli*.\(^{(18)}\) At the same time, *Lactobacilli* act as a mechanical barrier by binding to the surface of vaginal epithelial cells, preventing the binding of other bacteria that might influence these epithelial cells.

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

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

Further research is needed to elucidate if women with an unfavourable microbiome profile can actually switch to a favourable profile and whether their chance of becoming pregnant subsequently increases. Earlier research investigating the stability of the vaginal microbiome revealed the dynamics of five major classes of bacterial communities (CST I-V) and showed that some communities can change markedly over short time periods, whereas the majority is relatively stable over several months.\(^{(21)}\) However, there might also be women with fairly constant microbiota that do not change over time.
One of the potential opportunities to improve results after IVF or IVF-ICSI with the use of microbiome profiling is to counsel women to postpone ART and await a favourable profile. The women could sample the vaginal microbiome over a certain period of time awaiting a switch from an unfavourable profile into a favourable one. Another option is to postpone the first fresh ET, freeze the resulting embryos and transfer them later in unstimulated cycles with a favourable microbiome profile. It is however yet unknown how often, when and why the local microbiota composition changes spontaneously. There might also be therapeutic options aiming at modulation of unfavourable vaginal microbiota towards a more favourable profile, such as antibiotics, pre- or probiotics.(22)

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

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

DEclarations

Acknowledgement
The authors would like to acknowledge the collaborating centres: Radboud University Medical Centre, Erasmus University Medical Centre, Isala Fertility Centre, Amsterdam UMC Vrije Universiteit Amsterdam, Sint Elisabeth Hospital, VivaNeo Medical Centre Kinderwens, University Medical Centre Utrecht, Maastricht University Medical Centre+, and MVZ VivaNeo Kinderwunschzentrum Düsseldorf GmbH.
Sources of funding
This study was financed by NGI Pre-Seed 2014-2016, RedMedTech Discovery Fund 2014-
2017, STW Valorisation grant 1 2014-2015, STW Take-off early phase trajectory 2015-2016,
Eurostars VALBIOME grant (reference number: 8884).

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

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

SS reports no conflict of interest.

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

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

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

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

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

JSEL reports grants from Dutch Heart Foundation, Ferring, Metagenics Inc.. He received personal consultancy fees from Titus Health B.V., Danone, Euroscreen, Roche during the conduct of the study. In addition, JSEL is a co applicant on an Erasmus MC patent (New method and kit for prediction success of in vitro fertilization), that predicts IVF outcome based on the urinary microbiome. This particular patent is licensed to ARTPred B.V..

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

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

BJC has nothing to disclose.

KF reports personal fees from Ferring (commercial sponsor), grants from Ferring (commercial sponsor), grants from Merck-Serono (commercial sponsor), personal fees from GoodLife (commercial sponsor), grants from GoodLife (commercial sponsor), outside the submitted work.
CBL received speakers fee from Ferring; his department receives unconditional research grants from Ferring, Merck and Guerbet.

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

The other authors declare that they have no competing interests.

**RESEARCH IN CONTEXT**

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

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

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


### Supplementary Table 1: Reasons for study participants to drop out.

<table>
<thead>
<tr>
<th>Stage of treatment</th>
<th>Reason of drop out</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone stimulation</td>
<td>Too few follicles (n=34)</td>
<td>n=46</td>
</tr>
<tr>
<td></td>
<td>Too many follicles (n=10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stagnant growth (n=2)</td>
<td></td>
</tr>
<tr>
<td>Puncture</td>
<td>Risk of ovarian hyperstimulation syndrome (n=8)</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>Escape intrauterine insemination (n=1)</td>
<td></td>
</tr>
<tr>
<td>Laboratory</td>
<td>No oocytes (n=4)</td>
<td>n=19</td>
</tr>
<tr>
<td></td>
<td>No sperm cells (n=3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insufficient cleavage (n=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No embryo (n=3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total fertilisation failure (n=8)</td>
<td></td>
</tr>
<tr>
<td>Embryo transfer</td>
<td>Embryo(s) primary frozen (n=2)</td>
<td>n=2</td>
</tr>
<tr>
<td>Other</td>
<td>Intercurrent disease (n=6)</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>Relationship ended (n=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spontaneous pregnancy (n=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No IVF or IVF-ICSI treatment (n=1)</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary table 2: Baseline characteristics of the study patients.

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

<table>
<thead>
<tr>
<th></th>
<th>All women (n=303)</th>
<th>Inclusions (n=192)</th>
<th>Exclusions (n=111)</th>
<th>p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>23 (7.6%)</td>
<td>17 (8.9%)</td>
<td>6 (5.4%)</td>
<td>0.303†</td>
</tr>
<tr>
<td>No</td>
<td>277 (91.4%)</td>
<td>175 (91.1%)</td>
<td>102 (91.9%)</td>
<td></td>
</tr>
<tr>
<td>Missing*</td>
<td>3 (1.0%)</td>
<td>0 (0%)</td>
<td>3 (2.7%)</td>
<td></td>
</tr>
</tbody>
</table>

### Duration of infertility

<table>
<thead>
<tr>
<th></th>
<th>All women (n=303)</th>
<th>Inclusions (n=192)</th>
<th>Exclusions (n=111)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.39 (0.00-13.80)</td>
<td>2.35 (0.43-13.80)</td>
<td>2.40 (0.12-20.0)</td>
</tr>
</tbody>
</table>

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

† By Chi-square test.
‡ By Fisher’s exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
*these subcategories are excluded from analysis.
**comparisons are made between analysed category and the other categories combined.
Supplementary table 3: Univariate analysis of clinical characteristics of the IVF or IVF-ICSI procedure in women who became pregnant and women who did not become pregnant.

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

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

† By Chi-square test.
‡ By Fisher’s exact test.
§ By Independent Samples T-test.
¶ By Mann-Whitney U test.
*these subcategories are excluded from analysis.
**comparisons are made between analysed category and the other categories combined.
“There's only X amount of time. You can do whatever you want with that time. It's your time.”

Lou Reed
CHAPTER 8
The profiling of microbiota in vaginal and urine samples using 16s rRNA gene sequencing and IS-pro analysis

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ABSTRACT

16s rRNA gene sequencing is currently the most common way of determining the composition of microbiota. This technique has enabled many new discoveries to be made regarding the relevance of microbiota to the health of the host. However, compared to other diagnostic techniques, 16s rRNA gene sequencing is fairly costly and labor intensive, leaving room for other techniques to improve on these aspects. This study aimed to compare the output of 16s rRNA gene sequencing to the output of IS-pro analysis, using both vaginal swabs and urine samples from 297 women. 16s rRNA gene sequencing and IS-Pro analyses yielded very similar vaginal microbiome profiles, with a median Pearson's R$^2$ of 0.97. The low bacterial DNA loads of the urine samples prevented adequate analyses using 16s rRNA gene sequencing. However, comparing vaginal and urine samples from individual patients characterized by IS-pro with Pearson's R analysis showed a high correlation (median R$^2$ = 0.78). This indicates a high level of similarity between 16s rRNA gene sequencing and IS-pro results and between vaginal and urinary microbiota. From this we conclude that 16s rRNA gene sequencing and IS-pro can both be used to determine the microbiota composition. Interestingly, due to having only one amplification step in its pipeline, IS-pro appears to process lower load samples better than can be done through conventional 16s rRNA gene sequencing. Conversely, the quantity and format of output of 16s rRNA gene sequencing is better suited for deeper analysis of the results using currently available bioinformatics tools.

Importance

The microbiota in the vagina has been extensively studied through the use of 16s rRNA gene sequencing. However, compared to other diagnostic techniques, this takes a relatively long time and can be a labor intensive process. In this study we compared 16s rRNA gene sequencing with another technique called IS-pro. We found that, even though IS-pro analysis was comparatively easier and quicker to execute, results of the analysis were comparable to those of the 16s rRNA gene sequencing. Additionally, analyzing the urine samples was only possible with IS-pro, as the process is more capable of handling samples that do not contain large amounts of bacteria. We conclude that IS-pro is a suitable alternative for the profiling of vaginal and urinary microbiota in women.
INTRODUCTION

Bacterial microbiota in humans has received increasing attention over the past decade. Although links between microbiota and host health have been made for a long time, advances such as 16s rRNA gene sequencing have only recently made it possible to properly characterize an individual’s microbiome. This has led to many new links between host microbiome and disease(1, 2). Something that is less studied compared to the gut microbiome, but that is more relevant to women’s health, is the vaginal microbiome. Commensal microbiota in the vagina control pH levels through the production of lactic acid which is thought to provide a barrier to opportunistic pathogens.

Previous studies have shown that the vagina of healthy women is usually dominantly colonized by a large amount of one out of a limited number of different lactobacilli (3). The four most common of these lactobacilli dominant vaginal microbiome profiles are characterized by either L. iners, L. crispatus, L. gasseri, or L. jensenii. However, not all women have a Lactobacillus dominant vaginal flora, a condition which is associated with the clinical condition of Bacterial Vaginosis (BV). BV is a disruption of the ecological vaginal balance by overgrowth of typically non-Lactobacillus anaerobic bacteria (4). This results in an alteration of the milieu and composition of the vaginal microbiome (4, 5). BV is the most common vaginal disorder in women and occurs in up to 20% of pregnant women (6). Notable BV related bacteria are Gardnerella vaginalis, Mobiluncus spp., and Atopobium vaginae (7-9). Symptoms of BV include itchiness and a grey, watery discharge with a fishy odor. However, roughly 50% of the women who have BV are asymptomatic or have less obvious symptoms (10).

For decades the consensus has been that urine is sterile under normal circumstances. However, recently this claim has been disputed, suggesting that a distinct microbiome exists in the urinary tract with potential effects on host health (11). This urinary microbiome shows similarities with that of the vagina, however the amount of bacteria is generally much lower. The bacteria found in the urinary tract can be related to the vaginal flora of the same individual (12).

Microbiome profiling is becoming a highly important tool for diagnosis and prediction for a range of clinical phenomena. Currently, 16s rRNA gene sequencing are seen as the standard way of obtaining microbiome profiles. 16s rRNA gene sequencing, however, is still a relatively
expensive, and labor intensive procedure. As a cheaper and faster alternative, we have also included the IS-pro technique which is based on analysis of the length of the 16S–23S rRNA intergenic spacer (IS) region, which is indicative for bacterial species (13). IS-pro has the additional benefit of being able to process low load samples very efficiently.

In this study we aimed to perform an accurate comparison of microbiome profile outputs produced through 16s rRNA gene sequencing and IS-pro analysis, using vaginal swab and urine samples sequentially taken from women prior to IVF or IVF-ICSI treatment. The resulting data show to what extent 16s rRNA gene sequencing and IS-pro analysis are comparable in their ability to determine the microbiota compositions of women from both high and low load samples. Additionally, it gives insight into the microbiota found in the vagina and urine of women that are about to undergo IVF or IVF-ICSI.

**MATERIALS AND METHODS**

**Sampling**

Included in this study were 297 women attending reproductive clinics for IVF or IVF/ICSI treatment. Informed consent forms were obtained from all participants. Midstream urine samples and vaginal swabs were self-collected at one of eight participating reproductive health clinics from June 2015 until March 2016. Urine samples were stored at room temperature for a maximum 2 hours until further processing or storage at 2-8°C. Within 24 hours urine samples were vortexed and concentrated by centrifugation of 10 ml of urine for 10 min at 1500 RCF followed by resuspension in 1 ml of urine which was stored at -20°C. Vaginal swabs were collected by subject at the collection sites and directly placed in 0.5 ml of reduced transport fluid (RTF, Microbiome, Amsterdam, The Netherlands) at 2-8°C for a maximum of 2 hours, after which the swab is stored at -20°C. Samples were transferred on dry ice and stored at -20°C until further processing.

**DNA extraction and sample preparation**

DNA was extracted from concentrated urine and vaginal swabs suspensions with the Chemagen (Perkin-Elmer, Baesweiler, Germany) automated DNA extraction machine using the buccal swab extraction kit according to the manufacturer’s instructions. In short, swab suspensions and urine were thawed and vortexed. 200 µl of sample was incubated with
200 µl Chemagen lysis buffer and 10 µl Proteinase K at 56°C while shaking at 500 rpm. DNA was extracted with the protocol buccal Swab Prefilling. Elution of DNA was in 100µl of Chemagen Elution buffer.

Formation of the library
Sample DNA concentration was measured with the Picogreen dsDNA assay (Thermofisher, MA, USA). A PCR amplifying the V3/V4 region of the 16S rRNA gene region was performed with individually distinguishable dual index primer sets, which were developed to distinguish low diversity microbiomes, on each sample as has previously been described by Fadrosh et al. (2014) (14). The universal primer set 319F/806R, altered to also encode the Illumina sequencing primer and barcode labelling sequences, was used during the PCR. PCR conditions were as follows: 30 seconds at 98°C, then 30 cycles of 10 seconds at 98°C, 15 seconds at 58°C, and 15 seconds at 72°C and a final step of 3 minutes at 72°C.

The amplified DNA was purified with the AMPure XP magnetic bead assay (BeckmanCoulter Genomics, Danvers, MA, USA) quantified as above, recalculated into nM with the formula: 
\[
\text{[nM DNA]} = \text{DNA concentration (ng/µl)} \times 1e6 \text{ (µl/L)} / (\text{Sample fragment size in bp} \times 656.4 \text{ (g/mole)})
\]
and equalized to 12 nM. To ensure quality, pooled DNA that did not reach at least 8 nM was not used for 16s rRNA gene sequencing analysis.

16s rRNA gene sequencing 16s rRNA gene sequencing of the pooled samples was performed by the Tumor Genome Analysis Core group of the Department of Pathology at the VU University Medical Center in Amsterdam, The Netherlands with a Miseq tabletop sequencer (Illumina, San Diego, CA, USA).

Sequencing data analysis
Data generated through the 16s rRNA gene sequencing was processed with QIIME to remove primer and index sequences. A minimum Phred quality score threshold of 5 was upheld throughout the processing. Paired end reads with no errors in the barcode matching, a minimum overlap of six nucleotides, and a minimum combined length of 400 nucleotides were assembled to produce identifiable sequences. Operational Taxonomic Units (OTU) were picked with the Usearch method (15)@.. During this process the sequences were sorted based on length and abundance of identical reads, checked for chimeric sequences, and clustered at 97% identity to denoise the data. These OTUs were aligned to the reference
database with the PyNAST method for sequence alignment and subsequently assigned with the RDP classifier method which uses a Naïve Bayes classification. The assignment of OTUs used the database previously described by Srinivasan et al. (2012), assigning sequences on a genus to species level (16). The remaining sequences were BLASTed, and included if the sequence in question could be identified at a genus or species level.

**Intergenic spacer profiling (IS-pro)**
Amplification of 16S-23S rRNA intergenic spacer (IS)-regions was performed with the IS-pro assay (IS-diagnostics, Amsterdam, The Netherlands). IS-pro differentiates bacterial species by the length of the 16S–23S rRNA IS-region with taxonomic classification by phylum-specific fluorescently labeled PCR primers (13). The assay consists of two multiplex PCRs: one PCR contains two different fluorescently-labeled primers: one for the phyla Actinobacteria, Firmicutes, Fusobacteria and Verrucomicrobia and a second color for the phylum Bacteroidetes. A separate PCR is performed for the phylum Proteobacteria. The assay was performed according to the protocol provided by the manufacturer. Amplifications were carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). After PCR, 5 μl of PCR product was mixed with 20 μl formamide and 0.5 μl Mapmaker 1500 ROX-labeled size marker (BioVentures, Murfreesboro, TN, USA). DNA fragment analysis was performed on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). Species were assigned to peaks by using a database compiled of IS-pro fragments obtained from in-silico and in vitro IS-pro PCRs of known urine and vagina associated bacterial species. An internal amplification control (IAC) was used to control the PCR reaction for inhibition. A sample passed the quality control when the IAC signal was present in sufficient amount (3 of 5 IAC peaks >500 Relative Fluorescence Units (RFU)) or when a sufficiently high bacterial signal was present (at least one bacterial peak >20,000 RFU).

**Data analysis**
Alpha diversity of the microbiome per sample was measured by calculating the Shannon diversity index of individual samples. Relative abundance of microbiome per sample was used to perform a correlation clustering of all sample profiles according to the UPGMA method. Relative abundance for 16s rRNA gene sequencing data was calculated as a percentage of reads from total reads; for IS-pro, relative abundance is given as fluorescence intensity per peak as a percentage of total fluorescence. This data was then used to identify the major clusters making up the datasets. Pearson’s R linear regression was used
to compare abundance of species between samples. For Pearson’s R calculations only species that were available in both the 16s rRNA gene sequencing and IS-pro databases were included.

RESULTS

16s rRNA gene sequencing quality control
After sequencing of the vaginal samples, all reads were monitored for quality control purposes. A total of 294 (of 297) vaginal samples produced sequences matching the quality criteria. Sequencing of the DNA resulted in 17,947,706 reads. 8,374,321 reads passed quality control and were assigned to a taxon. Vaginal samples yielded a median of 9,661 reads per sample. During OTU calling, 75 species and 22 genera were assigned to the samples. An average of 29 (Stdev: 21.7) species or genera were assigned per vaginal sample. The two included sequencing controls yielded on average 9 reads after processing. Resulting taxonomic distributions can also be seen in Table S1. Pooled DNA from the urine samples did not meet the required 8nM of DNA for proper 16s rRNA gene sequencing analysis and was therefore not included in the 16s rRNA gene sequencing analysis.

16s rRNA gene sequencing results of vaginal samples
The heatmap in Figure 1 shows the microbiome profiles of the vaginal samples displaying relative abundance of bacterial species, clustered based on cosine-correlation. The Shannon diversity index of the samples shows a clear increase in diversity of the microbiome in profiles that are not clearly dominated by a single species, in most cases L. crispatus and L. iners.

Notable clusters identified through hierarchical clustering of the vaginal microbiome profiles included a L. crispatus dominant cluster including 132 samples, a L. gasseri dominant cluster including 17 samples, a L. iners dominant cluster including 74 samples, a diverse microbiome profile including 38 samples, and a L. jensenii dominant cluster including 22 samples. Eleven vaginal samples could not be ascribed to any cluster according to our clustering. These samples were characterized by a number of non-lactobacillus dominant bacteria, e.g. Leptotrichia or Prevotella that were not defining for the observed clusters.
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IS-pro results of vaginal samples

All of the 297 vaginal samples yielded results that passed the quality control as described in the Materials and Methods. The heatmap in Figure 2 shows the microbiome profiles of these vaginal samples characterized with IS-pro, displaying relative abundance of bacterial species clustered based on cosine-correlation. All IS-pro taxonomic distributions can be found in Table S2. Notable clusters identified through hierarchical clustering of the vaginal microbiome profiles were generally similar to clusters found with 16s rRNA gene sequencing for characterization and included a *L. crispatus* dominant cluster with 133 samples, a *L. gasseri* dominant cluster with 14 samples, a *L. iners* dominant cluster with 129 samples, a diverse microbiome profile with 12 samples, and a *L. jensenii* dominant cluster with seven samples. Two vaginal samples could not be ascribed to any specific cluster. These samples were characterized by a number of non-lactobacillus dominant bacteria, e.g. *Leptotrichia* or *Prevotella*.

IS-pro results of urine samples

The heatmap in Figure 3 shows the microbiome profiles of the urine samples characterized with IS-pro, displaying relative abundance of bacterial species, and clustered based on cosine-correlation. Notable clusters identified through hierarchical clustering include: a *L. crispatus* dominant cluster with 75 samples, a *L. gasseri* dominant cluster with 12 samples, a *L. iners* dominant cluster with 125 samples, a *L. jensenii* dominant cluster with six samples, and an *E. coli* dominant cluster with eight samples. A diverse microbiome was found in four samples. 26 urine samples could not be ascribed to any specific cluster. Internal amplification control was sufficient in 41 samples that did not provide any other signal and were therefore determined to be negative.
Figure 1: Heatmap of relative microbiome abundance found in vaginal samples obtained from 294 women through 16s rRNA gene sequencing. Column correlation clustering was performed with the UPGMA method to cluster microbiome profiles according to similarity. Row hierarchy clustering was performed to identify and order the most prominent taxa related to the microbiome profiles. Shown in the figure are the 20 most abundant taxa found in this correlation. The alpha diversity is shown in the bar graph using the Shannon diversity index in a sample order correlating with the above heatmap profiles.
Figure 2: Heatmap of relative microbiome abundance found in vaginal samples obtained from 297 women with IS-pro. Column correlation clustering was performed with the UPGMA method to cluster microbiome profiles according to similarity. Row hierarchy clustering was performed to identify and order the most prominent taxa related to the microbiome profiles. Shown in the figure are the 19 most abundant taxa found in this correlation. The alpha diversity is shown in the bar graph using the Shannon diversity index in a sample order correlating with the above heatmap profiles.
Figure 3: Heatmap of relative microbiome abundance found in urine samples obtained from 256 women attending reproductive clinics for IVF or IVF/ICSI treatment through IS-pro. Column correlation clustering was performed with the UPGMA method to cluster microbiome profiles according to similarity. Row hierarchy clustering was performed to identify and order the most prominent taxa related to the microbiome profiles. Shown in the figure are the 19 most abundant taxa found in this correlation. The alpha diversity is shown in the bar graph using the Shannon diversity index in a sample order correlating with the above heatmap profiles.
Comparison of 16s rRNA gene sequencing and IS-pro profiles of vaginal samples

Table 1 shows a cross table depicting distribution of vaginal sample profile clusters between 16s rRNA gene sequencing and IS-pro. The two methods yielded almost completely consistent L. crispatus cluster assignments. Sixty-eight samples were assigned to the L. iners cluster by both methods. To statistically determine the comparability of the cluster compositions of 16s rRNA gene sequencing and IS-pro, we further compared the results of 16s rRNA gene sequencing and IS-pro by calculating Pearson’s R correlation in paired samples from the same patient. This comparison showed a high correlation of the IS-pro and 16s rRNA gene sequencing results, with a median R² of 0.97 (Figure 4A and 4B).

Table 1: Distribution of vaginal sample cluster profiles between 16s rRNA gene sequencing results and IS-pro results, respectively. Only samples successfully analyzed by both techniques are shown.

<table>
<thead>
<tr>
<th>IS-pro</th>
<th>Divers</th>
<th>L. crispatus</th>
<th>L. gasseri</th>
<th>L. iners</th>
<th>L. jensenii</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divers</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. crispatus</td>
<td>4</td>
<td>113</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>L. iners</td>
<td>21</td>
<td>16</td>
<td>4</td>
<td>68</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>L. jensenii</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Comparison of vaginal and urine profiles in IS-pro

Next, we compared urine and vaginal profiles from individual patients using the IS-pro generated profiles. The Pearson’s calculations yielded a R squared of 0.78 (Figure 4C), indicative of a strong correlation between vaginal data and urine data. Furthermore, Figure 5 shows the total (5A) and relative (5B) abundance of bacteria for both samples of each subject on a horizontal line. In general, dominant species, such as L. crispatus (blue bars), L.gasseri (pink bars), and L. iners (yellow bars) are shared between vaginal and urine profiles of the same individual. Urine profiles of L. crispatus dominated individuals appear to contain a higher diversity of other bacteria than those of L. iners dominated individuals. E. coli has a high abundance in a number of urine samples.
Figure 4: Outcomes of Pearson’s R correlation where blue bars represent outcomes from analyses based on paired sample numbers, and green bars represent the same analyses where samples were not paired based on sample number. A & B Boxplot featuring R squared values of IS-pro vaginal sample outcomes correlated to those of 16s rRNA gene sequencing vaginal sample outcomes when samples are paired based on sample number (A) vs no pairing (B). C & D Boxplot featuring R squared values of IS-pro vaginal sample outcomes correlated to IS-pro urine sample outcomes when samples are paired based on sample number (C) vs no pairing (D). (Q1 = 1st quartile, Q3 = 3rd quartile)
Figure 5: Tornado plots where each horizontal line represents one subject with urine profiles on the left and vaginal profiles on the right of the vertical axis. White bars indicate samples that failed to yield data during analysis of one sample type. (A) IS-pro intensity distribution per subject depicting found taxa per color, and (B) the relative intensity of species signals found with IS-pro per subject. Included in the figure is a key clarifying which color depicts which taxa and showing the most relevant taxa found through IS-pro.

DISCUSSION

16s rRNA gene sequencing is currently the most common way of determining the composition of microbiota. However, compared to other diagnostic techniques, 16s rRNA gene sequencing is fairly costly and labor intensive, creating space for other techniques. In this study we show that 16s rRNA gene sequencing and IS-pro analysis produce comparable outcomes when profiling microbiota from vaginal swabs and urine samples. When sufficient DNA was present for IS-pro analysis, we found that microbiome profiles of urine samples were highly comparable to vaginal swabs, albeit with lower total loads.

The focus of our study was the comparison of two techniques used for microbiome profiling. 16s rRNA gene sequencing is currently seen as the gold standard for the profiling of microbiome. This is despite a lack of reproducibility between laboratory settings which is described in literature to be caused by, among other factors, the use of different DNA extraction procedures, library creation protocols, and/or sequencing equipment (17). In this study we show that vaginal microbiome profiling using the IS-pro technique creates outcomes highly comparable to those of the 16s rRNA gene sequencing. In addition, we show that the IS-pro is more capable of dealing with lower load samples, as urine samples could only be analyzed using the IS-pro due to load limitations that are set for 16s rRNA gene sequencing. This highlights the IS-pro technique as a test to be used in lieu of 16s rRNA gene sequencing for microbiome profiling, as was also shown in a previous study (13).

Even though the analyses were highly comparable, if we expand on the comparison of 16s rRNA gene sequencing and IS-pro techniques, we still observed a number of differences between them. Although the data produced by either 16s rRNA gene sequencing or IS-pro produced similar profiles there are a number of practical differences that are significant depending on the needs and limitations of the user. Microbiome profiling through 16s rRNA gene sequencing allows for the use of a vast array of bio-informatics tools that are the result
of many years of development as demand grew. This makes it more likely that complex custom demands can be applied on the user data. In contrast, IS-pro was developed with the goals of cost-effectiveness and simplicity in mind. This means that both the laboratory processing of the sample and the data-analysis were completed faster than for the 16s rRNA gene sequencing analysis, and at reduced cost.

In the results of the vaginal profiling using both 16s rRNA gene sequencing and IS-pro, profiles were found to be highly similar. However, when looking at the clustering data, there are still a number of differences in the amount of samples per cluster. This is especially apparent in the more diverse clusters. These differences are generally caused by small differences in abundance of species in profiles per technique. No formal criteria were used for clustering samples, but clusters were formed by performing a UPMGA clustering on a cosine correlation matrix. Therefore, small differences between samples may lead to different clustering outcomes.

The similarity of the vaginal and urinary samples can perhaps be attributed to contamination of urine samples by passage through the urethra and vulva, however this is a highly controversial topic. There are a number of fairly recent studies that do suggest there is a distinct urinary microbiota (18-22). The difficulty of obtaining samples from the urinary tract that are certain not to be contaminated by other microbiota as proof of the existence or non-existence of these microbiota has hindered clarifying research on this topic. In a study by Wolfe et al. suprapubic aspirates were obtained from patients specifically to avoid downstream contamination in the urinal tract (23). Even through use of this technique, a number of bacteria related to the vaginal microbiota were still found in the urine. These results were later confirmed in a study by Jacobs et al. who found that most bacteria in the urine were either Lactobacillus spp. or Gardnerella vaginalis (24). These findings, in combination with the results from our study, indicate that the microbiota found in the urine samples of this study are likely due to spillover of bacteria from the vagina and urethra.

Looking back at this study, some strengths and limitations should be discussed. The parallel analyses of 16s rRNA gene sequencing and IS-pro give a unique opportunity to compare the techniques with regards to output quality which are factors that could not be properly assessed if only one technique had been used. Furthermore, this serves as a check to see if any one technique obtains unexpected results compared to the other.
As a first limitation, the samples collected for this study were collected from women who were about to undergo an IVF or IVF-ICSI treatment due to subfertility. Although vaginal microbiome composition distributions found in this study showed similar distributions as other studies into the vaginal microbiome, it is still possible that this sampling group instigated a sampling bias for certain profiles. Second, the databases used for the 16s rRNA gene sequencing and IS-pro data processing did not completely overlap and were specifically composed for the vaginal microbiome. This may have resulted in missing bacteria species in either technique. Finally, it is likely that potential PCR bias plays a more significant role in the 16s rRNA gene sequencing procedure than in the IS-pro analysis as the former entails two PCR reactions. The strict 16s rRNA gene sequencing input criteria aiming at negating the effect of the resulting amplification competition is the reason why urine samples analyzed through 16s rRNA gene sequencing could not be included in this study.

The low loads of bacterial DNA prevented us from using 16s rRNA gene sequencing to profile urine samples in this study. In fact, the median DNA concentration in urine samples was 108 ng/ml, compared to 7007 ng/ml in the vaginal samples (data not shown). Besides the fact that this made the urine samples impossible to use in 16s rRNA gene sequencing analysis, we can also interpret from this data that the bacterial abundance in the urinary tract is very low.

In conclusion 16s rRNA gene sequencing and IS-pro analysis produce highly comparable results when analyzing microbiota collected with vaginal swabs. We also conclude that IS-pro is more suitable for analysis of microbiota collected through urine sampling, as the low bacterial load does not allow for analysis with standard 16s rRNA gene sequencing protocols. IS-pro analysis has the potential to increase speed and reduce costs of these analyses while maintaining the quality of the profiling, hopefully allowing for more research to take place into the vaginal microbiome.
DEKLARATIONS

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Author’s contributions
MS performed the 16S rRNA gene sequencing and analyses. RK performed the sample collection. MPB performed the DNA extraction and assisted with the 16S rRNA gene sequencing data analysis. LP performed IS-pro analysis and data processing. JSEL, SAM, PHMS and AEB conceived and designed the experiments. Additionally, AEB performed the majority of IS-pro data analysis. MS, MPB and AEB drafted the manuscript and all authors read and approved its final version.

Competing interests
The authors would like to state a number of competing interests. The authors P.H.M. Savelkoul and A.E. Budding are co-owners of IS-diagnostics Ltd., which is the company that developed the IS-pro technique. The author R. Koedooder reports that she is an employee at ARTPred B.V. during her PhD at Erasmus Medical Centre. Joop S.E. Laven reports consultancy fees from Titus Health Care.

Ethics approval and consent to participate
The medical ethics testing committee Erasmus MC has approved the ethicality of the study under reference MEC-2014-455. All participants were informed of the study contents and signed an informed consent form before inclusion into the study.

Availability of data and materials
The datasets generated and/or analysed during the current study are available in the SRA database repository, https://www.ncbi.nlm.nih.gov/sra/SRP133380.
The database that supports the IS-pro findings of this study are available from IS-Diagnostics Ltd. but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of IS-Diagnostics Ltd.

**Supplemental Files**

Due to their size, the supplemental files of this article are not shown in this thesis. They are available upon request, and will also be available at the time this article is published.
REFERENCES


“The first principle is that you must not fool yourself and you are the easiest person to fool.”

Richard P. Feynman
DISCUSSION
GENERAL DISCUSSION

This thesis explores the interactions between bacterial STIs present in the female genital tract, the vaginal microbiota, and the response of – and consequences to – the host. A focus is put on clarifying the underlying biological causes of differences in the clinical presentation of bacterial STIs. In addition to this focus, we assess the impact of the vaginal microbiota on the outcome of in vitro fertilisation (IVF) treatments. In the future this information may allow us to modulate the vaginal microbiota in order to affect IVF treatment outcomes. In the discussion of this thesis we compare data resulting from the previous chapters with the state of art data. Moreover, possible consequences and opportunities of the aggregated chapter results are discussed, after which recommendations are made.

The three chapters making up Part 1: “Pathogens in the vaginal tract and their interaction with the immune system” examine cytokine expression, bacterial genetics, and host genetics as three immunological factors that influence the immune response to – and clinical presentation of – bacterial STIs. Through meta-analysis of the current knowledge regarding cytokine expression during infection with bacterial STIs, we showed TLRs and pro-inflammatory cytokines that have significant effect on the severity of infections with bacterial STIs in Chapter 1. In Chapter 2 we show that the potential of bacterial STIs to stimulate or inhibit the TLR9 NF-κB pathway through CpG DNA motifs present in the bacterial DNA is highly variable between pathogens, with N. gonorrhoeae and T. pallidum being clear outliers. Finally, Chapter 3 shows that TLR9 and IL-10 are significantly associated with the severity of experimental infections with H. ducreyi.

Part 2: “The interaction between the vaginal microbiota and host reproductive health” comprises four chapters showing the interactions of the vaginal microbiota with pathogen infection processes, as well as with the host reproductive process. Dysbiotic microbiota compositions such as bacterial vaginosis are of particular interest in these chapters. Chapter 4 shows, through review of the current literature, a difference in the interactions of C. trachomatis and M. genitalium with microbiota related factors such as pH, H₂O₂, and tryptophan. Additionally, it highlights that knowledge of M. genitalium and its interactions with the host and microbiota lags behind the better studied C. trachomatis. In Chapter 5 a review and meta-analysis shows that dysbiotic microbiota profiles negatively affect early pregnancy development stages. Subsequently, the effect of the vaginal microbiota
on the outcome of IVF treatments is examined in Chapters 6 and 7. These two chapters provide an algorithm that can determine the likelihood of an IVF treatment to fail based on the microbiota composition at the start of the treatment. Finally, in Chapter 8 we showed that 16s rRNA sequencing and IS-pro analysis can both be used to determine the vaginal microbiota profile in vaginal swabs, while IS-pro analysis was more capable of determining the microbiota profile from urine samples.

Part 1: Pathogens in the vaginal tract and their interaction with the immune system

The effect of variety in expression of cytokines after infection with bacterial STIs

The connection between cytokines expression variations and the impact of this expression on the severity of clinical infections is a well-established fact. From mental disorders like depression, to infections with for example Streptococcus, these variations have been linked to the severity of disease (1-4). In chapter 1 we found that for C. trachomatis in particular, an extensive knowledgebase allowed us to make several strong associations between cytokine expression and complications such as tubal factor infertility. Similar associations could be made for N. gonorrhoeae and T. pallidum. As an alternative to the host mediated differences found in this chapter, differences in bacterial antigenic factors can also lead to increases and decreases in immune response of the host. The most common antigenic factors that are known to cause these differences in immune response are the virulence factors of bacteria, which often lead to an increase in symptom severity in the host (5). Nonetheless, every part of the bacteria that comes into contact with the immune system has the potential to produce a disproportionate immune response instead of the commonly expected and desired immune response.

Initial contact of antigens with the innate immune system commonly occurs through interaction of the bacteria with the pattern recognition receptors (PRR) such as Toll like receptors. In chapter 2 we examine bacterial unmethylated CpG DNA, which acts as the ligand to the PRR TLR9. Previous research has already shown that specific CpG DNA motifs can affect the host immune response (6-8). For example, the Herpes simplex virus DNA was shown to contain a high amount of immune-stimulatory CpG DNA motifs, which elicits more pro-inflammatory cytokine production after binding with TLR9 (8). The lowest CpG index we found was -77.1 for N. gonorrhoeae, showing a strong inhibitory potential for TLR9 activation by N. gonorrhoeae and making it the only included pathogen to reach an inhibitory index value. Interestingly, a study by Sanders et al. has shown a CpG index of -106.8 for N. meningitides, indicating that
a such a low CpG index is genus-specific (7). The highest CpG index found for an STI was 17.7 for *T. pallidum*, the bacteria which causes syphilis. The height of the index score brings *T. pallidum* close to the CpG index of *E. coli* at 21.1, which was shown *in vitro* to stimulate the production of relatively high amounts of TLR9 related cytokines compared to other common bacteria (9). CpG DNA is not the only antigenic factor with the potential to affect the innate immune response, as variations in a number of other bacterial antigenic factors have been shown to affect the expression of cytokines through other PRR pathways.

One other example of an antigenic factor that can affect the innate immune response is Lipid A, which is the specific section of lipopolysaccharide (LPS) that interacts with TLR4 (10). Lipid A shows a high diversity, with characteristics such as its location in the LPS structure and molecular build being highly variable depending on the bacterial species and strain (11-13). An example of this variability is shown in figure 1, where immunogenic differences between Lipid A molecules found in *Escherichia coli* and *Helicobacter pylori* are highlighted. This variability has been shown to affect host TLR4 in recognition of LPS as its target (11). Subsequently, the ability to properly recognise LPS through TLR4 has been shown to directly impact the clinical presentation of infection, leading to differences in symptom severity in patients (11, 12, 14, 15). A similar effect, in which differences in bacterial peptidoglycans affect the stimulation of TLR2, exists as well (16, 17). It is important to note the various redundancies in the host immune system. Bacterial antigenic factors impacting the stimulation of one PRR do not affect the interactions of other bacterial antigens with the respective PRR for that antigen. Therefore the inhibition of one PRR pathway does not necessarily translate into an increased or decreased immune response. Nevertheless, this thesis has added to the knowledge of how bacterial STI genetics can affect the expression of cytokines through TLR9 activation to affect the host immune response to - and clinical presentation of- bacterial STIs.
The impact of host genetics on the immune reaction to bacterial STIs

In concordance with the immune response expression affected by bacterial factors, host genetic factors also play a major role in the expression of cytokines during the initial immune response. In chapter 3 we show to what extent host genetics affect the severity of infections with the lesser known STI *H. ducreyi*. Single nucleotide polymorphisms (SNP) in the genes coding for TLR9 and IL10 were related to pustule formation during the disease. This chapter is the first to show a relation between host genetics and *H. ducreyi* severity. The discovered relations are in line with relations that have been made previously for other bacterial STIs.

Notably, the much more extensively studied *C. trachomatis* has a large number of host genetic relations with both susceptibility and severity of the infection. Currently, sixty-nine different genetic relations have been made to the STI chlamydia. These relations include a wide variety of PRRs, cytokines, chemokine receptors, and other genes related to the innate immune response of the host. The relations with TLR2, TLR4, IL10, and TNFA have
the most studies devoted to them (18-22). These relations partially overlap with those found for \textit{H. ducreyi} infections, where TLR9 and IL10 are the strongest contributors to the immune response and disease presentation. The host genetics related to the susceptibility and severity of \textit{C. trachomatis} infections have also been shown to affect the development and presentation of later complications of chlamydia. There is an opportunity for future studies to examine whether \textit{H. ducreyi} and recurrence of infection or tissue scarring after a patient has suffered from chancroids are similarly affected by host genetic factors.

Another well studied STI with regards to host genetic relations is gonorrhoea, which is caused by \textit{N. gonorrhoeae}. There are nine genes reported to be related to susceptibility and/or severity of the disease. Studies confirm that during \textit{N. gonorrhoeae} infection, as with \textit{C. trachomatis} and \textit{H. ducreyi}, PRRs and inflammatory cytokines are the most relevant to the outcome of the disease. Most host genetic relations between \textit{N. gonorrhoeae} infections and susceptibility or severity of the disease have been reported for TLR1, TLR2, TLR4, and TLR6 (23, 24). Regrettably, many other bacterial STIs that are not as well-known as \textit{C. trachomatis} or \textit{N. gonorrhoeae} do not have the same amount of knowledge available with regards to host genetic variation in relation to susceptibility to and severity of the infection. Our study in chapter 3 reduced this gap of knowledge for \textit{H. ducreyi} infections.

The methodology of chapter 3 is relatively unique, and provided us with a valuable angle of approach for host genetic analysis. Severity of \textit{H. ducreyi} infections is known to vary between individual patients. By experimentally infecting volunteers, we took away the possibility for the difference in severity to be caused by bacterial load or strain of the bacterium, leaving only host genetics as a potential influencing factor. By analysing selected inflammation related genes we showed the first relations between host genetics and \textit{H. ducreyi} infection. Furthermore, the results of the study are found for subdermal experimental infections in the arm. These results can likely be carried over to infections in or near the genitals due to the similar functional effect of the genes in which the SNPs were found. Nevertheless, potential differences of expression of these genes in the genital tract should still be accounted for in future studies.

The increasing interest in using host genetics in medical practice means that there is significant support for medical application of host genetic knowledge and relations with infectious diseases. Already, a test using host genetics for prediction of later complications of \textit{C. trachomatis} infections would be valuable.
trachomatis is in development (25). The similarity of related host genes between bacterial STIs may be used as basis for experiments examining other STIs. However, all current studies point towards bacteria specific interactions being crucial for relations to form. In light of our findings in chapter 2 of this thesis, N. gonorrhoeae was shown to have an especially low stimulation potential for TLR9. Such bacterium specific characteristics are one reason that overlap of genetic relations between bacterial STIs should not be assumed. Another reason is the varying level of impact host genetics may have. For C. trachomatis it has been described, through the in vitro study of infections in cells of twins, that approximately 40% of the variation between disease severity is caused by host genetic variation (26). Another study using this twin study design noted that for respiratory syncytial virus infection the host genetics accounted for 16% of the infection severity (27). Although this infection is not very comparable to C. trachomatis, and is only included here due to lack of similar study designs for more relatable diseases, it shows that the role of host genetics in severity of infections can be highly variable and should therefore not be easily assumed.

In the previous paragraphs the associations between host genetics and STIs have been discussed from a viewpoint primarily focussed on the meaning for the host immune system. Interestingly, the host genetic relations can also tell us something about specific aspects of the pathogenesis of the studied bacteria. In the case of H. ducreyi, the relation between the infection and TLR9, even though other PRRs were also investigated, shows that there may be characteristics of H. ducreyi that actively work against recognition by other PRRs in the host. This potential evasion of detection by the innate immune system is especially notable in the interaction between H. ducreyi and TLR4. H. ducreyi is a gram negative bacteria and therefore consist of a significant amount of LPS. Nevertheless, mutations affecting the functionality of TLR4 did not show a significant influence on the course of the infection. The fact that TLR4 does mediate infections of the genetically closely related H. influenza could indicate that the composition of the LPS molecule is not the primary reason for the lack of TLR4 activation (28-30). Further research may find that H. ducreyi carries a species specific mechanism for minimizing interactions with TLR4.

Through the work in the first part of this thesis we have shown the extent of current knowledge into cytokine expression during bacterial STIs, the differences in inflammatory potential between the DNA of bacterial STIs, and have for the first time shown an association between host genetic variations and the outcome of H. ducreyi infections. For C. trachomatis and
N. gonorrhoeae our results support further translational research. For lesser known and studied bacterial STIs the results of this part of the thesis especially support further research concerning host genetic variation and the relation the host genetics have with the outcome of the bacterial STIs.

**Part 2: The interaction between the vaginal microbiota and host reproductive health**

*The extent of the microbiota in the female genital tract*

In chapters 4 to 8 of this thesis the vaginal microbiota is studied as a proxy marker for effects that the female genital tract microbiota can have on pregnancy and IVF outcomes. Indeed, most of the knowledge we have about the microbiota in the female genital tract comes from previous research into the vaginal microbiome. It is often posited that microbiota found further up the female genital tract, such as the endometrial microbiota, can more accurately portray the effects on the reproductive process because these microbiota are in closer contact with the reproductive process (31, 32). Unfortunately, whereas compositions of bacteria in the vaginal microbiota are well studied with regards to composition and potential impact on host health, research into other microbiota in the female genital tract is generally lacking. Contamination during sampling is an important reason that studies into these microbiota are less reliable. For instance, during the process for sampling the endometrial microbiota the tools for sampling are passed through the vagina/cervix where high bacterial loads are present. For this reason, sampling is most often done through the use of catheters that are also used for placement of embryos during IVF treatment (33). Even then, contamination is an issue in these studies. Additionally, studies such as the one performed in chapter 7 of this thesis show that the use of the vaginal microbiota as a proxy measurement is sufficient for determining reproductive effects, lowering the need for more complicated sampling methods.

As is described in a study by Tao et al, proper sampling and analysis of the endometrial microbiota is often hindered by potential contamination by the vaginal microbiota (33). Regardless, there is significant interest from the scientific community in research into the microbiota in the upper female genital tract. One reason for this is that, unlike what was found for microbiota in urine in chapter 8, significant differences have been found in the composition of these microbiota when compared to the vaginal microbiota (31-37). Previously, it was commonly thought that the microbiota found in the upper female genital tract was a diluted run off from the microbiota in the vagina (38). This was proven to be
incorrect, as the microbiota in the upper female genital tract can be divided even further. Unique compositions have been found on the endometrium, in the uterus in general, and even in the fallopian tubes (32, 34-36). The presence of these differing microbiota suggests a complex situation regarding interactions between microbiota and host, as multiple significantly different microbiota appear capable of impacting the host reproductive health at the same time. This situation creates opportunity for further research to elucidate the relative impact of each microbiota on the host reproductive health. Regardless, the vaginal microbiota will likely continue to be studied as a proxy for microbiota in the female genital tract for the foreseeable future.

**The role of the vaginal microbiota in infection with bacterial STIs**

The vaginal microbiota has an effect on the susceptibility to a number of STIs, both bacterial and viral (39-42). An example of this interaction featuring the vaginal microbiome and *C. trachomatis* or *M. genitalium* is described in chapter 4 of this thesis. Chapter 4 also reviews the current knowledge on the microbiota interacting with bacterial STIs, and how it is focussed primarily on the best studied STIs. We showed that there appears to be a common theme in the negative effect on the host when there is a dysbiotic vaginal microbiota. That said, specific interactions between pathogens and bacteria making up the microbiota are often pathogen specific (43-48). An example of one such specific interaction is the presence of tryptophan producing *Prevotella* being beneficial specifically for *C. trachomatis*. Addressing the gap in knowledge for less studied STIs may lead to discovery of interesting bacterium specific interactions with the host.

The knowledge of interactions between the vaginal microbiota and bacterial STIs can be used to further our understanding of the pathogenesis of these STIs. A recent study has comprehensively charted the immune response to dysbiotic microbiota in the vaginal tract (49). In this study, the markers IL1ra and IL2 were found to be specifically increased during symptomatic bacterial vaginosis (BV), while the biomarker expression profile of intermediate microbiota was characterised by increases in expression of IL1β, IL8, MIG, MIP1α, and RANTES. This information opens up opportunities to specifically study bacterial STIs that have host genetic associations to disease progression, in patients that have microbiota compositions that increase the expression of these biomarkers. One such example can be derived from chapter 3 of this thesis. None of the factors that were associated to dysbiosis in the study of Campisciano et al. were shown to have an effect on the severity
of *H. ducreyi* infections (49). Through the lack of these associations we can speculate that the immunological factors related to the vaginal microbiota do not significantly affect *H. ducreyi* during infection.

As associations between the vaginal microbiome and susceptibility and severity are present for most major STIs there may be potential to use this knowledge for intervention. A number of strategies for modulation of the vaginal microbiome have gained in popularity in the past decade (50-52). Notably, there are a number of studies that describe successful long term change of the vaginal microbiome composition from dysbiotic states to healthy states (53-58). The modulation of the microbiome is achieved by treating the patient with the standard treatment for BV, which is Metronidazol, and following this up with a *Lactobacillus*-based probiotic. Table 1 gives a more detailed overviewed of the treatment protocols in the studies. It would be interesting to examine a potential relation between the modulation of the vaginal microbiota through for instance probiotics, with a positive effect on the susceptibility to – or severity of – STIs. Microbiome modulation strategies are further expanded upon in a later part of this discussion.
How the reproductive process affects the vaginal microbiota

As touched on in chapter 4 of this thesis, the vaginal microbiota is constantly influenced by the hormonal levels of the female host (59). Most notably estradiol and progesterone. This influence is so outspoken that the use of hormonal contraceptives can noticeably change the vaginal microbiota composition towards a more Lactobacillus dominated state (60). Interestingly, the strong increase of these hormones during pregnancy can also lead to a lower diversity, and a generally higher abundance of Lactobacillus bacteria (61). Reference values for estradiol and progesterone are more than 100 times higher in pregnant women than in non-pregnant women (62). The height of these values appears to lead to a defensive effect in women whose immune system is less effective due to pregnancy.

The move away from a dysbiotic microbiome composition makes the host less susceptible to STIs. Nevertheless, a direct association between pregnancy and a decreased susceptibility
to bacterial STIs has yet to be made. Conversely, the use of hormonal contraception has directly been associated to reduced susceptibility to STDs (63). One factor that can affect a potential association between hormonal levels and bacterial STIs in pregnant women, is that during pregnancy the host immune system is significantly downregulated. This downregulated immune system leads to an impaired ability to combat infections in the host. Kourtis et al. produced a list of infections that women are either more susceptible to during pregnancy or which produce a more severe infection during pregnancy (64). Notably Kourtis et al.’s study did not include bacterial STIs. Nevertheless, they found that an increase in severity of infection was far more common than an increase in susceptibility to the infections. There is a clear opportunity for a study investigating the associations between hormonal levels in pregnant women and susceptibility to bacterial STIs, and we recommend researchers to take special note to control for various aspects of risk behaviour and other confounders.

**How the vaginal microbiota affects the reproductive process**

The effect that microbiota in the female genital tract has on pregnancies is a subject that has received much attention from clinical professionals. Because of this attention the effects microbiota have on pregnancies have been well documented. Most notable are the effects of BV on the outcome of pregnancies (65-67). BV has been strongly related to pre-term birth, maternal infectious morbidity, and miscarriage (65, 66). These characteristics are well described for normal pregnancies, but recently additional effort has been made to find the associations between microbiota compositions and assisted reproductive technology. **Chapter 5** discusses the current knowledge related to the vaginal microbiota and its relation with IVF outcomes. Showing that there is a commonly found negative effect on IVF outcome when affected by BV. Furthermore, the results presented in **chapter 7** show that specific microbiota compositions influence the outcome of IVF in such a way, that a prediction can be made of the outcome of the IVF treatment based on the microbiota compositions.

The main differences between normal pregnancies and IVF induced pregnancies are the strictly defined periods of hormonal variations during IVF. These strict periods have made the microbiota easier to study during the IVF process. Interestingly, a negative association has been made between the contamination of the IVF catheter tip and outcome of IVF treatments (68, 69). This contamination of the catheter tip occurs during the passage of the tip past the vagina and cervix, where resident microbiota are highly abundant. This contamination has such an impact on the pregnancy rates after IVF that some treatment
centres administer prophylactic antibiotics to reduce the chance of contamination (70, 71). However, the clinical usefulness of these prophylactic antibiotics, especially concerning non-discriminatory use of prophylaxis, is still under debate (71, 72).

There is still uncertainty about the source and mechanisms behind the influence of microbiota on the reproductive process. As discussed in an earlier section of this discussion, microbiota can be found in the uterus and in the fallopian tubes as well (31, 32, 34-36). Although these microbiota have not been as extensively studied as the vaginal microbiota, associations between the presence and compositions of these microbiota and conception have been described (73, 74). Specifically the inflammation related to the presence of these microbiota has been pointed at as the main mechanisms through which conception and pregnancy outcomes are affected (75). The novelty of these topics makes it so that opportunity for further research focussing on confirmation and discovery is still plentiful.

The opportunities for vaginal modulation before pregnancy
The concept of adjusting or replacing an unwanted vaginal microbiome has been a focus point for a number of recent studies. The most frequently researched way of altering the vaginal microbiome is through the administration of probiotics, which are usually introduced to the vagina through vaginal tablets. In one study *Lactobacillus* based vaginal tablets were able to replace BV with a normal or intermediate vaginal microbiome, while only 12% of non-treated patients showed improvement (76). Treated patients were also less likely to have recurring instances of BV. More effective and long-term adjustment to a *Lactobacillus* dominated microbiome was obtained through the application of a combination of antibiotics and probiotics (56, 77, 78). Additionally, improvement of the vaginal microbiome during the later stages of pregnancy was also seen in women taking a probiotic mixture of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains (79). Most notably, there was an overall increase of anti-inflammatory effects observed in the vaginal microbiome. Local inflammation during pregnancy has long been related to pre-term births, so probiotics promoting an anti-inflammatory state likely reduce the amount of morbidity and mortality related to pre-term births.

Another way to adjust the vaginal microbiota is through the use of antimicrobials, often primarily used as a treatment of infection or BV. Giving a patient with BV the antibiotic Rifaximin over a course of five days has been shown to decrease BV related bacteria in the vaginal microbiome (80). Notably it was also shown that there was an increase in *Lactobacillus*
during and after the Rifaximin treatment. Similarly, treatment of BV using Metronizadol secreted by an intra-vaginal ring showed decrease of BV related characteristics in the vaginal microbiome (81). More recently it was shown that a combination of tinidazole treatment and probiotic *L. rhamnosus* treatment increased the amount of other *Lactobacillus* species in the vagina (51). Probiotic treatment is important, as BV related bacteria quickly recolonize the vaginal space after antibiotic treatment has stopped (82).

Although in a previous section of this discussion it was shown that hormonal contraceptives have been related to healthy microbiota compositions, the use of these hormonal contraceptives with the intent of modulating the vaginal microbiota did not result in the shift from a dysbiotic to a healthy microbiota. These results suggest that a strategy of hormonal change may not be the best avenue to alter the vaginal microbiome (83). Vaginal application of a sucrose based gel does show a capacity to change the vaginal microbiota from BV to a *Lactobacillus* based microbiota composition (84). Besides these methods a number of other female hygiene products, such as vaginal douches, have the unintended side-effect of altering the vaginal microbiome. Unfortunately, this alteration was shown to make the user more susceptible to BV (85).

It is important to note that, even though modulation of the microbiota in theory should have a positive effect on pregnancy rates and outcomes, there is still some controversy on this topic. One study has shown that, even though there was successful modulation of the vaginal microbiota, there was no noticeable effect on clinical pregnancy rates (73). It is likely that the topics covered in this discussion, such as the relation between microbiota in the upper female genital tract and pregnancy, make intervention strategies more complex than they may first appear.

The second part of this thesis has shown that there is a significant association between the composition of the vaginal microbiota and the way the immune system of women interacts with STDs. Additionally, it shows an important association between the composition of the vaginal microbiota and the outcome of IVF treatments. The contributions of this thesis have led to better definitions regarding the vaginal microbiota, but through them we have also taken valuable steps towards implementing the current knowledge of the vaginal microbiota into clinical practice.
Overview: Bacterial interactions in the female genital tract seen at a glance

Figure 2A: To clarify our findings, the interactions discussed in this thesis are illustrated here as a vaginal tract featuring the vagina and the cervix. Figure 2B gives a more detailed view of the microbiota at the cervix and the interactions with the reproductive process. Figure 2C on the right expands on interactions between STIs, and the microbiota protecting the host.

Figure 2B (Chapter 5-8): A microbiome dominated by a variety of BV related bacteria reduces the chance for IVF-induced pregnancy to occur, and for a positive pregnancy outcome. Interestingly, increased hormones during IVF treatment and pregnancy benefit Lactobacillus. Leading to a healthier microbiome in pregnant women.

Figure 2C (Chapter 4): A Lactobacillus dominated microbiome acts as barrier and keeps the pH value low to combat STI growth and infection. BV creates a weakened barrier and a higher pH. Additionally, BV related Prevotella produces tryptophan, which C. trachomatis needs for growth and reproduction.

Figure 2D (Chapter 1-3): TLR9 stimulation leads to secretion of cytokines. Inhibitory CpG DNA motifs or less functional TLR9 can lessen the secreted cytokines, weakening the inflammatory response.
CONCLUSIONS, RECOMMENDATIONS, AND FUTURE PERSPECTIVES

Part 1: Pathogens in the vaginal tract and their interaction with the immune system

Conclusions

In conclusion, the research described in part 1 of this thesis has led to an increased understanding of the interactions between bacterial STIs and the host. Our knowledge of these interactions has contributed on the following three levels. First, we revealed that differences in cytokine expression of hosts impacts the severity of *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* infections. Second, we have shown the inflammatory potential of bacterial STIs based on the presence and abundance of CpG DNA motifs in bacterial STI DNA. Third, the current understanding of host genetic factors was expanded by our study showing that TLR9 and IL10 are innate immune response related genes that influenced the production of pustules during *H. ducreyi* infections. Depending on the SNPs present in TLR9 and IL10, the influence could both be an increased or decreased production of pustules through polymorphisms in either gene.

Recommendations

Based on our findings, we recommend more attention is paid to the interactions between the host and lesser known STIs. Chapter 1 and chapter 3 showed that there is a lot of potential for discovery regarding the interactions between the host and less common bacterial STIs. These interactions are likely to affect the severity of the disease, as is shown for *C. trachomatis*, *N. gonorrhoeae* and *T. pallidum* in chapter 1. As an example of a lesser known STI, the influence of host genetics on the severity of *H. ducreyi* is shown in chapter 3. Studying these interactions may lead to improvement of the treatment of the lesser known infections in the future by taking the potential severity of the diseases into account.

Future perspectives

There are many future opportunities regarding the interactions between bacterial STIs and the host immune system. An effort is currently being made to implement a host genetic test which determines the potential for tubal factor infertility due to a previous *C. trachomatis* infection (25). If this test proves successful, similar tests may be developed for late complications of other bacterial STIs as well. As a possible example, a similar test may be
used to determine the likelihood of a *T. pallidum* infection to develop into the most severe stage of neurosyphilis.

Besides potential clinical benefits, opportunities for basic science exist as well. The *in silico* CpG index data from chapter 2 can be followed up in an *in vitro* experiment to corroborate the inflammatory potential of the bacterial DNA. Using available strictly TLR9 expressing cell lines, the expression of inflammatory cytokines after adding bacterial DNA and oligonucleotides of the inhibitory and stimulatory CpG motifs can be effectively measured. This would provide a nice direct comparison of *in silico* and *in vitro* data.

**Part 2: The interaction between the vaginal microbiota and host reproductive health**

*Conclusions*

From our research into the host and pathogen interactions with the vaginal microbiota we can conclude that these interactions are important factors for the general and reproductive health of the host. We reviewed current literature showing that *C. trachomatis* and *M. genitalium* are differentially affected by the microbiota, primarily based on molecular differences such as the pH or H₂O₂ levels, which can be related to dysbiosis of the vaginal microbiome. To experimentally point out another risk related to dysbiosis in the vaginal microbiota, we showed in a cohort of women attempting an IVF treatment that BV can be linked to the failure of IVF. The effect is so well-defined, that we were able to produce an algorithm that accurately predicts the failure of IVF based on the vaginal microbiota.

*Recommendations*

A need for additional study of upper female genital tract residing microbiota, such as microbiota in the uterus and fallopian tubes, was shown in the discussion of this thesis. Currently, there is a small number of studies showing potential associations between these microbiota and the reproductive process. More research is needed to clarify if these associations are different from the currently known associations between the vaginal microbiota and the host’s reproductive health. Additionally, we recommend that additional studies be performed to confirm current understandings regarding upper female genital tract residing microbiota. The associations between the vaginal microbiota and host reproductive health were, over time, found to be highly complex. Chapter 7 exemplifies this by showing that specific bacterial species impact the host reproductive health, rather than the entire composition
of (dysbiotic) microbiota. It is likely that similar specific interactions also apply in the case of microbiota in the upper female genital tract.

**Future perspectives**

Finally, we must now look into translating the results found in chapter 7 into a standard health care practices. Before this can be done, we need to know how long a (dysbiotic) microbiota composition remains stable over time. This will help us determine when a new IVF treatment and ReceptIVFity test can be offered to a patient after a dysbiotic microbiota has been found initially. A study to examine this is currently being set up, and is expected to start inclusion of patients in 2019. Additionally, we would like to be able to offer women with a microbiota composition that predicts a negative IVF outcome a solution to this problem. Therefore, a strategy for vaginal modulation is being devised, to help women obtain a vaginal microbiota that is more likely to lead to a successful IVF treatment. This study is expected to finish in 2020. Lastly, efforts are being made to include the prediction test into insurance covered health care in 2019.

Clinically there are exciting new prospects on the horizon. Implementation of microbiota related diagnostics and interventions are just now starting to become available. Vaginal microbiome modulation is likely to be a topic that garners increasing attention in the coming years. The treatment of recurring BV, which is estimated to occur in roughly 30% of the treated BV cases, using *Lactobacillus* based probiotic vaginal modulation has already shown a number of successes.
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SUMMARY

This thesis examines and describes interactions between pathogens, microbiota, and the host as they happen in the female genital tract. Additionally, it looks into the effects of these interactions on the risks of being affected by infections, disease, and fertility complications. In Part 1 a specific focus is put on the interactions of invading pathogens and the immune system of the host, while in Part 2 the relations between the vaginal microbiota, potential pathogens, and the host’s reproductive health are further examined.

There is often a large discrepancy between patients in their clinical presentation while having the same infection in the vaginal tract. This discrepancy is most noticeable in the difference between symptomatic and asymptomatic patients. Although there are many potential reasons for the different presentation of these diseases between patients (e.g. diet, physical health, smoking, the composition of the vaginal microbiota), in Part 1 we focus on the immune response as it is produced by the host against invading pathogens. This is done by studying potential factors influencing the ability of the host to effectively deal with the invading pathogen. The genetic makeup of the host may not always accurately reflect his or her potential of fighting of an infection. Therefore, we look at the variation in expression of cytokines upon infection between patients. The levels of actually expressed cytokines give a more direct picture of this potential and how this affects the clinical presentation of the most common bacterial sexually transmitted infections (STI).

The vaginal microbiota is an integral part of the health of the female genital tract. Its immunological function has been clearly defined as a capable mechanical barrier warding off opportunistic pathogens. However, the study of complex interactions between the microbiota and outside factors has clarified multiple other roles in the health of the host. Part 2 of this thesis first describes current knowledge of these complex interactions between the microbiota and Chlamydia trachomatis and Mycoplasma genitalium. Additionally, the extent of the effect the vaginal microbiota has on in vitro fertilisation (IVF) during various stages of pregnancy is described.

Furthermore, it describes the vaginal microbiota composition of subfertile women. Finally, to clarify the extent of anatomical coverage of the vaginal microbiota a comparison is made between these vaginal microbiota compositions and microbiota found after sampling urine from the same person.
Part 1: Pathogens in the vaginal tract and their interaction with the immune system

Analysing the expression of cytokines is the most direct way of studying the extent of an immune response to an infection. Host specific differences in cytokine expression can directly lead to differences in the progression and clinical presentation of bacterial STIs. Conversely, bacteria specific differences can also lead to an increased cytokine expression in the host. In Chapter 1 we perform meta-analyses on the available literature covering the expression of cytokines during infection with *C. trachomatis*, *Neisseria gonorrhoeae*, and *Treponema pallidum*. The results of these meta-analyses show that increases in interleukin (IL)-1, IL-6, IL-8, IL-10, tumor necrosis factor α (TNFα), or interferon (IFN)-γ significantly affect the clinical outcome of *C. trachomatis* infections. All of these effects, except for the ones caused by increased IL-10 expression, caused more severe infections with *C. trachomatis*. Interestingly, an increase in *C. trachomatis* linked complications such as tubal pathology and pelvic inflammatory disease was also found in people that had an increased expression of IFN-γ and TNFα. An increased expression of IL-10 had reduced the severity of the infection, which is in line with the immune-regulatory function of this cytokine. Similarly to *C. trachomatis*, multiple studies into IL-1 and IL-8 expression during *N. gonorrhoeae* infection showed aggregated effects that significantly increase the severity of the disease. Unfortunately, these results could not be linked to specific late complications of *N. gonorrhoeae* infections. Lastly, for *T. pallidum* infection, aggregated study data showed that IFN-γ expression variation in hosts could be linked to an increase in severity of this disease. Most notably, the increased expression of this cytokine facilitated the progression of the disease into neurosyphilis, which is an extremely damaging and potentially lethal stage of this disease. An important limitation found in the meta-analyses was that multiple potentially relevant cytokines could not be included in a meta-analysis, as not enough studies into the described infections had included the expression levels of these cytokines.

Expression of inflammatory cytokines is for a large part initiated through the NF-κB pathway. Toll like receptor (TLR) 9 is one of the pathogen recognition receptors (PRR) that triggers the NF-κB pathway after binding with unmethylated CpG DNA. In Chapter 2 we use the previously shown fact that the hexameric configuration of these CpG DNA motifs has either an increased or reduced stimulating effect on the activation of the NF-κB pathway through TLR9. Using an *in silico* setup, we calculate the CpG index of 11 bacterial species which are commonly found in the vagina in either a pathogenic role or as commensal bacteria. Most notably, *N. gonorrhoeae* shows a CpG index of -79.5 and is the only included bacterial species.
to show an inhibitory effect. G. vaginalis showed the highest CpG index with a value of 26.2. Lactobacillus species showed a near neutral CpG index value of 4.2, which may be linked to the commensal role of this species in the vagina. However, no link between CpG index values and pathogenicity or commensality could be found.

Having covered the genetic makeup of bacterial DNA in the previous chapter, Chapter 3 discusses genetic differences between hosts. The characteristic symptom of an infection with Haemophilus ducreyi are painful genital ulcers known as chancroids. However, the risk of development of these ulcers differs per person, with some infected hosts remaining asymptomatic. This suggests a genetic factor in the severity of this disease. In this chapter 105 volunteers residing in the USA are infected at 3 sites on the arm with H. ducreyi. The number of developed ulcers is then taken as a measure of severity of the disease. Single nucleotide polymorphism (SNP) analyses on the host DNA, targeting 14 SNPs related to 7 PRR pathway related genes, created a link between the severity of the infection and the host genetics. In European American volunteers, the SNP TLR9 +2848 GG has a protective effect on the severity of the disease, and the TLR9 TA haplotype of TLR9 -1237 and TLR9 +2848 has a risk-enhancing effect. Results differed in African American volunteers, where a protective effect was found for IL10 -2849 AA, and a risk enhancing effect was found for the IL10 -2849 A, IL10 -1082 A, and IL10 -819 C haplotype. Larger studies into the effect of host genetic differences could discover even more associations during infection with H. ducreyi, as a clear link between host Immunogenetics and infection severity has now been made apparent.

Part 2: The interaction between the vaginal microbiota and host reproductive health
The interactions between the vaginal microbiota and pathogens are a crucial initial step in the host susceptibility to a disease. The current state of knowledge regarding these interactions between the microbiota and C. trachomatis as well as M. genitalium are reviewed and discussed in Chapter 4 of this thesis. The role of oestrogen as a host factor with indirect effects on the microbiota and pathogen interactions is of specific interest. Other points of discussion include the effects of lactic acid levels and H₂O₂ on C. trachomatis and M. genitalium. Lactic acid has been shown to be a competent inhibitor of C. trachomatis, while H₂O₂ inhibits bacterial vaginosis (BV) associated bacteria such as M. genitalium. Generally though, the presence of a large abundance of Lactobacillus spp. in the vagina is an indicator for reduced susceptibility to either of the studied pathogens. A notable result is the
identification of the gap in current knowledge regarding interactions between *M. genitalium* and the vaginal microbiota. *C. trachomatis* is extensively studied, however for *M. genitalium* we often needed to examine the mechanisms related to other *Mycoplasma* species to derive answers. Additionally, interactions of *M. genitalium* with the vaginal microbiome and the host often need to be speculated about through their functionality with other pathogens, such as *C. trachomatis* in the case of this chapter.

The relation between vaginal microbiota and early pregnancy development process during IVF treatment is discussed in Chapter 5. Due to the timing, the potential effect of the vaginal microbiota in this early pregnancy process is mostly apparent during the conception. A meta-analysis aggregates six studies to show that the presence of abnormal vaginal microbiota, represented as BV, leads to 1.4 times as much likelihood for IVF failure in this early stage of pregnancy. However, during the systematic review of literature, it has become clear that methodologies in studies examining the vaginal microbiota during IVF treatment often varies at important points. For instance, microbiota profiles are likely to differ between studies if the vaginal sample is taken at the same time as an antibiotic treatment compared to no antibiotics. This chapter suggests a number of methodological factors that deserve specific attention from readers and researchers interested in the topic of microbiota during IVF.

In Chapter 6 we ask whether the vaginal microbiota of a woman undergoing IVF treatment can give an indication about the outcome of the IVF treatment. Current success rate of IVF treatments is close to 30% per treatment, so the outcome of this study can help prevent treatments that are unlikely to succeed. Additionally, if specific microbiota are related to the outcome, modulation might prove a viable tactic to improve the chances for successful IVF treatments. To obtain an answer to the research question of this chapter vaginal and urine samples were taken from a group of 297 women receiving IVF treatment, to subsequently perform a microbiota profiling. Our results showed that women with a low abundance of *Lactobacillus* spp. in their vaginal sample were less likely to have a successful embryo implantation. Surprisingly, and in contrast to the previous statement, an abundance of below 60% of *L. crispatus* was a predictor for becoming pregnant. These factors and various factors of smaller impact were worked into a predictive algorithm named the ReceptIVFity test. This test showed a predictive accuracy of 94% (sensitivity 26%, specificity 97%) in predicting a negative IVF outcome. This finding paves the way for potential interventions
in the future, however those would require further studies into this topic. For now this test can help couples make a better decision regarding the timing of their IVF treatment cycles.

Another point of interest that has become a point of debate recently is the existence of a urinary microbiota. Although the bladder is traditionally thought of as a sterile space, multiple studies have discussed finding microbiota closely resembling the vaginal microbiota through urine sampling. In Chapter 7 an effort is made to clarify this topic. Samples and data from the women examined in the previous chapter were used for this. Vaginal and urinary microbiota found in these women bore a striking resemblance. Notable deviations occurred as a large abundance of \emph{E. coli} in the urine samples of some women, without showing up in the respective vaginal samples. These are likely due to subclinical cystitis in the urinary tract of the woman. We conclude from this, in combination with the relevant current literature, that there is no distinct urinary microbiota. Rather it is a dilution of the vaginal microbiota, possible acting as a reservoir for the vagina at times, which contaminates conventionally taken urine samples.

**Concluding remarks**
This thesis highlights the complexity of the interactions between pathogens, microbiota, and the host. The studies included in this thesis shed light on the interactions and prove the relevance of these interactions in the various infectious and reproductive processes. The findings in this thesis related to pathogen interactions with the host give clear insight into the pathogenesis of the pathogen. But perhaps more importantly, it sheds light on the biological background model of the clinical presentation in STIs. This is a topic that has consistently been linked to the presence and severity of later complications related to these infections. With the results of this thesis we hope to create a foundation not only for further research into these subjects, but also for translational efforts to put this knowledge to work in clinical settings.

However, this thesis also shows that there is still much work to do. In the case of infectious processes, the impact of microbiota on pathogens is often only described for the best studied pathogens. In the case of reproduction, current knowledge appears quite extensive, but significant pitfalls in study methodologies create a situation where even experts might have difficulty interpreting the information correctly. For all these current shortcomings though, these interactions are also fields where discoveries are happening at a lightning
fast pace. The potential impact of the implementation of vaginal microbiota diagnostics in healthcare, and especially in assisted reproductive therapies is already being noticed. To the point where complete adoption of the concepts is perhaps already inevitable. We recommend settings that plan to adopt the vaginal microbiota diagnostics to take extra care when researching the literature, but also to realize that benefits are likely to be great.
SAMENVATTING
SAMENVATTING

Dit proefschrift bestudeert en beschrijft de interacties tussen pathogenen, microbiota, en de gastheer zoals ze voorkomen in de vrouwelijke genitale kanaal. Daarnaast wordt gekeken naar de effecten die deze interacties hebben op de risico’s voor de gastheer om beïnvloed te worden door infecties, ziekte, en vruchtbaarheidsproblemen. In deel 1 van de thesis wordt specifiek aandacht besteed aan de interacties tussen binnendringende pathogenen en het immuunsysteem van de gastheer, terwijl in deel 2 de relaties tussen de vaginale microbiota, potentiële pathogenen, en de reproductieve gezondheid van de gastheer verder worden onderzocht.

Er is vaak een grote discrepantie tussen patiënten in de klinische presentatie van dezelfde infectie in het vaginale kanaal. Deze discrepantie is het meest merkbaar tussen symptomatiche en asymptomatische patiënten. Hoewel er veel potentiële redenen zijn voor de verschillende klinische presentaties van deze ziekten (bijv. voeding, lichamelijke gezondheid, roken, de samenstelling van de vaginale microbiota), richten we ons in deel 1 op de immunorespons zoals deze door de gastheer wordt geproduceerd tegen pathogenen. Dit wordt gedaan door het bestuderen van potentiële factoren die van invloed kunnen zijn op het vermogen van de gastheer om effectief te reageren op de binnenvallende ziekteverwekker, zoals de genetische aanleg van de gastheer. De genetische samenstelling van de gastheer weerspiegelt echter mogelijk niet altijd het potentieel van de gastheer om te vechten tegen een infectie. Daarom kijken we ook naar de variatie in expressie van cytokines na infectie zoals die zich voordoet tussen patiënten. De niveaus van tot expressie gebrachte cytokinen geven een beter beeld van dit potentieel en hoe dit de klinische presentatie van de meest voorkomende bacteriële seksueel overdraagbare aandoeningen (SOA) beïnvloedt.

De vaginale microbiota is een integraal onderdeel van de gezondheid van het vrouwelijke genitale kanaal. De immunologische functie van de vaginale microbiota is duidelijk gedefinieerd als een effectieve mechanische barrière die opportunistische pathogenen afweert. De studie van complexe interacties tussen de microbiota en externe factoren heeft echter meerdere andere rollen voor de vaginale microbiota in de gezondheid van de gastheer verduidelijk. Deel 2 van dit proefschrift beschrijft eerst de huidige kennis van deze complexe interacties tussen de microbiota en Chlamydia trachomatis en Mycoplasma genitalium. Daarnaast wordt de omvang van het effect van de vaginale microbiota op in-vitrofertilisatie (IVF) tijdens verschillende stadia van de zwangerschap beschreven. Vervolgens beschrijft
het de samenstelling van vaginale microbiota van subfertiele vrouwen. Ten slotte wordt, om de mate van anatomische dekking van de vaginale microbiota te verduidelijken, een vergelijking gemaakt tussen deze vaginale microbiota-samenstellingen en microbiota gevonden na het nemen van monsters van urine van dezelfde persoon.

Deel 1: Pathogenen in het vaginale kanaal en de interactie met het immuunsysteem

Analyse van de expressie van cytokines is de meest directe manier om de omvang van een immuunrespons op een infectie te bestuderen. Gastheer specifieke verschillen in cytokine-expressie kunnen direct leiden tot verschillen in de progressie en klinische presentatie van bacteriële SOA’s. Omgekeerd kunnen bacterie specifieke verschillen ook leiden tot een verhoogde cytokine-expressie in de gastheer. In Hoofdstuk 1 voeren we meta-analyses van de beschikbare literatuur uit betreffende de expressie van cytokines tijdens infectie met *C. trachomatis*, *Neisseria gonorrhoeae*, en *Treponema pallidum*. De resultaten van deze meta-analyses tonen aan dat stijgingen in interleukine (IL) -1, IL-6, IL-8, IL-10, tumornecrosefactor alfa (TNFα) of interferon (IFN) -γ significant de klinische uitkomst beïnvloeden van infecties met *C. trachomatis*. Al deze effecten, behalve die veroorzaakt door verhoogde IL-10-expressie, veroorzaakten ernstigere infecties met *C. trachomatis*. Interessant is dat een toename van *C. trachomatis* gerelateerde complicaties zoals tubaire pathologie en kleine bekken ontsteking (in de Nederlandse literatuur wordt hier vaak de Engelse term "Pelvic Inflammatory Disease") voor gebruikt) ook werd gevonden bij mensen die een verhoogde expressie van IFN-γ en TNFα hadden. Een verhoogde expressie van IL-10 verminderde juist de ernst van de infectie, wat in overeenstemming is met de immuunregulerende functie van dit cytokine. Net als bij *C. trachomatis* toonden meerdere onderzoeken naar IL-1- en IL-8-expressie tijdens *N. gonorrhoeae* infectie geaggregeerde effecten die de ernst van de ziekte aanzienlijk verhogen. Helaas konden deze resultaten niet worden gekoppeld aan specifieke late complicaties van *N. gonorrhoeae* infecties. Tenslotte toonden voor *T. pallidum* infectie geaggregeerde onderzoeksgegevens dat IFN-γ expressievariatie in gastheren kon worden gekoppeld aan een toename van de ernst van deze ziekte. De verhoogde expressie van dit cytokine versnelde ook nog eens de progressie van de ziekte naar neurosyphilis, wat een uiterst schadelijk en mogelijk dodelijk stadium van deze ziekte is. Een belangrijke beperking van het onderzoek gevonden in de meta-analyses was dat meerdere potentiële relevante cytokines niet konden worden opgenomen in een meta-analyse, omdat niet genoeg onderzoeken naar de beschreven infecties de expressieniveaus van deze cytokinen hebben bestudeerd.
Expressie van cytokinen gerelateerd aan ontsteking wordt voor een groot deel geïnitieerd via de NF-κB-route. Toll-like receptor (TLR) 9 is een van de pathogeen herkenningsreceptoren (PRR) die de NF-κB-route induceert na binding aan ongemethyleerd CpG DNA. In Hoofdstuk 2 gebruiken we het eerder aangetoonde feit dat de hexamere configuratie van deze CpG DNA motieven een verhoogd of verlaagd stimulerend effect heeft op de activering van de NF-κB route doordat TLR9 stimulatie. Met behulp van een in silico opstelling berekenen we de CpG-index van 11 bacteriesoorten die gebruikelijk in de vagina worden aangetroffen als pathogeen of als commensale bacterie. In het bijzonder vertoont *N. gonorrhoeae* een CpG-index van -79,5 en is het de enige opgenomen bacteriesoort die een remmend effect vertoont. *G. vaginalis* toonde de hoogste CpG-index met een waarde van 26,2. *Lactobacillus* soorten vertoonden een bijna neutrale CpG-indexwaarde van 4,2, die kan worden gekoppeld aan de commensale rol van deze bacterie soort in de vagina. Er kon echter geen verband worden gevonden tussen CpG-indexwaarden en pathogeniciteit of commensaliteit van de bacteriën.

Nadat in het vorige hoofdstuk de genetische samenstelling van bacterieel DNA is behandeld, wordt in hoofdstuk 3 de genetische verschillen tussen gastheren besproken. Het kenmerkende symptoom van een infectie met *Haemophilus ducreyi* zijn pijnlijke genitale ulcera die bekend staan als zachte sjankers. Het risico van de ontwikkeling van deze ulcera verschilt echter per persoon, waarbij sommige geïnfecteerde gastheren compleet asymptomatisch blijven. Dit suggereert een genetische factor die effect heeft op de ernst van deze ziekte. In dit hoofdstuk zijn 105 vrijwilligers die in de VS leven experimenteel besmet op 3 plaatsen op de arm met *H. ducreyi*. Het aantal ontwikkelde ulcera is genomen als een maat voor de ernst van de ziekte. Single nucleotide polymorfisme (SNP) analyses op het DNA van de gastheer, gericht op 14 SNP’s gerelateerd aan 7 PRR pathway gerelateerde genen, creëerden een verband tussen de ernst van de infectie en de genetica van de gastheer. Bij Europees-Amerikaanse vrijwilligers heeft de SNP *TLR9* +2848 GG een beschermend effect op de ernst van de ziekte en heeft het *TLR9* TA-haplotype van *TLR9* -1237 en *TLR9* +2848 een risico verhoogend effect. De resultaten verschillen in Afrikaans-Amerikaanse vrijwilligers, waar een beschermend effect werd gevonden voor *IL10* -2849 AA, en een risico verhogend effect werd gevonden voor het *IL10* -2849 A, *IL10* -1082 A en *IL10* -819 C haplotype. Grotere studies naar het effect van gastheer genetische verschillen kunnen waarschijnlijk nog meer associaties ontdekken tijdens infectie met *H. ducreyi*, deels omdat een duidelijke link tussen gastheer immunogenetica en ernst van de infectie nu duidelijk is geworden.
Deel 2: De interactie tussen de vaginale microbiota en de reproductieve gezondheid van de gastheer

De interacties tussen de vaginale microbiota en pathogenen zijn een cruciale eerste stap in de ontvankelijkheid van de gastheer voor een ziekte. De huidige kennis over deze interacties tussen de microbiota ende pathogenen C. trachomatis en M. genitalium wordt beschreven en besproken in hoofdstuk 4 van dit proefschrift. De rol van oestrogeen als een gastheerfactor met indirecte effecten op de microbiota en pathogene interacties is specifiek merkbaar. Andere punten van discussie zijn de effecten gerelateerd aan niveau verschillen van melkzuur en H₂O₂ op C. trachomatis en M. genitalium. Van melkzuur is aangetoond dat het een competent remmer is van C. trachomatis, terwijl H₂O₂ bacteriële vaginose (BV) gerelateerde bacteriën zoals M. genitalium remt. Over het algemeen is de aanwezigheid van een grote hoeveelheid Lactobacillus spp. in de vagina een indicator voor verminderde gevoeligheid voor allebei de bestudeerde pathogenen. Een belangrijk resultaat is de identificatie van de kloof in de huidige kennis met betrekking tot interacties tussen M. genitalium en de vaginale microbiota. C. trachomatis wordt uitgebreid bestudeerd, maar voor M. genitalium moesten we vaak de mechanismen gebruiken die in verband zijn gebracht met andere Mycoplasma-soorten om antwoorden te vinden. Bovendien moet vaak gespeculeerd worden over interacties van M. genitalium met het vaginale microbioom en de gastheer door te kijken naar functionaliteit van andere pathogenen, zoals C. trachomatis in het geval van dit hoofdstuk.

Als onderdeel van de invloed van de vaginale microbiota op de IVF-behandeling wordt het ontwikkelingsproces voor de vroege zwangerschap besproken in hoofdstuk 5. Vanwege de timing is het potentiële effect van de vaginale microbiota in dit vroege zwangerschapsproces het meest duidelijk tijdens de conceptie. Een meta-analyse is uitgevoerd om zes studies te aggregeren, om vervolgens aan te tonen dat de aanwezigheid van abnormale vaginale microbiota, weergegeven als BV, 1.4 keer zoveel kans op IVF-falen in deze vroege zwangerschapsfase oplevert. Tijdens de systematische literatuurstudie is het echter duidelijk geworden dat methodologieën in onderzoeken naar de vaginale microbiota tijdens IVF-behandelingen vaak op belangrijke punten variëren. Microbiota-profielen worden bijvoorbeeld waarschijnlijk verschillend gedetecteerd tussen de onderzoeken als het vaginale monster tegelijkertijd met een antibioticabehandeling wordt ingenomen in vergelijking met geen antibiotica. Dit hoofdstuk suggereert een aantal methodologische factoren die specifieke aandacht verdienen van lezers en onderzoekers die geïnteresseerd zijn in het onderwerp microbiota tijdens IVF.
In hoofdstuk 6 vragen we of de vaginale microbiota van een vrouw die een IVF-behandeling ondergaat een indicatie kan geven over de uitkomst van deze IVF-behandeling. Het huidige succespercentage van IVF-behandelingen ligt dicht bij 30% per behandeling, dus de uitkomst van dit onderzoek kan behandelingen helpen voorkomen die waarschijnlijk niet zullen slagen. Bovendien, als specifieke microbiota gerelateerd zijn aan het resultaat, kan modulatie een effectieve tactiek blijken te zijn om de kansen op succesvolle IVF-behandelingen te verbeteren. Voor de analyses werden vaginale en urinemonsters genomen van een groep van 297 vrouwen die een IVF-behandeling kregen, om vervolgens een microbiota-profielerende uit te voeren. Onze resultaten toonden aan dat vrouwen met een lage hoeveelheid *Lactobacillus* spp. in hun vaginaal monster hadden minder kans op een succesvolle embryo-implantatie. Verrassend, en in tegenstelling tot de vorige verklaring, was een hoeveelheid van minder dan 60% van *L. crispatus* een voorspeller om zwanger te worden. Deze factoren en verschillende factoren met een kleinere impact werden verwerkt in een voorspellend algoritme genaamd de ReceptiVFity-test. Deze test toonde een voorspellende nauwkeurigheid van 94% (gevoeligheid 26%, specificiteit 97%) bij het voorspellen van een negatieve IVF-uitkomst. Deze bevinding maakt de weg vrij voor mogelijke interventies in de toekomst, maar die zouden verdere studies over dit onderwerp vereisen. Voorlopig kan deze test helpen door paren een beter geïnformeerde beslissing te laten nemen met betrekking tot de timing van hun IVF-behandelingscycli.

Een ander onderwerp dat recent een punt van discussie is geworden, is het bestaan van een urinaire microbiota. Hoewel de blaas van oudsher wordt beschouwd als een steriele ruimte, hebben meerdere studies besproken dat microbiota gedetecteerd door middel van urinemonstering nauw lijkt op de vaginale microbiota. In hoofdstuk 7 wordt dit onderwerp verduidelijkt. Om deze duidelijkheid te krijgen hebben we de microbiota en IVF-behandelresultaten van de vrouwen in het vorige hoofdstuk gebruikt. De vaginale en urinaire microbiota die bij deze vrouw werden aangetroffen, hadden een opvallende gelijkenis. Opmerkelijke afwijkingen deden zich voor als een grote hoeveelheid *E. coli* in de urinemonsters van sommige vrouwen, zonder te verschijnen in de respectieve vaginale monsters. Deze zijn waarschijnlijk te wijten aan subklinische cystitis in de urinewegen van de vrouw. We concluderen hieruit, in combinatie met de relevante huidige literatuur, dat er geen duidelijke urinair microbiota is. De gevonden microbiota is zeer waarschijnlijk een verdunning van de vaginale microbiota, en zal soms optreden als een reservoir voor de vagina, welke ervoor zorgt dat urinemonsters worden vervuilt.

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Concluderende opmerkingen

Dit proefschrift benadrukt de complexiteit van de interacties tussen pathogenen, microbiota, en de gastheer. De studies opgenomen in dit proefschrift werpen licht op de interacties en bewijzen de relevantie van deze interacties in de verschillende infectieuze en reproductive processen. De bevindingen in dit proefschrift gerelateerd aan de interacties van pathogenen met de gastheer geven duidelijk inzicht in de pathogenese van het pathogen. Maar misschien nog belangrijker, werpen ze licht op het biologische achtergrondmodel van de klinische presentatie in de bestudeerde SOA's. Dit is een onderwerp dat consequent in verband is gebracht met de aanwezigheid en ernst van latere complicaties in verband met deze infecties. Met de resultaten van dit proefschrift hopen we een basis te creëren welke gebruikt kan worden voor verder onderzoek naar deze onderwerpen, maar ook voor translationele inspanningen om deze kennis in klinische settings te laten werken.

Dit proefschrift laat echter ook zien dat er nog veel werk op ons wacht. In het geval van infectieuze processen wordt de impact van microbiota op pathogenen vaak alleen beschreven voor de best bestudeerde pathogenen. In het geval van reproductie lijkt de huidige kennis vrij uitgebreid, maar significante valkuilen in studiemethoden creëren een situatie waarin zelfs experts problemen zouden kunnen hebben om de informatie correct te interpreteren. Voor al deze tekortkomingen zijn de interacties echter ook onderwerpen waarin ontdekkingen in een razendsnel tempo gebeuren. De potentiële impact van de implementatie van vaginale microbiota diagnostiek in de gezondheidszorg, en met name bij kunstmatige voortplantingstechnieken, wordt al opgemerkt. Dit zelfs tot het punt waar volledige acceptatie van de concepten misschien al onvermijdelijk is. We bevelen instellingen aan die van plan zijn de vaginale microbiota-diagnostiek over te nemen om extra voorzichtig te zijn bij het onderzoeken van de literatuur, maar ook om te beseffen dat de voordelen potentieel groot zijn.
ABOUT THE AUTHOR

Martin Singer was born in Blokker, the Netherlands on the 26th of July 1989. He attended secondary school at the Copernicus SG in Hoorn, and graduated from there in 2008. After this he attended the Inholland University of Applied Sciences in Amsterdam, where he studied for a bachelor’s degree in Biology and Medical Laboratory Science until he graduated in 2012. For his graduate internship he investigated the links between host genetic SNPs and the severity of *Haemophilus ducreyi* infections at the laboratory of Immunogenetics at the VU Medical Centre in Amsterdam. In the summer of 2012 year, he was briefly employed at the same laboratory as a research analyst. In September of 2012 he started the Biomedical Sciences Master degree study at the Vrije Universiteit in Amsterdam, from which he graduated with a dual specialization into Immunology and Infectious Diseases in 2015. His graduate internship was performed at the parasitology department of the Royal Tropical Institute in Amsterdam, where he examined the conditions required for optimal experimental infections of human cell lines with *Cryptosporidium parvum*. In 2015 Martin started working as a PhD student within the department of Medical Microbiology and Infection Control of the VU Medical Centre in Amsterdam and the spin-off company related to this department called TubaScan Ltd. In this setting, and under the guidance of his promotor Prof. Dr. Servaas A Morré and his co-promotor Dr. Sander Ouburg he produced the work presented in this thesis.
Vrije Universiteit in Amsterdam, waar hij in 2015 afstudeerde met een dubbele specialisatie in Immunologie en Infectieziekten. Zijn afstudeerstage werd uitgevoerd op de afdeling parasitologie van het Koninklijk Instituut voor de Tropen in Amsterdam, waar hij de voorwaarden onderzocht die nodig zijn voor optimale experimentele infecties van menselijke cellinen met *Cryptosporidium parvum*. Martin begon in 2015 te werken als promovendus bij de afdeling Medische Microbiologie en Infectiepreventie van het VU Medisch Centrum in Amsterdam en het spin-off bedrijf met betrekking tot deze afdeling genaamd TubaScan Ltd. Onder leiding van zijn promotor Prof. Dr. Servaas A. Morré en copromotor Dr. Sander Ouburg produceerde hij in deze setting het werk gepresenteerd in dit proefschrift.
LIST OF PUBLICATIONS

CpG DNA analysis of bacterial STDs.  

Quantitative analysis of Cryptosporidium growth in vitro culture- the impact of parasite density on the success of infection.  

Effect of cytokine level variations in individuals on the progression and outcome of bacterial urogenital infections – a meta-analysis.  

Host Polymorphisms in TLR9 and IL10 Are Associated With the Outcomes of Experimental Haemophilus ducreyi Infection in Human Volunteers.  

The two-sided role of the vaginal microbiome in Mycoplasma genitalium and Chlamydia trachomatis pathogenesis.  

The relation of the vaginal microbiota to early pregnancy development during In Vitro Fertilization treatment – A meta-analysis  
M. Singer, M. Borg, S. Ouburg, S. A. Morré. Submitted

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

The ReceptIVFity cohort study protocol to validate the urogenital microbiome as predictor for IVF or IVF/ICSI outcome 

The profiling of microbiota in vaginal and urine samples using 16s rRNA gene sequencing and IS-pro analysis  
DANKWOORD
DANKWOORD

Prof. dr. Morré, beste Servaas. Bij jou is het allemaal begonnen. Mijn eerste babystapjes in het onderzoek zette ik toen ik als jonkie bij je kwam solliciteren voor een HBO stage. Ondanks dat er af en toe gepiept en gekreund werd, zegt het genoeg dat ik 7 jaar na dato nog steeds bij je rondloop. Ik wil je oprecht bedanken voor alle mogelijkheden die je mij hebt aangeboden. Je commentaren en kritieken waren vaak het wachten waard, en je hebt mij een uniek voorbeeld gegeven van hoe tijd soms erg relatief kan zijn. Een week aan werk kan gedaan worden in een uur, maar een klusje van een uur... daar gaat soms wat meer tijd overheen. Daarnaast kan ik alleen maar hopen dat mijn volgende werkgever net zo'n geweldige smaak in films en humor heeft als je jij. Al met al heb jij voor de unieke sfeer gezorgd die ons lab "ons lab" maakte. Ook al scheiden onze wegen binnenkort, ik weet zeker dat een nieuwe samenwerking niet ver te zoeken is.

Dr. Ouburg, beste Sander. Als ik zeg dat alles bij Servaas begonnen is, dan weet jij natuurlijk wel beter. Want op de eerste dag van mijn afstudeerstage was het niet Servaas die toegang voor me regelde, of mij uitlegde wat mijn stage nou precies in zou houden. Zeven jaar lang heb jij er voor gezorgd dat plannen realiteit werden, eerste versies omgezet werden naar artikelen, en ongemotiveerde HBO studentjes tot onderzoekers werden gesmeed. Het spreekt voor zich dat zonder jou dit boekje er nooit was gekomen. Sander, ik wil je heel erg bedanken voor alle hulp die je mij hebt geboden. Jij was bij alle hoogtepunten, zoals de acceptatie van mijn eerste artikel, maar ook bij alle dieptepunten. Het geloof dat jij altijd in me had is zeker niet in dovemans oren gevallen. Misschien verdienen je wel het meeste lof om het feit dat je na 7 jaar nog geen mental breakdown hebt gehad nadat ik wederom een potje heb lopen klagen over het een of het ander. Hopelijk kunnen we in de toekomst vaker samen boven een artikel staan (in Nature ofzo).

Roel en Jolein, ook wel bekend als de ruggengraat van het lab. Aan jullie heb ik veel van mijn praktische skills te danken. In een setting waar constant nieuwe gezichten verschenen en weer verdwenen, waren jullie de vaste waarden die identiteit gaven aan de Immunogenetica en Tubascan. Maar daarnaast zorgden jullie er ook voor dat de gezelligheid erin bleef door middel van zang, spel, en eten. Het maakt me niet uit waar mijn toekomst me heen leid, als er weer pulled chicken of appeltaart gemaakt wordt wil ik hier graag van op de hoogte worden gehouden! En met appeltaart bedoel ik natuurlijk die van jou Jolein. Alle anderen staan daarbij niet in vergelijking. Roel, de Chlamydia-song dag was er één om nooit te vergeten. Misschien zet ik hem wel op mijn CV... onder "Other skills". Het was weer gezellig. Tot de volgende keer.
Je bouwt een speciale band op met mensen die hetzelfde pad bewandelen. Daarom wil ik in het speciaal al mijn co-promovendi bedanken voor hun steun en vertrouwen. Eleanne, ik denk dat niemand beter weet dan jij wat de frustraties en pijnpunten van mijn promotie waren. Je gesprekken waren altijd zeer interessant, of ik er nu actief aan mee deed of niet. Nu je weg bent, is de werkkamer er alleen maar kouder op geworden. Ik wil je niet alleen bedanken voor je steun tijdens de zware tijden, en de gezelligheid tijdens het mooie weer, maar ook heel veel succes wensen met je eigen promotie! Als iemand het kan, dan kan jij het vast ook. Beste Dewi, het HBO ligt alweer ver achter ons, en voor een tijdje leken we allebei vast te zitten bij Servaas. Nu zijn we allebei eindelijk verlost. Ik wens je het beste, en dank je voor je hulp met het artikel, en het sfeer maken. Beste Melissa, ik wil je heel erg bedanken voor je geweldige review hulp. Je promotie is helaas niet gelopen zoals je zou willen, maar ik hoop dat je nu je pad naar geluk hebt gevonden. Beste Omaima, bedankt voor je aanwezigheid in de groep. Ik hoop dat je promotie voorspoedig gaat wanneer ik weg ben. Beste Rivka, dank je voor de zeer uitgebreide hulp en samenwerking aan de Valbiome artikelen. Beste Bernice, jou stukje is toepasselijk aan de late kant. Maandagen en woensdagen werden een stuk verdraagzamer door jouw aanwezigheid. Dank je dat je er altijd was om tegen aan te klagen, en de sfeer te verbeteren.

Ook wil ik graag al mijn andere labcollegas bedanken. Stuk voor stuk hebben jullie aan de geweldige sfeer in onze groep bijgedragen. Anne, Mansi, and Karen, you set a great example for me to follow into the future. I hope I become just as skilled, fun, and ambitious as you three. Wies, dank je voor de gezelligheid. Hopelijk begin je snel aan je eigen PhD avontuur. June, als frisse wind ben je door de groep komen waaien. Ik kijk met plezier terug naar de boottochtjes. Bedankt voor de leuke tijd.

Beste Maria en Thirza, nadat ik vele stagairs voorbij heb zien komen kan ik met een gerust hart zeggen dat jullie bovengemiddeld zijn. Jullie hebben me in laten zien dat studenten begeleiden geen straf is. Naast dat ik jullie allebei wil bedanken voor alle inzet en gezelligheid, wil ik Maria extra bedanken voor haar geweldige werk aan het artikel dat in dit boekje te lezen is.

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meest invloedrijke persoon geweest tijdens mijn promotie. Het dubbelchecken van datatables zal ik nooit meer vergeten (beloofd). Heel erg bedankt, en hopelijk kunnen we in de toekomst vaker samenwerken.

Beste Coen en Janneke, vanaf dag één kon ik altijd met mijn kweek vragen bij jullie terecht. Ik waardeer jullie hulp onvoorwaardelijke hulp oprecht. Sanne en Savannah, ook al is het onwaarschijnlijk dat jullie dit boekje ooit onder ogen krijgen, wil ik jullie toch bedanken voor jullie gezelligheid en de geweldige hulp bij het celkweken.

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Elke pad in het leven heeft zijn obstakels. Zonder namen te noemen wil ik toch diegene bedanken die mij hebben geleerd dat frustratie en motivatie vaak hand in hand gaan.

En als laatste: lieve leden van de filmcommissie. Met creativiteit en humor hebben we geprobeerd wat meer licht te brengen in de soms saaie kamers van het MF en O|2 gebouw. En hoewel die artistieke visie niet altijd werd begrepen door de massa, ben ik trots op wat wij hebben gecreëerd. Gelukkig is afscheid voor ons een onwerkelijk begrip. Onze commissie zal in de toekomst wederom nodig zal zijn. En zoals altijd sta ik paraat!
“Citation needed”