UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Pelvic inflammatory disease in women; improving diagnosis and better understanding the disease process.

by Rami Mazraani
Bachelor of Medical Science (Honours)

Thesis submitted in fulfilment of the requirements for
the degree of

Doctor of Philosophy

under the supervision of Associate Professor Wilhelmina Huston
and Dr. Catherine Burke

University of Technology Sydney
Faculty of Science

February 2021

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Rami Mazraani declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy in the school of life science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Signature: Production Note:
Signature removed prior to publication.

Date: 11/01/2021

List of keywords

Pelvic inflammatory disease, vaginal microbiome, host immune response, *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium,* idiopathic

Acknowledgements

Firstly, I am forever grateful for these valuable opportunities that have been given to me throughout my tertiary education. However, when asked why I chose to do my PhD? The answer is still a mystery. My time as a PhD student has likely been the most difficult period in my life. It all started on an information night for research where I saw a new academic to the University of Technology, Sydney munching down on a muffin without anyone else talking to her. Maybe it was destiny or I to was hungry, but since that moment a relationship have developed where I discovered a fond interest in female sexual health research. I honestly feel it would have been difficult without the amazing encouragement, personal and technical help to persevere and work through this, and teachings of Associate Professor Willa Huston. I am forever grateful!

Secondly to my partner Brad, you should have not let me continue my PhD, what were you thinking, I lost 80% of my hair, 80% of wrinkles grew, and I think I discovered a white hair when writing this. However, your support in listening about my research and issues has been non-stop. Thank you! Of course, I would like to extend my sincerest thanks to my family for helping me throughout the years of education. The friends I have made along the way have also been of great assistance, and I wish to personally thank Mark Thomas for teaching me cell culture, and my dearest friend William King for helping me consistently through correct analysis. I would like to thank my friends who have dealt with my complaints and rants, Jessica Pederson, Natalie Strange and Nicole Lima.

“Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world” – Albert Einstein

Disclaimer: Although this study and its works focus on PID in cis women, I would like to show my respect to my fellow LGBTQA+ community and understand that trans men can have pelvic inflammatory disease.

Table of Contents

List of Figures

List of Tables

Abstract

Pelvic inflammatory disease (PID) is a condition that involves inflammation of the upper genital tract in women that can be debilitating. This condition can result in reproductive health sequelae such as tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. Diagnosis of PID is a complex and subjective clinical process. This includes a bimanual examination to test for cervical, uterine, and adnexal motion tenderness which is considered a critical diagnostic criterion to indicate PID. The current treatment protocol for PID raises concerns as it is immediately treated with several antibiotics prior to and regardless of infections diagnosed. The increase of antibiotic resistant bacteria in the world and the rise of multiple drug-resistant sexually transmitted infections may be at further risk from this treatment protocol. The antibiotics used for PID include ceftriaxone, cefotaxime, metronidazole, doxycycline, azithromycin and moxifloxacin, with a minimum of three used in conjunction. PID can be associated with sexually transmitted infection, respiratory pathogens, instrumentation (e.g. IUD), and/or an idiopathic aetiology.

This project conducted a prospective pilot study of women with PID, by comparing reproductive tract specimens from women with PID to healthy controls (Case-Control study design) and also further compared the findings in against a separate test group of women with asymptomatic or mild sexually transmitted infections. This involved three main objectives, the first being examining the bacterial compositions of

the female genital tract and their potential relationship with PID. Secondly, investigating expression of a selection of human immune genes for associations with PID. Lastly, the third objective involved characterising the pathogenicity of chlamydial variants using *in vivo* and *in vitro* methods, with a goal to determine if a mouse model could show large pathogenic differences that will establish a system of mouse infections ready to be adapted to looking for traits in chlamydial isolates from PID Cases or Controls.

Human research ethic committee approvals were granted and the recruitment resulted in 15 Cases, 31 Controls, and 13 Test group members (total of 59). Self-completed questionnaire data on behaviour and relevant medical history was received from a total of 59 participants. Biospecimens were successfully collected and received from 59 participants (resulting in 249 bio-specimens, consisting of 177 cervical and 59 vaginal swabs). The demographic, self-reported questionnaire and medical record review datasets were compiled and analysed for each of the groups to evaluate any differences in known risk factors for PID, or other substantial differences between the groups and no confounding epidemiological data was found. DNA was successfully extracted from cervical and vaginal biospecimens. Based on 16S rRNA gene amplicon sequencing, the cervicovaginal microbial composition was found to be variable across women regardless of disease status However, quantitative PCR confirmed that a higher bacterial load of *Atopobium vaginae* and *Prevotella species'* was associated with PID.

RNA was successfully extracted from the cervical swabs of participants. Attempts to extract RNA from the cervical swabs from the test group were unsuccessful, likely due to contamination and extended storage. The gene for lysozyme was found to be expressed

at higher levels in PID cases than controls, as the sole significantly different gene. Albeit, pathway analysis was conducted to identify pathways that had different gene expression levels between cases and controls, and significantly different pathways between cases and controls included those with cell communication and detection of bacteria and viruses.

Chlamydia trachomatis variants were examined to establish a framework approach to evaluate pathology of isolates. Fatty acids were successfully extracted from the bacterial strains and analysis found that there were different concentrations of four unsaturated acids relative to wild-type. Variants were found to have distinct susceptibilities to the fatty acid synthesis inhibitor type II (triclosan) compared to wildtype. Analysis of the *in vivo* properties (survival and reproductive tract pathology) of the variants found no significant differences compared to the wildtype in C57BL/6 and C3H/HeJ mouse vaginal infection models. Establishment of a suitable animal model for *C. trachomatis* virulence to profile if certain strains or isolates have distinct pathogenic traits requires further development.

Overall, this pilot study has established that the recruitment, specimen processing and analysis protocols are effective. The results indicate that there are potential microbiota that may influence the disease. Secondly, human immune gene expression profiles were found to correlate with PID, and these biomarkers could be used for future molecular diagnostic of PID with further supporting data. Finally, this study results and conclusions support that there is a need for a in vivo model of PID, especially in terms of investigating different organisms and potential pathogenic factors

Chapter 1 - Introduction

1.1. PELVIC INFLAMMATORY DISEASE

1.1.1. Definition of Pelvic inflammatory disease

Pelvic inflammatory disease (PID) is a condition that involves inflammation of the upper genital tract in women that can be debilitating [1, 2]. This condition can result in reproductive health sequelae such as tubal factor infertility, ectopic pregnancy, and chronic pelvic pain [3]. In a proportion of cases, the condition is attributed to pathogenic infection spreading from the vagina or cervix to the upper genital tract resulting in inflammation leading to PID [4]. The symptoms of PID are, but not limited to; acute onset of lower pelvic pain, odorous vaginal discharge, abnormal uterine bleeding, postcoital bleeding, dyspareunia, dysuria, and fever PID can be classified one of three aetiology. The first is pathogen associated that is defined as PID caused by a sexually transmitted infection. A second grouping is idiopathic associated, is defined as PID with no known pathogens. Finally, instrumentation (IUD) associated PID, defined as PID caused by recent insertion of instrumentation (e.g. IUD). These are further discussed in greater detail in section 1.3.

1.1.2. Prevalence of PID

The prevalence of PID is reported to be 4.4% in sexually experienced women of reproductive age (18 – 44 years) in the USA [5]. However, the global prevalence of the disease is unknown. A study of 38 193 women from New South Wales (NSW) found that

rates of PID hospitalisations were higher in those that have previously been infected with *Chlamydia trachomatis* or *Neisseria gonorrhoeae* than a general population (females of reproductive age)[6]. Figure 1.1 shows the incidence ratio of women hospitalised with PID and detected STIs as reported in this study. In another study [7], *N. gonorrhoeae* had the highest incidence for those hospitalised with PID (96.61%) when compared to *C. trachomatis* and the general population. This study, however, did not take into account idiopathic PID. Figure 1.2 shows a study of PID hospitalisations due to *C. trachomatis* cases over six countries conducted by Bender *et al* [7]. This results of the study suggested an overall decrease of PID events except for New Zealand (in women aged 15-19). However, this decline in rates might not correlate with an actual reduction in PID incidences, as hospitalisations reflect only the most severe cases of PID. In Australia, the majority of PID cases are diagnosed and managed through a general practitioner or sexual health clinics where 100 000 – 300 000 cases are seen per year compared to 10 000 hospitalisations [8].

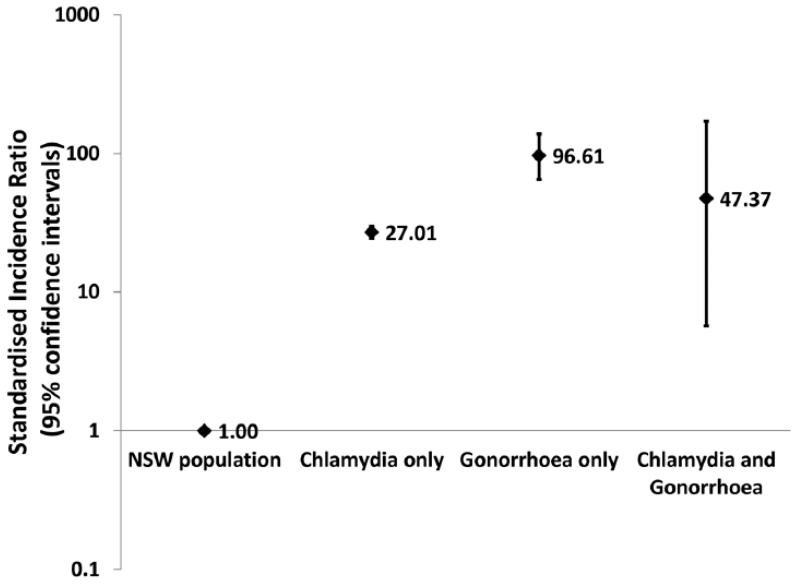


Figure 1. Standardised* incidence ratio (SIR) for hospitalisation for pelvic inflammatory disease (PID) in women in New South Wales (NSW). Women were aged 15–30. *standardised for age and year of follow-up (reproduced directly from [7]).

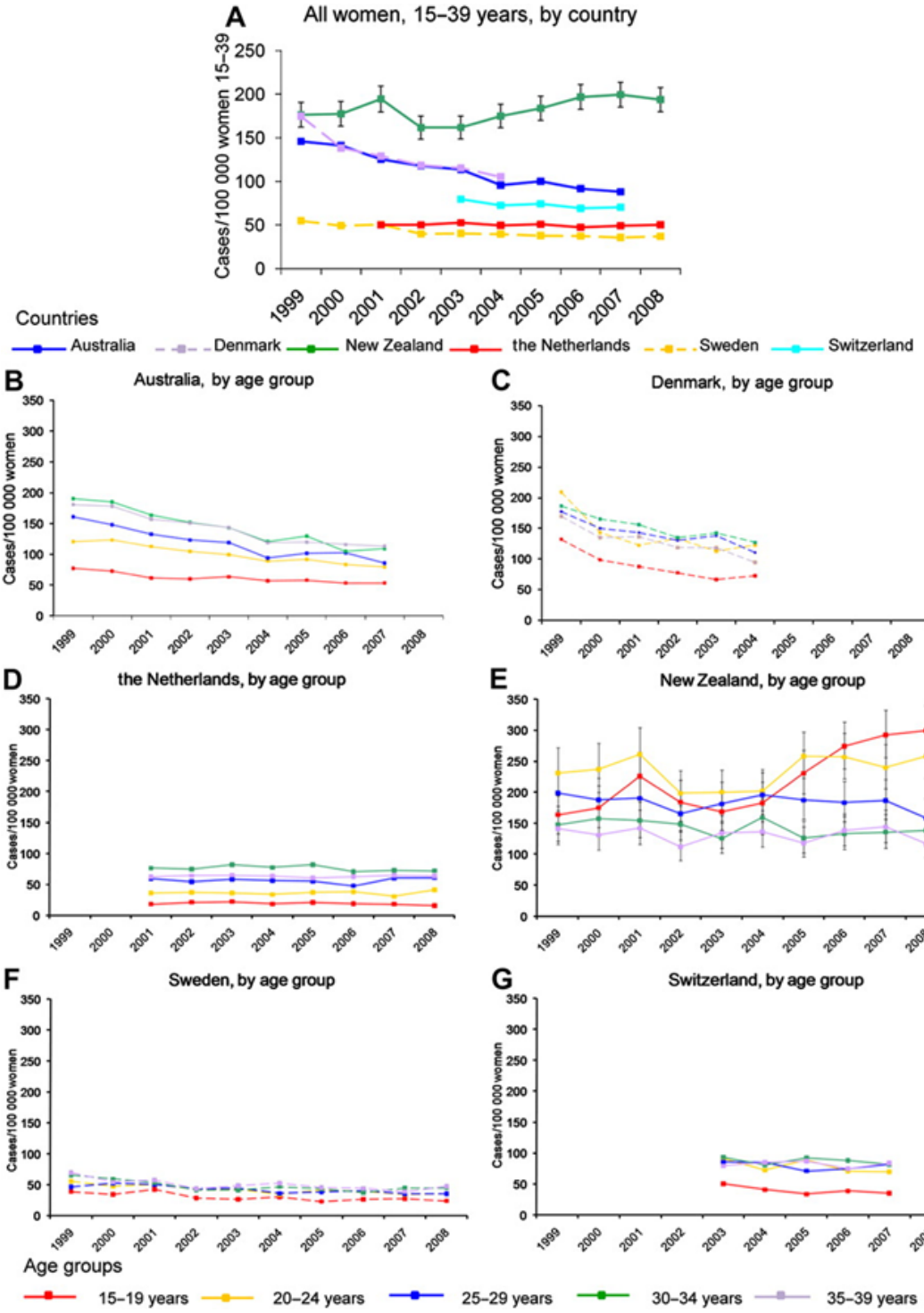


Figure 1.1 Rates of hospitalisation for pelvic inflammatory disease (PID) 1999-2008. (A) PID rate per 100 000 women aged 15-39 years, by country, age-standardised. (B-G) PID rate in each country per 100 000 women, this shows an overall decrease of PID

hospitalisations in Australia, whereas in New Zealand an increase occurs in the age group 15-19. Reproduced directly from [7]

1.1.3. Diagnosis of PID

Diagnosis of PID is a complex multi-step process (Figure 1.3) that involves patient history, elimination of other potential conditions, and examination findings. The gold standard was introduced in the 1960s for diagnosis; this was that for a presumptive diagnosis of PID the patient must have lower abdominal pain and two of the following criteria: abnormal vaginal discharge, fever, vomiting, menstrual irregularities, urinary symptoms, rectal pain, pelvic tenderness or swelling [9]. After presumption, either clinical or surgical investigations (laparoscopy) can be conducted for confirmatory diagnosis. An evaluation comparing laparoscopic and clinical diagnosis of PID suggested that there is a low sensitivity for clinical diagnosis ranging from 60-70% [10-12]. In a study of 95 women with PID (laparoscopically diagnosed) further evaluation using endometrial biopsy's supported that only 44 of these women had PID (defined by histological detection of leukocytes). This study concluded that laparoscopy had a sensitivity for 46%, and the authors suggested this method should be accompanied by histological studies [13].

Further diagnostic options have also been utilised such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and hysterosalpingography (HSG). Ultrasound is becoming more frequently used than laparoscopy, especially in the

presence of a pelvic swelling in order to eliminate pelvic cysts [11]. It has been demonstrated that ultrasound (either abdominal or transvaginal) lacks specificity in diagnosing PID with a sensitivity of 81-93% and specificity of 5-100% [11, 14, 15]. The other techniques of imaging are either too expensive or not readily available in the clinical settings where most PID is diagnosed. One of these imaging techniques showing promise is MRI with a reported sensitivity and specificity of 95% and 93% (compared to laparoscopy) [16]. Unfortunately, this study size was small, consisting of only 30 women with severe PID [16]. A further study of MRI needs to be conducted and include comparisons with a control group and more acute PID cases, although again MRI is only accessible via hospitals so likely has limited utility in PID diagnosis. Whilst numerous methodologies have been in trial; there are no accurate and specific diagnosis for PID.

The current diagnostic approach in Australia focuses on a common collection of parameters which includes the elimination of other reasons, with the first sign of a woman of reproductive age experiencing abrupt emergence of lower pelvic pain (see Figure 1.3 for the outline of the diagnostic protocol). A human chorionic gonadotropin (Hcg) pregnancy test is typically to rule out an ectopic pregnancy. A urinalysis is also conducted to differentiate the possibility of urinary tract infection or PID. A speculum examination is conducted where any sign of cervical mucopurulent discharge is one further indication of PID. The clinician then conducts a bimanual examination to test for cervical, uterine, and adnexal motion tenderness which is considered a critical diagnostic criterion to indicate PID. If indicative of PID the patient is immediately administered antibiotics, whereby a rapid response to treatment, meaning a decline in pain and symptoms within

1-3 days on follow up, supports the diagnosis of PID. During the examination, cervical swabs are taken to conduct molecular tests for sexually transmitted infections, but treatment is administered regardless of this result, in part, because the studies are invasive and complicated to conduct and because the condition is difficult and subjective to diagnose. Criteria for recruiting PID in this study was based on the diagnostic approach in Australia.

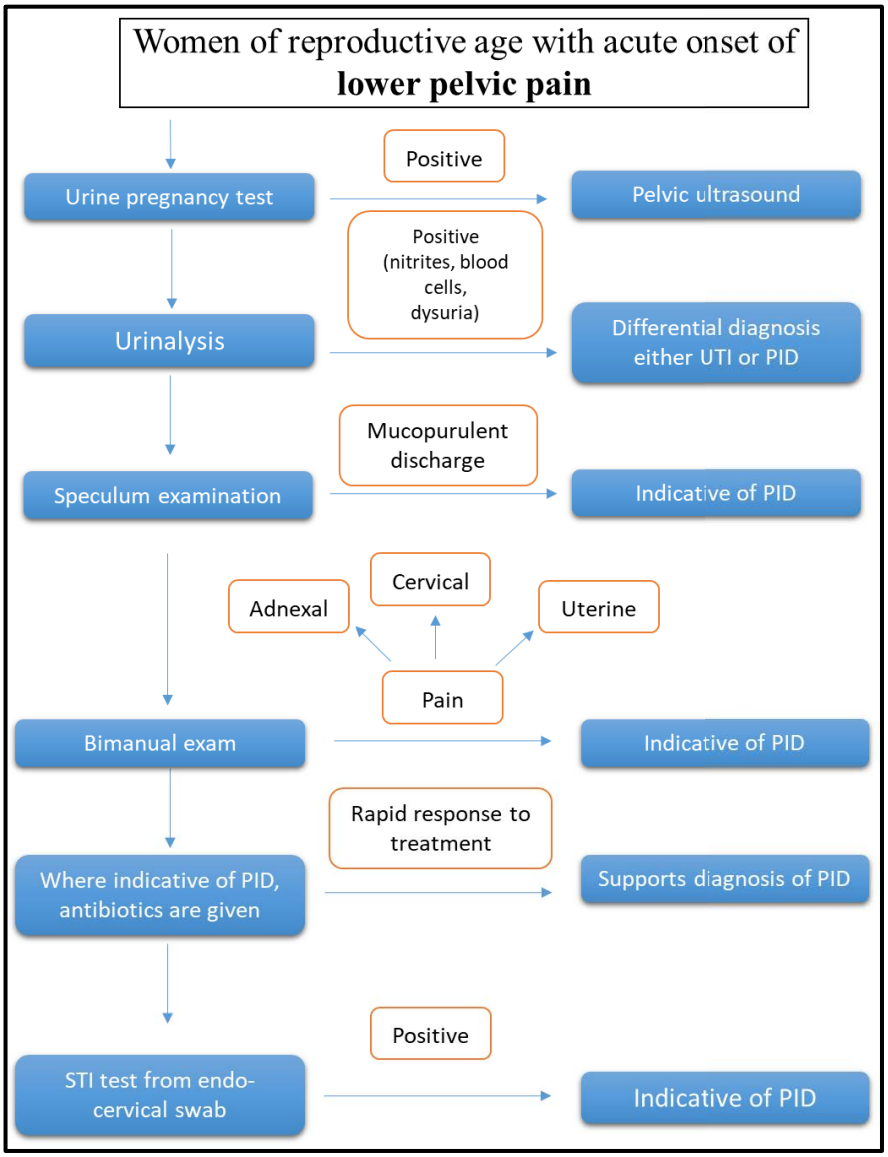


Figure 1.2. The criteria for a diagnosis of PID. The flow chart depicts the typical diagnostic work flow conducted by clinicians in Australian settings. Currently PID is difficult to confirm so during a physical examination a practitioner uses pressure to determine patient's pain. The patient must have tenderness in the lower abdomen, adnexal and cervical motion tenderness, plus a pathophysiological evidence as above.

1.3.1.1. Complexity of diagnosis

As described in section 1.3.1, diagnosis can be complex and is time consuming compared to a standard clinical consult for a sexual health check-up, for example. Besides, a bi-manual examination may raise certain cultural sensitivities or may constitute a barrier to the conduct of a bi-manual examination. Moreover, the bi-manual examination is subjective because it relies on a pain scale after a clinician has placed their hands in order to examine cervical and uterine tenderness (this process is already painful). For example, one study found that only 80% of male clinicians provided a chaperone during the examination [17]. In a study of medical clinicians, 98 clinicians were asked about their attitude towards the bimanual examination (regardless of purpose), many were sceptical about the exam due to its subjectivity and that it was time-consuming [18]. In a study of over 400 women, a large proportion (41.8%) of women indicated that pelvic inspection contributed to feelings of diminished modesty and shyness [19]. There are many studies indicating women's feelings of discomfort, embarrassment, and shame when it came to gynaecological examinations, whether it be the gender of the clinician, cultural issues or availability of a chaperone [17]. This leads to a worrying issue whereby women are delaying examinations or potentially ignoring subtle symptoms [20]. In a medical record audit conducted at the Melbourne Sexual Health Clinic, there was a 62% variance rate between doctor diagnosis of pelvic inflammatory disease and presence of PID factors in the medical record of the consultation [8]. In another study of the deviation between doctor diagnosis, PID was compared to genital warts (positive control for diagnosing a condition), whereby the diagnosing ability was found to vary three times more than genital warts [8]. In a cross-sectional study of 3804 women assessed by 39

clinicians at a United Kingdom sexual health clinic, it was reported that inexperienced clinician (defined by nurse practitioners who have worked for less than 1 year and medical doctors who are interns or have just started working for less than 4 months) were three times more likely to miss PID cases than experienced clinicians (defined by a minimum of 1 year of full-time equivalent experience of assessing symptomatic women in sexual health clinic) [21]. However, this study's limiting factor was that women were not assigned randomly to the various clinical grades but were examined by next available clinician in order of registration, which could lead to potential bias. It has been speculated that PID cases are missed regardless of who is in charge of diagnosis due to the subjectivity of the diagnostic protocol.

Thus the combination of a subjective and invasive bimanual examination, and the time consuming nature of the consultation, indicates that there is an unmet need for a molecular diagnostic that would improve the accuracy of diagnosis. Therefore, this project aims to evaluate the pathogen, microbial, and host immune responses that are significantly associated with PID, in order to identify a suite of molecular biomarkers that could potentially be developed into a molecular PID diagnostic test.

1.1.4. Treatment of PID

The current treatment protocol for PID (Table 1.1) raises concerns as it is immediately treated with several antibiotics regardless of infections diagnosed. The increase of antibiotic resistant bacteria in the world and the rise of multiple drug resistant sexually transmitted infections may be at further risk from this treatment protocol. The

antibiotics used for PID are ceftriaxone, cefotaxime, metronidazole, doxycycline, azithromycin and moxifloxacin, with a minimum of three antibiotics are used in conjunction [22]. Ceftriaxone is a cephalosporin of third generation that is used in a number of diseases, such as meningitis, gonorrhoea, and pneumonia [23]. It has a broad range of antibacterial activity targeting both Gram-positive and negative, and both anaerobic and aerobic organisms [24]. Many urogenital pathogens are known to be resistant to ceftriaxone such as *Escherichia coli*, *Streptococcus* group B and *Neisseria gonorrhoeae* [25]. Metronidazole has limited bacterial targets, mainly anaerobic Gram positive and negative bacterium, but also protozoans (*Entamoeba histolytica, Giardia lamblia,* and *Trichomonas vaginalis*) [26].

Antibiotic sensitivity for anaerobic organisms are not conducted in most pathology laboratories [27], but there has been limited evidence of oral pathogens showing resistance [28]. This is worrying as metronidazole is always prescribed for PID. Doxycycline is another broad spectrum antibiotic that targets both anaerobic and aerobic, Gram positive and negative organisms [29]. It is commonly used for nongonococcal urethritis, cholera, brucellosis, rectal chlamydial infections and several rickettsial infections. This is effective in the management of many sexually transmitted illnesses, stomach disorders, parasites, and specific infections in the respiratory tract [30]. Many organisms are now resistant to doxycycline, including some circulating strains of *N. gonorrhoeae* [31]. Cefotaxime is a relatively new semisynthetic cephalosporin administered intravenously or intramuscularly that acts against both aerobic and anaerobic, Gram positive and negative bacteria [32]. This antibiotic is commonly used for lower respiratory tract infection and in surgical prophylaxis [33]. Resistance against

cefotaxime is found in *Pseudomonas aeruginosa, Escherichia coli* and *Enterobacter spp* [34]. Azithromycin is a macrolide antibiotic with a primary target of Gram-negative organisms [35]. It is commonly used for *C. trachomatis* and is active against *Ureaplasma urealyticum*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Mycoplasma hominis* [36]. Resistance to this macrolide has been noted in pathogens such as *N. gonorrhoeae, Haemophilus influenzae, Staphylococcus aureus* and *Streptococcus pneumoniae* [37]. Moxifloxacin is only used in cases where *M. genitalium* is present. This antibiotic is from the fluoroquinolone family, with a broad spectrum of activity against Gram positive and negative organism, with superior activity against anaerobes [38]. It is most commonly used for people with a respiratory infection such as chronic bronchitis [39]. Paradoxically, resistance against this antibiotic has been found in *M. genitalium* [40] and *Pseudomonas aeruginosa* amongst others [41]. Presumptive and rapid treatment is administered routinely for PID in spite of these risks because of the known serious outcomes for the condition. Therefore, the clinicians must weigh up risk to the individual by not treating compared to relatively unknown risks of this individual contributing to development of antibiotic resistance and typically presumptive treatment makes sense and is recommended in current guidelines. Any diagnostic would need to have the capacity to be eventually rapid and point of care to potentially impact on these crucial practical considerations.

While I talk about the Australian guidelines for treatment of PID, it changes across the globe with these combinations of antibiotics used reviewed by Quentin *et al* [42] and listed below:

- amoxicillin and clavulanic acid 2–3 g/day, + doxycycline (200 mg/day),

- amoxicillin and clavulanic acid 2–3 g/day + ofloxacine 400 mg/day,
- cefotetan 2 g Intravenous (I.V.) every 12 hours or cefoxitin 2 g I.V. every 6 hours + doxycycline 100 mg I.V. or orally every 12 hours,
- clindamycin 900 mg I.V. every 8 hours + gentamicin loading dose I.V. or intramuscular injection (I.M.I) (2 mg/kg of body weight) followed by a maintenance dose (1.5 mg/kg) every 8 hours,
- ofloxacin 400 mg I.V. every 12 hours + metronidazole 500 mg I.V. every 8 hours,
- ampicillin and sulbactam 3 g I.V. every 6 hours + doxycycline 100 mg I.V. or orally every 12 hours,
- ciprofloxacin 200 mg I.V. every 12 hours + doxycycline 100 mg I.V. or orally every 12 hours + metronidazole 500 mg I.V. every 8 hours.

Table 1.1. Treatment protocol for PID in Australia

Situation	Treatment
Out-patient settings	Ceftriaxone (500 mg in 2 mL of 1% lignocaine IMI[a], or 500 mg IV[b], stat[c]) + Metronidazole (400 mg PO[d], BD[e] for 14 days) + doxycycline (100 mg PO[d], BD[e] for 14 days)
Hospital	Ceftriaxone (2 g IV[b], daily) or Cefotaxime (2 g IV[b], TDS[f]) + Azithromycin (500 mg IV[b], daily) + Metronidazole (500 mg IV[b], BD[e])
If *M. genitalium* positive	Moxifloxacin (400 mg, PO[e], daily for 14 days)

a: IMI; intra-muscular injection, b: intravenous, c: stat = immediately, d: PO= by mouth, e: BD = twice daily, f: TDS = three times a day

1.1.5. Reproduction morbidities associated with a history of PID

PID can lead to further complications, as has been evidenced in numerous studies. In one prospective study of 831 women, gynecological health was assessed for a period of time after diagnosis. The majority of women in the study had mild to moderate PID (90%) and were followed up every 3-4 months for 35 months. It was identified that 18.4% of PID cases developed infertility, 33.7% chronic pelvic pain, and 41.2% tubal obstructions (none of which were significant compared to controls) [43]. In this aforementioned study, significance was compared to the control group of women who were pregnant during the 35 months of follow up after the onset of PID. Defining by the outcome of pregnancy or not, after PID is not unreasonable but perhaps an under measure of the impact, potentially a control group of high risk sexually active group with no onset of PID may have provided better insights. In this study, infertility was defined on 12 consecutive months of sexual activity with no use of contraception. There was no significant difference between outpatient and inpatient treatment and the frequency of women reporting the serious reproductive outcomes. Therefore the findings suggest that less severe PID did not significantly differ in serious morbidities, so it is important not to underestimate the possible burden from General Practitioner (GP), or sexual health clinic diagnosed and treated PID in any settings.

In another earlier cohort study of laprascopically diagnosed (less subjective diagnostic method but invasive) pelvic inflammatory disease of 1844 women compared to control group that underwent laprascopy with normal findings (n = 657) [12]. The women with PID were followed for a mean of 94 months and controls for a mean of 80

months. It was reported that 9.1% of women had an ectopic pregnancy (p-value <0.001, compared to 1.4% in controls) and 16% infertility (2.7 % in controls), after PID diagnosis [12]. No statistical test was conducted on those women who developed infertility probably due to the multitude of infertility reasons (tubal factor, partner infertility, anovulation etc). Furthermore, the study did not analyse or report those that developed chronic pelvic pain. It is now understood that PID may lead to infertility, ectopic pregnancy, and chronic pelvic pain, as stated by the Australian sexual health guidelines [22] and in many other reviews [44-49]. While the exact costs of PID in Australia (and other developed countries) is unknown, the costs associated with urogenital *Chlamydia* and its complications (including PID) have been shown to be more than $2 billion US dollars annually in the USA (United States of America) [50].

1.2. EPIDEMIOLOGICAL AND DEMOGRAPHIC FACTORS ASSOCIATED WITH PID

Young age, low socioeconomic setting, sexually active and low education are all factors that correlated with increased risk of an episode of PID [5, 51]. Rates of PID are much higher in remote communities such as remote Indigenous communities in Australia, where a retrospective, cross-sectional analysis of medical files of 342 indigenous women determined that 32% have had PID [52]. In another study of medical files of 267 200 women hospitalised for PID in the USA, it was found that risk for hospitalisation was higher for women in their 20s [53]. In a case-control (140 PID cases and 241 control either attending a clinic for a blood test or tubal ligation) study, it was found that age at first sexual intercourse <20 years; non-white ethnicity; not having had children; a self-

reported history of a sexually transmitted disease all were associated with an increased risk of PID (all p-value <0.01) [54].

1.3. AETIOLOGY

PID can be associated with sexually transmitted infection, bacterial infections of other sources (e.g. respiratory pathogens), instrumentation (IUD), and or an idiopathic aetiology (although all studies that have examined microbes have demonstrated that PID is polymicrobial). Each aetiological form is described in great detail in subsections (1.3.1 – 1.3.4). In brief, STI agents are highly prevalent in PID, including *Chlamydia trachomatis* (30-50% of PID cases), *Neisseria gonorrhoeae* (30-50% of PID cases) [51], and *Mycoplasma genitalium* [55]. *Mycoplasma genitalium* has only recently been associated with PID and the incidence of PID cases associated with this bacterium is unknown. Additionally, many cases of idiopathic associated PID have also been reported with one study reporting almost 62-70% of women having no infectious agent [55, 56]. Procedures involving instrumentation have the ability to trigger inflammation in the upper genital tract and this has been associated PID, e.g. abortion, hysterosalpingography, IUD insertion or removal, dilation and curettage of the uterus, or embryo transfer as reviewed by Mears *et al* [57].

1.3.1. Idiopathic PID

Since aetiological agents are not identified in the majority of cases, polymicrobial agents such as natural flora of the vagina and anaerobic bacterium that are less frequently

found in the vagina are considered likely agents [51]. Bacterial vaginosis (BV) is a condition defined by the shift of the vaginal microbiome, noted by an odour. The incidence of BV in reproductive-aged Australians has been reported to be 9.4/100 person-years [58]. This dysbiosis of the vaginal flora has been associated with cervicitis and PID [59-62]. In one study among predominantly young, African-American women followed longitudinally, it was found BV-associated microorganisms cultured from the vagina increased the risk of developing PID, and that this was most significant after a new recent sexual partner [63]. However, no one organism has been associated with bacterial vaginosis and no causality with PID has been identified [64]. PCR testing for bacterial vaginosis-associated bacteria in endometrial samples detected bacteria such as *Sneathia sanguinegens*, *Sneathia amnionii, Atopobium vaginae* and *BV-associated bacteria 1* (BVAB1) from women with PID [65]. In a study of endometrial biopsies of 105 women with PID, 16% had *C. trachomatis;* moreover, more than half (56%) had *Prevotella spp.*, 38% had *Gardenerella vaginalis,* 18% had *Atopobium vaginae* and 22% had an unclassified anaerobic gram-positive cocci species using molecular and culture techniques [66]. Hence, it is likely the cervicovaginal microbiome plays an essential part in development and protection from PID and is suggested that a combination of organisms may be associated with the development of PID [67-69]. Whilst only recently, studies are being to investigate the polymicrobial factors that lead to PID, more rigorous approaches into genotypes/strains or sub-types, actual abundances, co-associated organisms should all be considered in future studies.

1.3.2. Pathogen associated PID

1.3.2.1. *Chlamydia trachomatis*

Chlamydia (C.) trachomatis is the most common bacterial sexually transmitted infection worldwide [70]. The organism is an obligate intracellular Gram-negative bacterium. *Chlamydia* has a unique biphasic lifecycle (termed developmental cycle, Figure 1.4). This biphasic developmental cycle includes a relatively dormant elementary body (EB), which is the infective, extracellular form, and the replicative, intracellular reticulate body (RB). The EBs attach to a host cell, enter, and form a unique niche termed the inclusion vacuole, where they then reorganise into the RB form as reviewed by O'Connel *et al* [71]. This form of the bacterium then multiplies within the inclusion vacuole consuming host metabolic intermediates. The multiplication occurs via binary fission, and the cells eventually condense to the EB form after 30 to 48 hours (asynchronously). The host's cell lyses or the bacterium can extrude from the host [72] and the bacterium can now infect other cells [73]. As shown in Figure 1.3, another possibility before host lysis is when the organism forms an aberrant body (AB), which is a relatively less active form and persists in this state due to an external stressor. After which the organism emerges and returns to EB form [71].

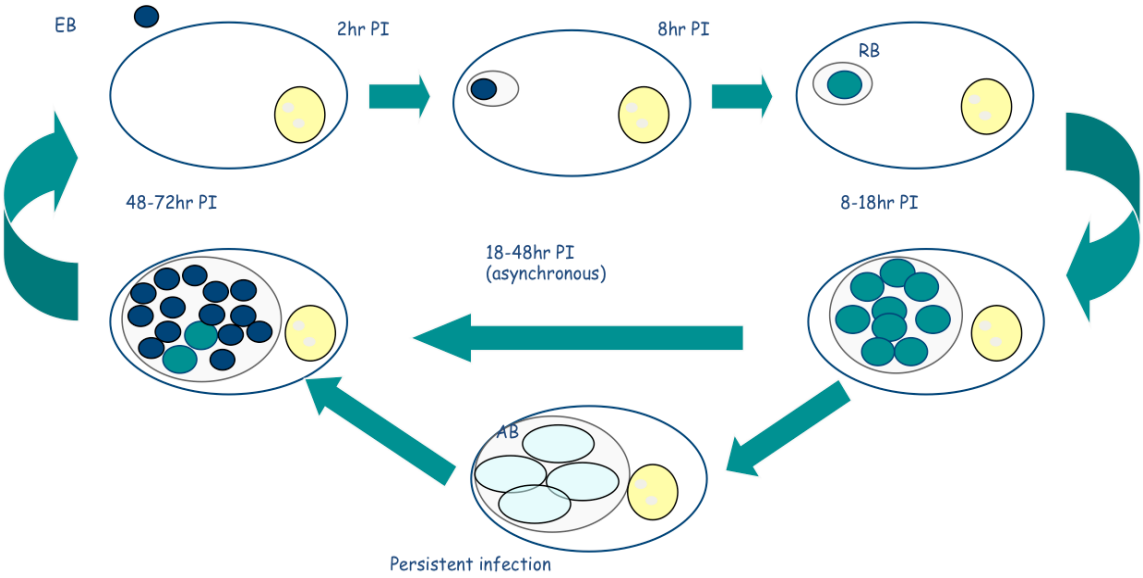


Figure 1.3. The chlamydial developmental cycle. Starting with elementary bodies (EB) that infect the mammalian host cell causing a vacuole to form. The EB's then transform in reticulate bodies (RB), the reproductive state of *Chlamydia.* Then within the vacuole, the organism divides and multiplies and can enter a persistent state where the organism is known as an aberrant body (AB). After the developmental cycle, the organism then reverts the EB's to lyse out of the host cell and infect other cells.

1.3.2.2. Clinical presentations for *Chlamydia*

This unique intracellular growth niche provides the bacterium with a protective niche to avoid host responses [74]. The infection process commences when the bacterium enters the columnar epithelium within the endocervix of the cervix [70]. While at the site of infection, oedema, redness, and discharge may occur, the patient may not be aware of these signs. Clinical symptoms of infection include dysuria, abnormal vaginal discharge, abnormal menstruation, lower abdominal pain and post-intercourse bleeding [70] However, 70% of female cases are asymptomatic.

1.3.2.3. Burden of disease

There is an estimated 131 million new annual cases of urogenital *C. trachomatis* infections; these infections have the potential to cause PID, tubal infertility, and ectopic pregnancies in women [75]. Global estimates of the prevalence of chlamydial infections in women were 4.2% in 2012 [76]. The burden of the chlamydial disease includes the substantial costs associated with screening, testing, and treating the primary infections, but also the costs of complications from the infection, and the social burden of infertility [77]. *C. trachomatis* is also the leading cause of preventable blindness and is currently endemic in developing countries such as Africa, Southeast Asia, and the Middle East, and also in remote Indigenous communities in Australia. It has been estimated that globally, 150 million people have ocular *C. trachomatis* infections, and another 6 million have irreversible blindness because of this pathogen [77].

In Australia, genital chlamydial infections have been notifiable since 1991 with notification rates showing a marked increase over the past decade. Incidence of this disease has been shown to be the highest amongst younger people (under 25) [78]. Notifications are the highest in the Northern Territory with a particularly high incidence in Indigenous persons [78]. However in a meta-analysis of the prevalence of *C. trachomatis* in the Australian population found that the burden is high not only in the Indigenous population, but also in young adults, and men who have sex with men [78].

1.3.2.4. Treatment of *Chlamydial* infections

C. trachomatis is resolved with the use of antibiotics. In the late 1990s, the antibiotic doxycycline was prescribed for seven days, with two tablets per day. This was followed by the introduction of a single dose of azithromycin (1 gram) treatment regimen [79]. In the San Francisco Department of health, after the new treatment protocol was introduced compliance was significantly increased from 61.8% in 1998 to 81% in 2000, and females patients under 20 years of age had an even higher compliance rate [79].

The treatment with 1g of azithromycin or seven days of doxycycline is relatively effective. However, recent controversy suggests there may be treatment failures for these drugs, due to a high rate of reinfection in the population [80]. The data suggests that this may be due to differing pharmacokinetics within different regions of the body, different organism load, and potentially different host immune responses [80].

1.3.2.5. Immunological responses to *Chlamydia*

The exact process that leads to the most damaging consequences (such as tubal infertility) from the infection are not yet fully understood, with several models or theories around immune or inflammatory processes proposed in the literature. The significant models will be described below and include the HSP60 (heat shock protein-60) model of hypersensitivity, the cellular paradigm, ascension, and persistence models. The HSP60 model of hypersensitivity to *Chlamydia* is supported by high titres of antibodies against the protein being found in the serum of women; however, these antibodies found in other

human sera may not be indicative to tubular infertility due to *Chlamydia* as they cross-react with other bacterial HSP60 protein [81-84] Also, the antibody response and higher titres may simply indicate that an immune response is involved in the development of tubal factor infertility and this antigen is one of the dominant antigens recognised, rather than an antibody response to this specific antigen being the causal mechanism of tissue damage.

Another model is the cellular paradigm that which proposes that the primarily infected epithelia in the upper reproductive tract and the immediate innate response prime the type of immune response that follows are responsible for the outcomes of the disease in each woman [85]. The cellular paradigm was supported by a study that demonstrates that a primary pro-inflammatory response from the infected epithelia triggers the immunopathology[86]. IL-6 (Interleukin-6) is produced as a product to chlamydial infection and in particular, the chlamydial stress response proteins (HtrA) and tail-specific protease (Tsp)[87]. Thus, these stress proteins likely have PAMPs, (pathogen-associated molecular patterns) that are recognised by the host cell and which induce IL-6 based inflammatory response leading to the pathology of the infection [87]. When a pro-inflammatory response occurs to a damaging degree, the magnitude of the initial response and type of response (driven by multiple factors) in each individual may determine the profile of the primary response and subsequent impact.

A third model for the development of infertility is the ascension model, whereby the pathogen is thought to travel up beyond the cervix to produce tubal pathology [88]. Verification for this model is by chlamydial deoxyribonucleic acid (DNA) recognition in

salpingectomy specimens from patients with ectopic pregnancies, and endometrial and cervix specimens from other women [89]. In one study, chlamydial DNA was observed in the ovaries, endometrium and fallopian tubes of 56% of women with ectopic pregnancies [90]. Further supporting this model are findings that suggest that the pathology of infertility only happens to some women, and maybe due to multiple factors that likely influence the disease progression from infection [90]. Moderating factors are likely to include; menstrual cycle stage, hormone levels at the time of infection, immune status, genotypic factors and the composition of the reproductive tract microbiome [90]. However, it is likely that ascension is not all that is required to result in the upper reproductive tract tissue damage, and that ascension of the infection may occur in the absence of pathology.

Another known plausible model is the persistence model whereby viable, but non-culturable forms of *C. trachomatis* develop due to various conditions and remain dormant for a lengthy period of time [90, 91]. It has been suggested that during this period of dormant infection, the immune response continues, and causes damage to the tissue and thus scarring in the fallopian tube [90, 91]. This model would rely on ascension to the upper reproductive tract prior to the development of persistence.

1.3.3.1. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae (*N. gonorrhoeae)* causes the disease gonorrhoea which can ascend into the upper genital tract leading to further sequelae [92]. This bacterium is

a Gram-negative diplococcus. It is non-spore forming, non-motile, non-acid-fast encapsulated bacteria [93].

1.3.3.2. Clinical presentations of gonorrhoea

N. gonorrhoeae is detected in the reproductive tracts of both females and males. Generally, as a localised infection of the epithelia within the vagina, rectum, cervix, urethra, and pharynx [94]. Symptoms include discharge, dysuria, lower abdominal pain and uterine bleeding. Since asymptomatic infections occur in 50-60% of women, the ascension of the bacterium is likely and problematic. The infection is associated with severe sequential problems such as ectopic pregnancies, PID, and infertility. 10-17% of women with a urogenital gonococcal infection develop PID [95].

1.3.3.3. Burden of gonorrhoeal infection

A 2012 study that attempted to estimate the prevalence of different STI's estimated the global incidence of gonorrhoea to be 78 million cases per annum [76]. The World Health Organization (WHO) has mapped the incidence of gonorrhoea by and identified the highest incidence to occur in the western pacific region (see Figure 1.5) [76]. This study methodology was very comprehensive and shows that gonorrhoea is most prevalent in developing countries. This aforementioned study used 79 published studies and the WHO's previous estimates in 2005, and then used the same methodology as published by WHO in 2005 [96] to generate the global estimates. Conversely, using

another estimation model with already published and population-aggregated data sets, gonorrhoea prevalence was determined to be 6.6% [97].

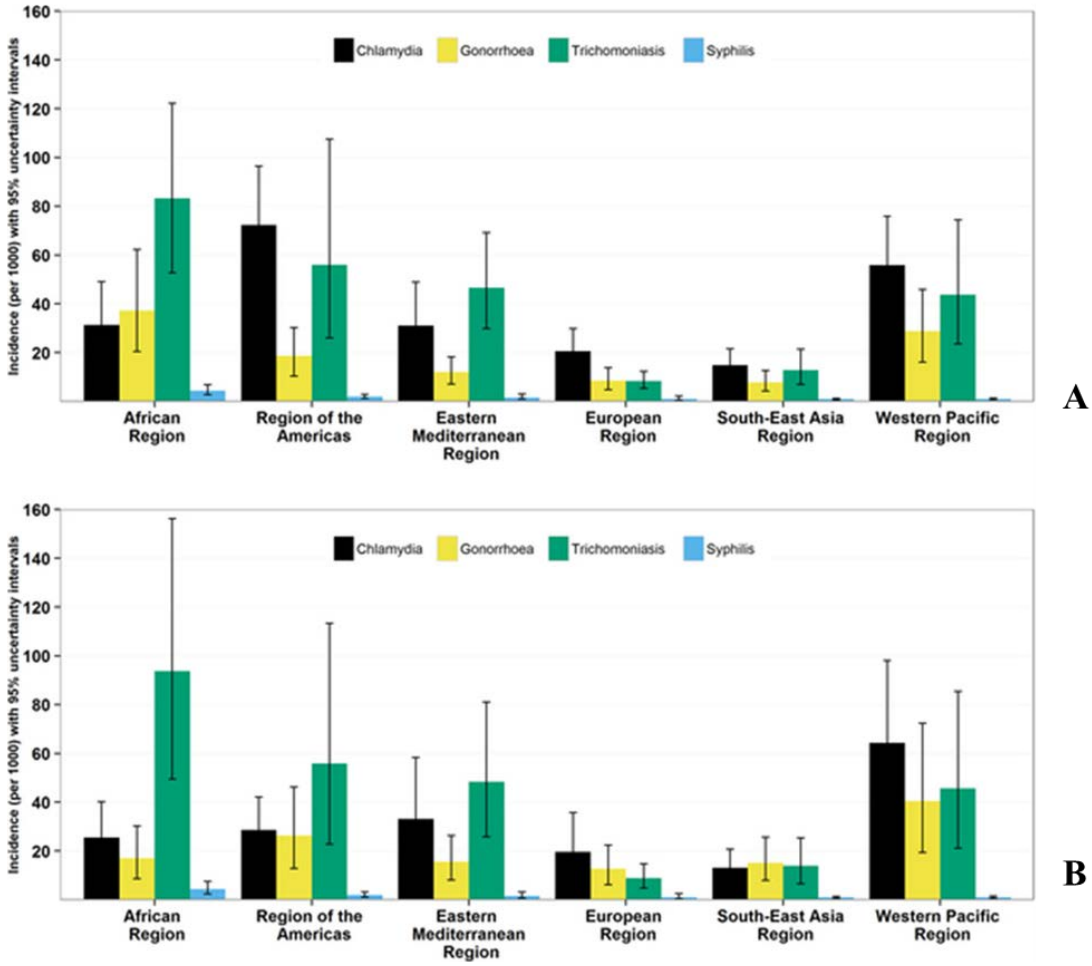


Figure 1.4 The incidence per 1000 people of common STI's including Chlamydia, Gonorrhoea, Trichomoniasis and syphilis by regions of the world. The incidence of Gonorrhoea and Chlamydia is highest in the western pacific region, trichomoniasis and syphilis are highest in the African region. Panel A is women only and B is men only, for both column graphs, the x-axis is the WHO region and y-axis is the incidence per 1000 with 95% uncertainty interval. The black is chlamydia, yellow is gonorrhoea, green is trichomoniasis and blue is syphilis (reproduced from [76]).

1.3.3.4. Diagnosis of gonorrhoea infection

Urogenital infections of *N. gonorrhoeae* can be detected using both culture and non-culture methods in the laboratory. Currently, the first line of diagnosis in most settings is nucleic acid amplification (NAAT) [98]. Culture methods were previously the standards for diagnosis of infection; however, these relied on stringent conditions needed for transportation and handling of patient specimens. The first non-culture methods included those that incorporated an enzyme immunofluorescent assay (EIA), which detects a host response based on the infection process [98]. These are currently not recommended to be used in clinical settings as they have a low sensitivity of 51% [99].

1.3.3.5. Current Treatment of gonorrhoea

Current WHO recommendations for treatment for anorectal and urogenital include a regime of dual therapy of intramuscular ceftriaxone (500 mg) and a single oral dose of 1 g azithromycin [100]. For patients that cannot undergo the above (allergy), it is recommended that a single oral dose of 400 mg cefixime and 1 g of azithromycin [100]. Failed primary treatments of infections are on the rise due to antibiotic resistance (discussed in 1.3.2.6). Secondary treatment dual recommendations include 500 mg intramuscular of ceftriaxone with a single oral dose of 2 g azithromycin or a single oral dose of cefixime (800 mg) and azithromycin (2 g) or 240 mg intramuscular injection of gentamicin with a single oral dose of azithromycin (2 g) or a 2 g intramuscular injection of spectinomycin with a single oral dose of azithromycin (2g) [100]. However, it is

recommended that reinfection is explored through strain typing before commencing secondary treatments to limit the selection for multiple drug resistance.

1.3.3.6. Antimicrobial resistance found in *N. gonorrhoeae*

Culture methods are commonly used for antibiotic sensitivity testing, and commonly the protocol uses an E-test strip which is a plastic carrier strip with an antibiotic gradient that is applied to the surface of inoculated agar [101]. The case of Multi-drug resistant *N. gonorrhoeae* is increasing at a rapid rate whereby the USA Center for Disease Control (CDC) has described it as an urgent threat that requires urgent public health intervention [102]. This bacterium owes its successes to being able to modify genetic material and rapidly transfer genetic material between bacterial cells [103]. This bacterium is now known to possess every method of antimicrobial resistance and thus poses a threat to become untreatable [103].

1.3.3.7. Virulence of *N. gonorrhoeae*

This bacterium has evolved virulence factors that allow it to survive within the host and evade the immune response. These factors include the pili, Por protein expression, lipooligosaccharide (LOS) expression, IgA1 protease production and opacity-associated protein (Opa) expression [104]. The Pili allows for the attachment to mucosal membranes such as the urogenital tract. These pili then retract, allowing for the closer junction between the bacterium and epithelium cell [105]. Opa proteins are variably expressed as

infection occurs, and a single gonococcal bacteria has 12 Opa genes that express different proteins that allow for secondary attachment on to different human cells [106]. It is the variable expression of these membrane proteins that allow for both different virulence capacity and immune evasion. As the cell attaches to the human epithelium, it can be internalised or membrane-bound, and that's when the Por proteins are vital for survival. Por proteins are responsible for 60% of total gonococcal proteins. They are required for the translocation of nutrients and waste within the bacterium [107]. LOS also play a part in defence as it they have properties that are unlike other Gram negative bacteria, gonococcal LOS is resistant to human serum killing aiding in pathogenicity [108]. LOS is one of the major factors responsible for inflammation as it is toxic to the fallopian tube, causing the human cilia to slough [109]. During bacterial infection, survival is attributed to the secretion of IgA protease which is an enzyme that cleaves the heavy chain region of the human IgA1 subclass immunoglobulin and lysosome-associated membrane protein 1 (LAMP1) [110, 111]. These factors are important protection against host defence mechanism.

1.3.4.1. *Mycoplasma genitalium*

Mycoplasma genitalium (M. genitalium) is one of the smallest free-living organisms that can be parasitic in humans, reptiles, and fish. Lacking a cell wall and intracytoplasmic structures, these organisms only have a plasma membrane [112]. Unlike other mycoplasma species, *M. genitalium* was found to be flask-shaped and not circular, which is proposed to aid in the specific attachment in the genital tract [113]. This bacterium is fastidious in growth and isolation and wasn't discovered until the technology

of polymerase chain reaction (PCR) was available where two strains were identified in 2 men with non-gonococcal urethritis [114]. This human bacterial parasites preferred site of habitation is the urogenital tract but can be found in the anorectal and respiratory tracts. It has been reported that *M. genitalium* can translocate through blood and affect other anatomical sites such as the joints [115].

1.3.4.2. Clinical presentations of *M. genitalium*

The association of *M. genitalium* with symptoms and disease is a recent and somewhat controversial development in the STI field. This organism has been detected in vaginal, cervical, and endometrial samples, including in association with inflammation [116]. However, the association between PID and this bacterium has been identified but is not yet well characterised [117]. Serological studies have displayed a strong correlation between this bacterium and tubal factor infertility (TFI) which is a known sequela of PID [118].

A study compared three groups of women; those infected with *M. genitalium,* those infected with *C. trachomatis,* and control group (no detected infections) to evaluate symptoms. It was established that signs of urethritis and/or cervicitis were significantly higher in women with *C. trachomatis* (p-value <0.01) and *M. genitalium* (p-value = 0.03) when compared to controls [119]. This study had a small sample size, with only 22 participants positive for *M. genitalium,* and there is a need for more extensive studies to confirm their findings. Vaginal discharge due to inflammation was also associated with *M. genitalium* infection from 20 independent clinical studies [120]. With the studies

present, there is considerable variation as to the role of *M. genitalium* in PID, mostly due to high asymptomatic positivity [120]. Also, the lack of a reliable diagnosis of PID could be one reason why there is not yet a robust independent correlation of *M. genitalium* and PID. Another possibility is that there has been a detection of variation in the MgPa gene (used as a target gene for clinical diagnosis) [121] and many strains are undetected. Furthermore, it is reported that up to 5% of the population can harbour the organism and be asymptotic [122, 123]

1.3.4.3. Burden of *M. genitalium*

Since the majority of cases are asymptomatic and diagnosis for *M. genitalium* is recently new, the global burden is not fully understood. It is also believed that co-infection with other STIs is common, but the dynamics of these co-infective states are yet to be studied [120]. In a meta-analysis comparing a group of low-sexual risky behaviour compared with high-risk sexual behaviour based on the definition of the number of sexual partners, the bacterium was more frequently present in those that partake in sexually risky behaviour (approximately 7.3% compared to 2% in low risk population) [119, 120, 124-126]. This comprehensive study was based on a total of 27 000 women diagnosed with *M. genitalium* with high risk participants known as STI clinic visitors, individuals involved in a study that included symptoms of urogenital disease, patients who were present at family planning termination clinics, or people listed as sex workers. Low-risk participants were not attending an STI clinic, participants to fertility clinics, chosen by randomization from a healthy population and all women enrolling in pregnancy studies [120]. However, due to the nature of comparing multiple studies, that have different case-

control definitions, different symptoms or disease presentation no statistical analysis was performed. Hence, the true prevalence of the bacterium and if it causes symptoms is still unknown as in a low risk population the organism is detectable.

1.3.4.4. Diagnosis and treatment of *M. genitalium*

Current diagnosis of *M. genitalium* is limited to NAAT on an endocervical or vaginal swab or first-pass urine [125]. Optimal treatment is not yet available for infection. In a study of *M. genitalium* positive men (n=34) and women (n=26) it was reported that tetracyclines (doxycycline 500mg for seven days) were not effective [127]. However, azithromycin (1 g single oral dose) resulted in *M. genitalium* PCR negative participants after five days [127]. Tetracyclines are part of recommended treatments for *C. trachomatis* and *N. gonorrhoeae* [100] and not effective for *M. genitalium,* in turn, causing this bacterium to flourish and persist in the host. Since the majority of infections are asymptomatic and tests are not commonly ordered by health practitioners, it is not clear as to when the infection is should be considered serious and treated. Concerningly, antimicrobial resistance is being detected in *M. genitalium,* this includes resistance to azithromycin, moxifloxacin (first line prescription), and josamycin (a macrolide antibiotic from *Streptomyces narbonensis*) as reviewed by Unemo *et al* [128].

1.3.4.5. Virulence of *M. genitalium*

The *Mycoplasma* family are all small in size (0.6-0.7 μm) and share common adherence properties [113]. *M. genitalium* is found on the surface of cells with an intimate junction between the host and organism. This junction is due to adhesion molecules found on the membrane (P1, MgPa, and P30), which allow survival in an area with fluid motion [112]. Other virulence factors are yet to be studied for this species due to difficult *in vitro* culture requirements. However, it is hypothesised that *M. genitalium* shares similar mechanisms to evade the host response as the mycoplasma family. This includes the direct death of T-cells by fusing with T lymphocytes [112]. Introduction of downregulating cytokines such as interleukin-10 is thought to be induced by *M. genitalium* inducting a pathway that results in down-regulation of T cell activation (evading the host response) [112].

1.3.3. *In vivo* animal models of STI's

Attempts to conduct *in vivo* animal models of STIs are currently limited. At least half of the animal studies widely cited have been calculated not to represent a human randomised control trial standard because of differences between models and humans [129, 130]. Hence the use of any animal model needs to be cautiously considered and statistical power needs to be calculated beforehand. While non-human primate models are the most accurate and translatable their use comes with many ethical concerns, physical concerns (handling etc), and monetary considerations. Therefore the mouse (*Mus*

musculus) is the most commonly used model and it represents the human immune system to some extent [131].

For pathogens, *C. trachomatis, N. gonorrhoeae* and *M. genitalium* mice are used to understand pathogenic and virulence factors. In a study of hormone's (progesterone and estradiol) effect on *M. genitalium,* mice were intravaginally infected with the pathogen and it was found in both cases that the pathogen colonies the upper genital tract within three days [132]. This study, however, doesn't take into account the viability of organism past four days due to clearance of organism. Furthermore, they compare post cull detection of the pathogen in the uterus to a vaginal swab, and this in itself isn't an accurate comparison. Many trials have tried infecting mice with *N. gonorrhoeae;* however, the murine model clears the bacterium rapidly due to adaptation of *N. gonorrhoeae* to humans [133]. The gonococcal murine infection only lasts for a maximum of two weeks and host response is limited to minimally activated neutrophils in vaginal smears as reviewed by Jerse *et al* [134].

Chlamydial murine infections, while discussed in Chapter 5, are certainly more advanced in technologies and more widely used than the previously mentioned murine models. However, there remain limitations for human chlamydia, *C. trachomatis,* as detectable organism clears after 21 days [135].

Many studies have investigated the host immune response after murine infection of *C. trachomatis* [136-139]. An example is a murine study that infects TLR4 depleted C3H/HeJ mice with *C. trachomatis* serovar A, D and L2 in order to study the adaptive

host response [140]. Comparing to *C. muridarum,* this study determined that *C. trachomatis* genital infection was far less dependent on CD4+ T cells (unlike *C. muridarum*) and that adaptive immune responses markers (IgG1, IgG2a, IgG2b, IgG3 and IgA) was delayed and not as prolonged as *C. muridarum* [140]. While host response is delayed, these studies are important in understanding the immune response towards *C. trachomatis.*

One advantage of a chlamydial murine model is that the mouse has mouse-specific *C. muridarum* which induces a sustained host response that can lead to PID and other complications [141]. It has been 70 years of chlamydial vaccine research and *C. muridarum* has played a vital role in acting as a vaccine test model in preclinical trials [142]. Considering the methods both *M. genitalium* and *N. gonorrhoeae* lack simple animal models, with limited success in the human specific *C. trachomatis* mouse model. Hence understanding the pathogenicity of STI's that lead to PID is very complicated and largely relies on *ex vivo* studies. An animal model of PID would highly benefit the field and maybe developed with the advent of humanised mouse strains.

With the evolvement of CRISPR-Cas9 genomic editing system, animals models have the potential to be developed to be susceptible to human pathogens [143]. Furthermore, ethical management of larger animals has evolved and the use of guinea pigs and mini pigs has been on the increase [144, 145]. In a humanised endometriosis mouse model, C57BL/6 mice were crossbred with McGreen (Cfs1r-EGFP) mice, which allowed for endometriosis was induced by intraperitoneal injection of donner uterine

horn, endometriosis developed for the duration of experiment [146]. Since endometriosis could develop from PID, mouse models like these should be considered.

1.3.4. Instrumentation induced PID

In 1998 the Royal College of Obstetricians and Gynaecologists (United kingdom) suggested the use of prophylactic antibiotics before uterine instrumentation be used (including but not limited to; curette, intra-uterine device (IUD), speculum, and endometrial pipelle) [147]. The most studied instrumentation is the contraceptive IUD, which raised concern over PID implications in the 1980s. In a meta-analysis of 12 randomised studies whereby women had one of each of the ten types of IUD's inserted [148]. Out of this analysis, it was found that the PID incidence after insertion was 1.58 per 1000 woman-years, and then established that in the first 20 days after insertion the rate was 9.6 per 1000 woman-years, where this rate decreased after 20 days (>20 days since insertion) to 1.38 per 1000 woman-years [148]. However, this analysis wasn't able to take into account the risk factors of PID, such as STI's and the number of recent sexual partners. Research into intrauterine device and pelvic inflammatory disease associations are challenging to conduct, meaning current understanding is constrained by these limitations. However, it is almost certainly a risk factor in the immediate time after insertion and most clinicians would rapidly treat women presenting with unresolved acute pelvic pain after instrumentation.

1.3.5. Occurrence of respiratory pathogens in PID

In recent times (2010 – current) the role of respiratory pathogen found within the genital tract of women with PID is beginning to be understood [149]. One reported cause was of a 44 year old woman who was found to have PID due to throat pathogen group A *Streptococcus* toxic shock syndrome [150]. However, this is a very rare occurrence and this organism was only tested for due to the negative results of common STI's or BV-associated organism. Other reports of PID associated with *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* that have colonized the lower genital tract as reviewed by Brunham *et al,* [4]. A more commonly studied respiratory pathogen in the female genital tract is *Mycobacterium tuberculosis* (TB) wherein one retrospective clinical study of 1,922 cases of PID in a developing region of China (where TB is common) the incidence rate was of TB caused PID was 42.1% [151]. However, TB caused PID is common in regions of the globe, whereby TB is circulating commonly in the population [152-154]. It is still not understood of the mode of transmission of the respiratory pathogen to the genital tract in order to cause PID, but it is speculated that it is due to oral sex.

1.3. THE MICROBIOTA

Briefly, the vocabulary in microbial community research is important. The microbiota is known as the assembly of microorganisms in a given ecosystem. The term flora is describing the organisms in this community, all these terms are reviewed by Marchesi *et al* [155].

1.3.1. The cervicovaginal microbiome

Bacterial communities in the human vagina are key components of a dynamic antimicrobial defence system that protects women against disease. The vaginal flora is typically dominated by the *Lactobacillus* species, these organisms lower the pH of the vagina through the production of lactic acid [156-158]. In order for the lactic acid to be produced, there is a need for free glycogen (the source of the fermentative reaction), and in a longitudinal study of 21 women for 8-11 years, it was found an increase of glycogen concentration is correlated with an increase in *Lactobacillus* abundance [159]. Furthermore, this lactic acid and *Lactobacillus* helps modulate the mucosal layer by communication to host epithelium [160, 161]. With the development of next-generation sequencing, vaginal pH and vaginal swabs were analysed of 396 asymptomatic, sexually active women who fairly equally represented four self-reported ethnic groups: white (n=98), black (n=104), Asian (n = 97), and Hispanic (n = 97) [162]. This major study set the standard for categorising the vaginal microbiome into five communities of microbes termed community state type (CST). CST I is dominated by *L. crispatus*, whereas CST II, CST III, CST V were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively, whilst CST IV had no dominant *Lactobacillus* and often had mixed proportions of anaerobic, Gram-variable bacteria (Figure 1.5.) [162].

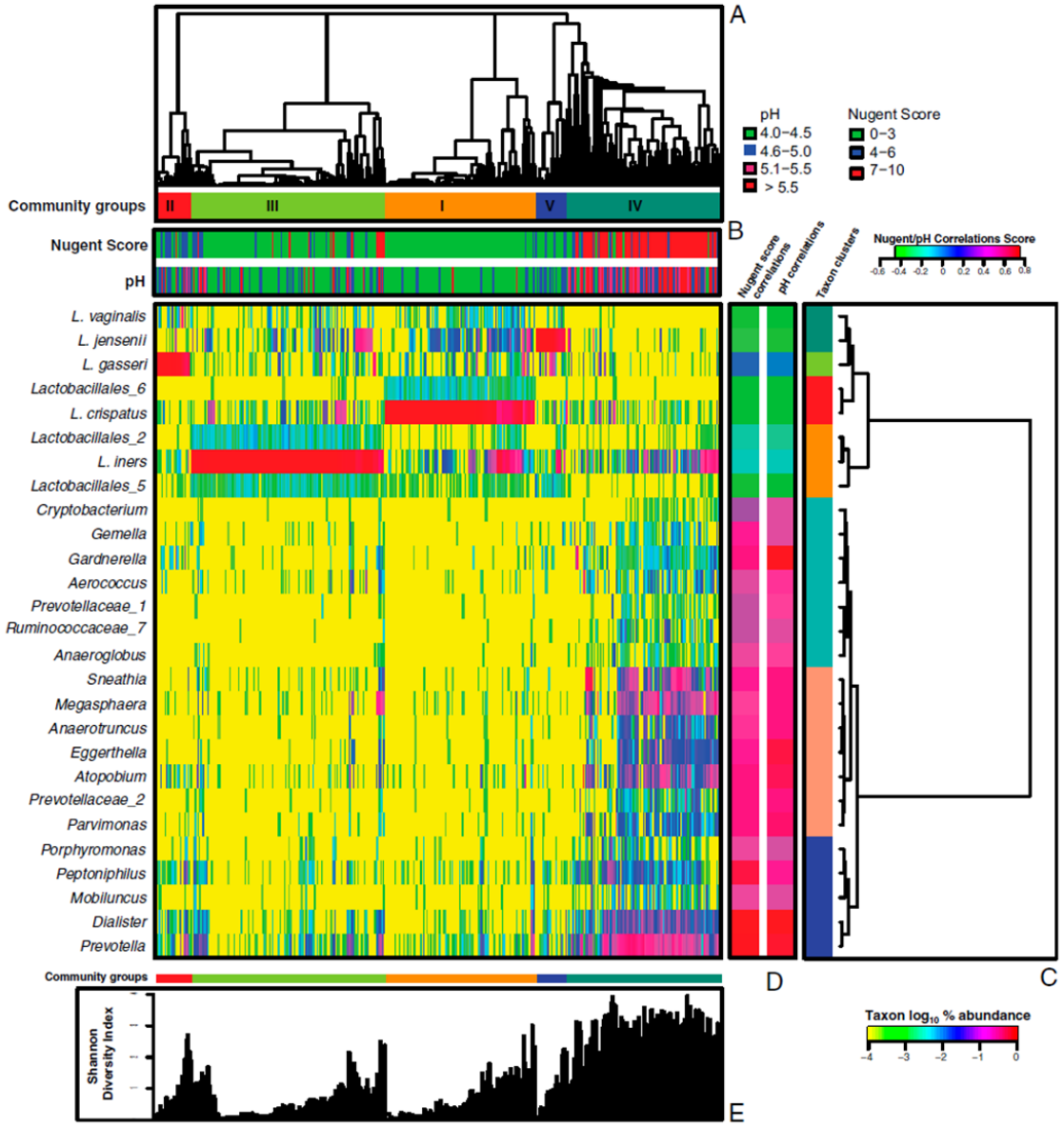


Figure 1.5. Log10-transformed proportions of microbial taxa contained in the vaginal bacterial population as a cluster dendrogram with a heat diagram. (A) Dendrogram association was dependent on vaginal bacterial species composition and abundance identifying population communities I to V. (B) Nugent scoring and pH estimation. (C) total taxa association clustering based on Spearman's coefficient. (D) Spearman's coefficients of association between the taxon's and the Nugent score or sample pH. (E) Measured Shannon index for diversity (reproduced from [162]).

1.3.2. The functional microbiome

Lactobacillus produces lactic acid and is directly correlated with the pH (Table 1.2), and in turn, the low pH provides protection against pathogens (STIs) [163-166]. As mentioned in section 1.2. a large amount of women can have PID with no STI present; this raises the question of PID being polymicrobial upper genital infection from vaginal flora or vaginal dysbiosis or possibly novel causative agents that remain to be identified. In a longitudinal study where vaginal flora was cultured from 1140 women, it was determined that a lack of hydrogen peroxide producing bacteria correlated with a significantly elevated risk of getting PID [63]. Furthermore, anaerobic BV associated bacteria were found to persist in women with PID after the recommended treatment was administered and were associated with the development of infertility [167] hence presence of anaerobes is a significant risk.

Table 1.2. Community state types, dominant bacterial species and vaginal pH associated with these microbial profiles in women

Species	pH
Lactobacillus crispatus	4.0
Lactobacillus gasseri	5.0
Lactobacillus iners	4.4
Lactobacillus jensenii	4.7
CST IV (some *Lactobacillus*, majority dominated by anaerobes such as *Gardnerella vaginalis, Prevotella spp, Atopobium vaginae, Sneathia spp)*	5.3

(adapted from [168])

1.3.3. The dynamic temporal cervicovaginal microbiome

Longitudinal studies conducted on vaginal swabs obtained twice weekly for 16 weeks or daily for ten weeks or four weeks demonstrated comprehensively that the vaginal microbiome is dynamic [169-172]. Some women were found to have a stable vaginal microbiota compositions longitudinally, while many others experienced changes, mostly towards CST IV [171]. It was found many things were associated with the timing of the shift of microbial compositions; these included but were not limited to; contraceptive use, ethnicity, menstrual cycle, sexual behaviour, pregnancy, bacterial infection, and host factors (Figure 1.6) [173]. This complex environment is not yet analysed in women with PID; in published studies documenting bacterial vaginosis in women with PID, only culture-based and directly targeted PCR techniques have been used in identification of bacterial vaginosis associated bacteria in women with PID (e.g. *Gardnerella spp, Prevotella spp* etc.) [174, 175].". In a culture-independent study of 38 women (+ 19 endometriosis controls) with PID receiving salpingectomy or salpingo-oophorectomy, and using 16S rRNA gene amplicon profiling, samples from cervical swabs and aspirates from the fallopian tube were categorised into two groups those dominated by one organism (*Acinetobacter, Escherichia, Sneathia, Streptococcus, Gardnerella or Lactobacillus*) or those polymicrobial [174]. The choice of a control group consisting of women with endometriosis might be a risk as there has been current association of PID and endometriosis, even if indirect due to physiological changes that may pre-dispose or some other factor [175]. Although relatively recent, this study did not group the microbial communities into the established community state type profiles (I-

V), however, extrapolations suggest that the organisms most frequently reported in PID cases forms part of CST IV.

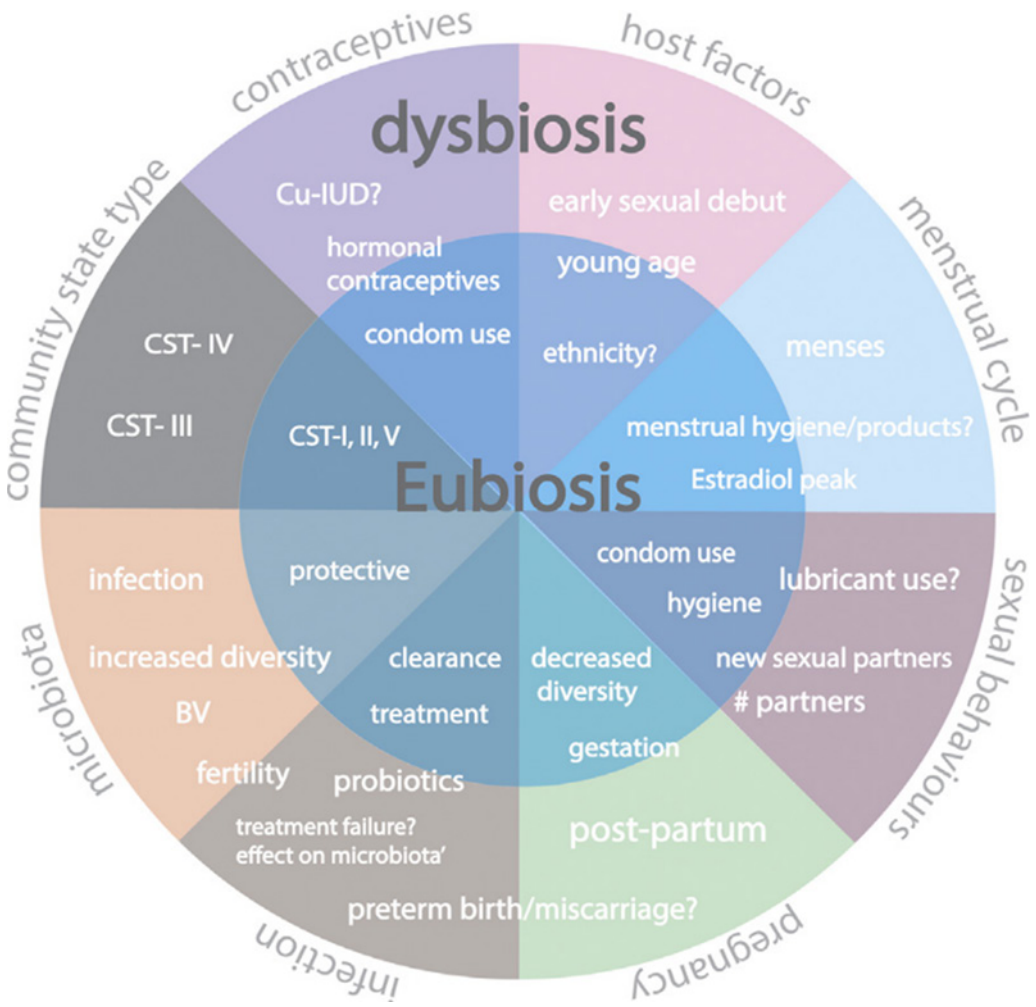


Figure 1.6. The influences on the cervicovaginal microbiome dysbiotic or eubiotic state. The inner circle represent a state of eubiosis or increased protectiveness (against pathogens), the outer circle is a state of dysbiosis and a state of less protectiveness (against pathogens) (reproduced from [173]).

1.4. HOST IMMUNE FACTORS IN PID

The immune response is undoubtedly a key component of PID and it may be that a molecular diagnosis of PID could include host immune factors. It is known that PID is an inflammatory condition and was initially characterised by the influx of neutrophils in endometrial tissue biopsies (Figure 1.7) [176]. Although known to be an inflammatory condition the PID host response has only been reported to be examined using molecular approaches in a relatively small number of papers. A number of cell biology papers focussing on PID, and the few investigations of host response are discussed in great detail Chapter 4. In brief, blood collection has been used to extract RNA and investigate numerous genes and pathways related to PID [177-179]. The only drawback is that blood is not collected during PID diagnostic protocols in Australia. As shown in section 1.1.3. the study by Zheng *et al* [177] compares three cohorts previously recruited for other studies, women with PID, women who were undergoing clinical trial for PID treatment, and women who were asymptomatic but are at high risk of contracting an STI. This resulted in a 21 gene biomarker array that was reported to be predictive of STI induced PID with sensitivity and specificity of 83.3% and 86.7% respectively [177]. Furthermore, sensitivity was only comparing those with chlamydia induced PID and those asymptomatic with a high burden of cervical *C. trachomatis.* This doesn't take into account the other microbial agents known to be associated with PID (Section 1.3.2). Authors also acknowledge that the biomarkers "don't discriminate between tuberculosis and STI induced PID" [177]. Another publication by the same authors using the same cohorts used mRNA to investigated pathways of *N. gonorrhoeae* and *C. trachomatis* induced PID [179]. Their investigations report that the STI-induced PID and chronic

endometritis blood transcriptional profile is characterized by overexpression of myeloid cell and IFN genes with suppression of T cell, protein synthesis, and mitochondrial oxidative phosphorylation genes and further add to the evidence that endometritis (inflammation of endometrium) is the first step of PID [179]. Once again, only two pathogens were concentrated on and no genes were mentioned due to Intellectual Property (IP). The other broad investigation was a cross-sectional study of adolescents presenting to the emergency room due to PID [178]. The pitfall of this is that majority of PID cases are diagnosed in outpatient settings (section 1.1.2). Similarly, as the previous studies, they too utilise blood for investigating a biosignature, cases were those diagnosed with PID and controls were adolescents undergoing surgery with non-abdominal problem. This study identified 170 deferentially expressed genes (not named due to IP) by comparing cases to control (p-value = 0.0001) [178].

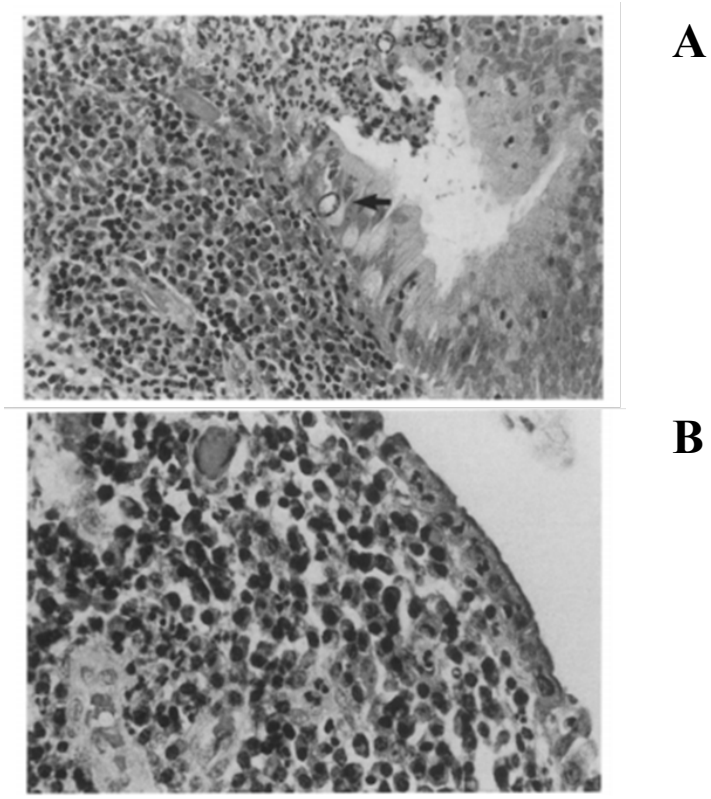


Figure 1.7. Histological sections of endometrial tissue. Panel A displays the section of endometrium at 10 × objective, panel B shows panel A(where the arrow symbol is) at 40 × objective. The neutrophils are seen clearly in the darkened nucleus amongst the tissue cells (reproduced from [176]).

Other examples of cellular biology investigations in terms of PID is the immune response to chlamydial heat shock protein (HSP60) that involves the upregulation of interleukin (IL) IL-10. PID has been correlated with high IL-10 levels in the peripheral blood, in a study of 62 women where 9 had PID and a history of *Chlamydia* [180]. Toll-like receptors (TLRs) are responsible for the detection of bacterial, viral, and fungal elements in the host. It was reported that TLR-2 was abundant in the genital tract and fallopian tubes and maybe the first trigger of the inflammatory response to PID inducing pathogens [181]. This receptor is also responsible for the activation of IL-8, triggering a

pro-inflammatory state. Cystatin C is a protease inhibitor that is associated with the regulation of inflammation and cell death [182]. Cathepsin B is another protease commonly found in acidic environments. Both these proteases show differences in women with PID where there is an increase in cathepsin B, a decrease in cystatin C and an imbalance in the equilibrium between these protease's [183]. This imbalance was proposed to causes the progression towards PID. In a study of 64 women with PID compared to 70 healthy controls, it was found that an increase in levels of plasma serum CD40L (a transmembrane glycoprotein) in PID cases (p-value = 0.0001). This plasma ligand has an essential function in enhancing inflammatory response by increasing the activity of monocytes and pro-inflammatory cytokines IL-1, IL-6, IL-8, TNF-α and monocyte chemo-attractant protein-1[184]. Recruitment of these cytokines (especially IL-1β and TNF-α) causes an increase of inflammatory response in turn resulting an increase of production of serum CD40L, then causes a positive feedback loop. Thus it is hypothesised that the concentration of CD40L in blood plasma could correlate to the severity of PID and may be helpful in diagnosis.

1.5. KNOWLEDGE GAPS

There is now some evidence that PID is associated with expression of genes related to innate and adaptive immune response, as demonstrated by the previously mentioned studies (section 1.5). However, there is currently very little known about the specifics of molecular and host immunological factors involved in PID, particularly at the local site of the reproductive tract. Immunological reactions, including immune cell infiltrates and a proinflammatory environment are present in the upper genital tract tissue including the

endometrium. However, profiling of the gene expression during PID in humans has only been conducted on the blood cells of a small group of women with PID (compared to controls) and an even smaller group using endometrial biopsies. No study of reproductive tract gene expression from cervicovaginal swabs in women experiencing PID has been published.

Generally, known to be STI associated, or respiratory pathogens, or polymicrobial, or instrumentation (e.g. IUD), but given this diversity in microbial associations, exactly why some women with these pathogens progress to PID and others do not is unknown. Furthermore, it is unknown if there are distinct pathogen virulence traits that are associate with PID compared to asymptomatic infections, as there has not yet been a detailed analysis of the genotypes of organisms isolated from PID. There have been associations of STIs and microbiota; generally, infections have been reported to be frequently associated with a microbiota consisting of CST IV organisms [170, 185, 186]. However, differing variants of pathogens commonly detected in women with PID have not been investigated in order to understand varying pathogenic factors that could lead to STI induced PID. Moreover, the long lasting effect on the cervicovaginal microbiome of a cleared STI infection is unknown.

The human microbiome has been associated with disease, especially in the field of gastrointestinal disease [187, 188]. There is a need to show how microbiota may be relevant for PID and the difference in the cervical and vaginal microbiome to aid better diagnosis and treatment. Furthermore, an analysis of microbiota and concurrent host response has not been reported in the context of PID.

1.6. PROJECT SCOPE

This project aims to conduct a prospective study of women with PID, by comparing reproductive tract specimens from women with PID to healthy controls (Case-Control study design) and also a test group of women with asymptomatic or mild sexually transmitted infections. Swabs will be collected from the cervix and vagina of women, with human research ethics committee approval and full informed consent from participants. Using the participant biological samples, pathogens, microbiota, and host immune factors will be examined to test for an association with PID compared to the controls. Ultimately, this study may identify biomarkers in either the pathogens, microbiota or immune response that could be developed into diagnostic tools for this important condition. Ultimately, the ability to generate a molecular diagnostic tool for the specific pathogenic/microbial agents responsible for an individual case of PID could support tailored antimicrobial treatment. This, in turn, would lead to the improved use of antibiotics in line with the global goal of improved antimicrobial stewardship.

This project is a pilot case-control study that aimed to prospectively recruit 60 women at Family Planning NSW Clinics: 30 women with a clinical diagnosis of PID (Cases) and 30 Controls undergoing routine cervical screening and were otherwise healthy. Additionally, a set of sexual health participants who are suspected of having mild a/symptomatic chlamydial or gonococcal infections (section 1.7.1) will be included as a test group. It is important to note that there is validity to having both symptomatic and

asymptomatic controls in the analysis so these participants will add valuable comparison group to the study (so long as the symptoms are not consistent with PID).

Data will be analysed to identify factors (microbiota, immune gene expression, or pathogen features or potentially patterns or profiles of multiple factors) that are significantly associated with PID. The first analysis will be to compare the microbiome, immunological and pathogen-specific features between the PID cases and controls using non-parametric t-tests to identify factors that are significantly associated with PID. While this pilot study may not be adequately powered to detect a profile or combination of factors, a multivariate analysis will be conducted to test for any combined factors that are significantly associated.

An initial study to build the tool of a murine model to investigate STI pathology differences in the human isolates and possibly investigating the sub-species or strains of other organisms that are found in PID. This will, in turn, allow for the investigation of the roles played by differing organisms and their pathogenic features.

1.7. HYPOTHESIS AND AIMS

There are four hypotheses to be tested in this project:

1. Women who develop PID possess a distinct microbiome (i.e. the cervical and vaginal microbiota) that is lacking in *Lactobacillus species*. (Chapter 2 and 3)

2. Women who develop PID possess a unique immune gene expression profile consisting of an inflammatory response. (Chapter 4)

3. PID is a multifactorial process that occurs from the interplay of the vaginal microbiota, pathogens, and the host response. (chapter 2-4 and 6)

4. Certain *Mycoplasma genitalium*, chlamydial or gonococcal strains have pathogenic traits more likely to be associated with development of PID that could be detected using an animal model of PID. (Chapter 5)

This is encompassed with five aims, all set to establish and validate protocols in a pilot study format to determine if a larger scale PID study following these principles is feasible and potentially valid.

Aim 1: Conduct a prospective pilot Case-Control and test group study recruiting women of reproductive age with similar demographic factors in each group (Chapter 2).

Aim 2: To determine whether the composition of microbial communities in vaginal and cervical microbiota is significantly between Case-Control participants, and identify any new microbiota, or microbiota profiles, associated with PID (Chapter 3) .

Aim 3: To determine whether cervical expression of host immune genes are significantly different in women experiencing PID (Chapter 4).

Aim 4: To investigate differing microbiota and concurrent analysis of the host response and demographic factors in terms of women experiencing PID compared to asymptomatic controls (Chapter 2-4 and 6).

Aim 5: To see whether variants of a pathogenic organisms have different pathogenic features to wild type that can be detected *in vivo* and *in vitro* that may help to establish a tool for animal model investigations of pathogenic variants associated with PID (Chapter 5).

Chapter 2 - Case-Control study to investigate factors associated with pelvic inflammatory disease in NSW

2.1. INTRODUCTION

2.1.1. Epidemiological associations of women with pelvic inflammatory disease

Pelvic inflammatory disease (PID) is defined as inflammation occurring in the female genital tract, uterus, fallopian tubes, and/or ovaries. Factors that are associated with increased risk for an incidence of PID include sexual and behavioural factors. These include an increased number of sexual partners, being of reproductive age, acquisition of an STI, smoking, and psychoactive drug use that leads to behavioural modification and increased risky behaviour such as unprotected sex [189-191]. Another factor is the act of douching, as reported in a case-control study of 425 women, those women that douched at least once a week had an odds ratio of 3.9 for the incidence of PID when compared to controls [192]. Epidemiological factors that are associated with PID are discussed in greater detail in Chapter 1.

2.1.2. Study design for the accurate diagnosis of cases for case-control studies

The recruitment methodology is a crucial part of the case-control study design, to ensure that cases and controls are correctly assigned and any factors identified in the biological specimens analysed from the participants are valid to compare. As outlined in Chapter 1, the diagnosis of PID is a challenge and this has been carefully considered in the study design. The ‘gold standard’ diagnosis of clinically suspected PID through laparoscopy and/or histological assessment [9], is not routinely available in Australian

settings, so recruitment relies on the clinical protocol that is currently used. There is a risk of recruiting women for the case group who do not really have PID. Furthermore, asymptomatic PID cases could be recruited in the controls. This is a similar problem that was faced in the PID Evaluation and Clinical Health (PEACH) study, which is a previous large study that tested the effectiveness of outpatient and inpatient treatment for PID [193]. This issue of misdiagnosis resulted in the reclassification of participants and is an unavoidable part of the current diagnostic criteria (Chapter 1). However, in awareness of this issue, frequent training of clinicians by the clinical leads was implemented in the study protocol, emphasising the key criteria of cervical, adnexal, and uterine tenderness was conducted throughout the study, and only clinicians receiving this regular training were eligible to recruit for this study.

2.1.3. Considerations for questionnaire design to increase accuracy and participation

Research has shown that responses to questionnaires vary by the way the question is displayed or phrased. Factors such as paper colour, mode of delivery, and content of the questionnaire all affect the participants understanding and willingness to be truthful. For example, in a study of college students, it was found that oral sex was not viewed as a sexual interaction to report [194]. In another study, the term “sexual partners” caused confusion amongst married participants where they thought the term was too casual, referring to their married partner, while in contrast, single participants felt the term was only referring to steady relationships [195]. However a questionnaire is worded, it will always be biased to particular populations and this is well understood amongst clinical

recruitment studies. While there is no validated survey tool specific for PID, a content-valid audio computer-assisted self-interview was developed and used in an emergency department (Seattle, USA) for STI risk assessment, 76.5% preferred this method than face to face questionnaires [196]. In another study in the United Kingdom, a computer based tool was used in sexual health clinics, many participants preferred the web based survey tool rather than verbal questions during the clinical consultation (82%) [197]. Implementation of electronic tools may increase response validity, possibly due to the privacy and sense of 'less judgment'.

In a second major PID study called the Prevention of Pelvic Infection (POPI), similar problems were identified in recruitment and wording of questions used to collect data. This was a community-based study with the aim of increasing non-invasive tests for chlamydial screening to reduce the incidence of PID. The POPI study investigators found that recruitment was more successful than their previous trial if the following strategies were used: recruiting participants in groups, targeting a specific population, avoiding the term "vaginal swabs" but instead using the words "small cotton tip" and finally also participant incentives increased participation [198]. It is important to note that in Australia incentives are not considered ethically appropriate in clinical and research studies, although reimbursement for time is considered appropriate. The data from these previous studies support that it is essential for investigators and clinicians to continually evaluate previous evidence and approaches to optimise the recruitment protocol.

2.1.4. Demographics of people attending sexual health clinics

The risk of recruiting subclinical PID in the control group could also be high, and this is defined as women with an absence of traditional signs and symptoms of PID, but actually have PID [199]. However, the control group recruited here are women presenting for 'well women's checks' which is typically cervical screening and these women may be quite different from PID Cases and have little sexual activity or other risk factors, which may not mean they are a risk for sub-clinical PID but may not represent the best control for PID. These issues will be evaluated by examining the demographic data in the questionnaire. However, due to these considerations, we conducted the recruitment of a group that is of high risk for PID, termed the test group, that may include some participants with subclinical PID. The demographics of attendees in sexual health clinics are generally considered high risk or priority persons. The Australian government (2018) recognises priority populations as; people in custodial settings, travellers and mobile workers, culturally and linguistically diverse people, young people, Aboriginal and Torres Strait Islander people, gay men and other men who have sex with men, and, sex workers [200]. In a recent study of priority populations, especially young people, it was found that priority populations were less likely to seek sexual health care from general practice compared with the general population [201]. In terms of Family Planning NSW, this is a not for profit organisation, that caters to all populations and are advocates for the provision of specialised services and programs to report the reproductive and sexual health and rights of all. Family Planning NSW, attracts the majority of female clients due to their specialties they offer, but do also consult on men's sexual health.

Thus, the aim of this chapter is to design and successfully implement a case-control study, recruiting a pilot sample of 60 female participants, in an Australian Family Planning setting and additionally recruit a group of women in a sexual health clinic setting (a test group). The key to successful recruiting, in this aim, is where the demographic, self-reported questionnaire and post-analysed medical record databases for any of the participants do not include large differences in risk factors for PID that may confound or influence the findings of the study.

2.2. STUDY DESIGN AND METHODOLOGY

2.2.1. Recruitment protocol and criteria

This pilot case-control study had a target sample size of 60. The sample size was arbitrarily selected to allow the pilot study to establish the protocols and also test the biological markers for findings that could inform the design and size of a future larger study based on the outcomes of this pilot study. Cases were being defined as women with a clinical diagnosis of PID; controls have been defined as women presenting for routine cervical testing or intrauterine device (IUD) insertion. The aim was to recruit 60 women at Family Planning NSW clinics (FPNSW): 30 cases and 30 controls. Women aged 18 to 29 years, English speaking and willing to give informed consent were the main criteria for inclusion for the study. The exclusion criteria included pregnancy, which was confirmed via a human chorionic gonadotropin (Hcg) test performed prior to registration. Participants in the case study were women who were confirmed and evaluated with

presumed PID (with other reasons excluded, e.g. ectopic pregnancy, polycystic ovaries, irritable bowel disease etc.) during their appointment, either from recent IUD insertion or other possible triggers (e.g., STI). PID was defined by current national guidelines (Chapter 1) including the following criteria: new onset of pelvic pain, confirmed lack of pregnancy, bimanual examination to elicit cervical motion tenderness and adnexal and uterine tenderness. Asymptomatic women of the same age group presenting for routine cervical screening were offered study participation in the control group. The exclusion criterion included any recent previous diagnoses of PID that had not been resolved.

Primary investigators (researchers and clinical team) held chart review meetings every four months for medical record review and to access to the pathology diagnostic results (STI tests) for all participants who consented and were recruited into the study. Clinical principal investigators trained all recruiting clinicians on a regular basis, providing a refresher on the study criteria (every four months). Only these trained clinicians were invited to recruit for the study. A medical chart review was conducted to identify any enrolled participants where the investigation in the follow up appointments did not confirm PID, and to confirm the definitive criteria of tenderness during the bimanual examination was noted in the initial appointment (defining characteristic of PID for the purpose of this study). Based on the chart information and test results the investigators also classified the cause of PID. Discussion and confirmation of participant grouping category based on the evidence in the medical record and outcomes of follow up meetings (i.e. case or control) was conducted in these audit sessions. These recruited cases identified with PID were categorised into the cause of PID (i.e. idiopathic, instrumental (IUD), or sexually transmitted infection associated). Women who were

classified as idiopathic had no STI detected, those that were classified as STI associated had an STI detected and those who were classified as instrumental associated were women who had a recent insertion of instrumentation (IUD). All participants recruited were assigned a de-identified code matched to them to ensure anonymity, with only clinicians able to determine the identity of codes.

2.2.2. Participant requirement protocol for Family Planning NSW study sites

The case and control groups had separate recruitment strategies (Figure 2.1), separate participant information sheets, and informed consent signature forms. Both groups had the same biological samples collected and completed the same questionnaire (see Appendix A for an example of the self-completed questionnaire). All study samples were collected immediately after any diagnostic samples on the day of recruitment. These samples were swabs from the posterior vaginal fornix and the endocervix collected into various buffers (listed in Table 2.1) and transported to pathology and stored at 4 ºC, where they were collected within an ice-cooled esky and stored at -80ºC (within 4-7 days of collection). The information sheet included consent to examine medical records as relevant to the study, with no follow up directly to participants from the research team for the purposes of this study. Any clinical issues requiring further intervention or discussion with the participant that emerged during medical record review that were identified in the chart review of the PID investigation were followed up by Family Planning NSW clinical staff.

A study kit (see appendix A for example) was prepared which included a participant information sheet and informed consent form (2 copies, one for the participant and one for the medical record), study withdrawal form, questionnaire, breakpoint swabs (Copan Diagnostics, Cat No, 520CS01, Murrieta, USA), pre-labelled study tubes (Table 2.1), and specimen storage bag to place all items. Each item and page in the study kit had a matching unique study code to ensure de-identification of all materials before they are provided to the study team. This de-identification code and signed consent form with participant name were stored a Family Planning NSW with the secure medical record storage system already established.

Table 2.1. Study tubes with storage buffer used and their purpose

Cryotube	Storage Buffer	Purpose	Amount
1- Cervical swab	RNAProtect[a]	16S rRNA gene amplicon sequencing and qPCR	1mL
2- Cervical swab	RNAProtect[a]	Host response immune profiling	
3- Cervical swab	SPG[b]	Bacterial storage buffer for Chlamydia	
4- Vaginal swab	RNAProtect[a]	16S rRNA gene amplicon sequencing	

a: Qiagen Cat No, 76526, Germany

b: SPG is 10 mM sodium phosphate, 250 mM sucrose and 5 mM L-glutamate

The medical practitioner performed a speculum examination and bimanual pelvic examination to confirm the presumptive diagnosis of PID and collected cervical swabs for suspected PID as per the national guidelines (*Chlamydia trachomatis*, *Neisseria gonorrhoea,* and *Mycoplasma genitalium* PCR swabs). After the routine diagnostic swab was collected, an additional three cervical swabs and one high vaginal swab was collected from the consented participant for the purposes of this study.

The participant completed the study questionnaire and placed it in the provided envelope at the clinical site. The signed informed consent form was kept on clinical records (one copy) and swabs were sent to the Family Planning NSW pathology provider (in a specific study pack) along with swabs taken as part of routine clinical care. The pathology provider (Douglas Hanly Moir) stored all specimens until UTS staff collected the specimens from the pathology lab every second day. This was repeated for control group participants with the exception that these participants did not undergo bimanual examination for PID confirmation.

It was noted during the routine cervical screening, or IUD insertion assessment consultation if there was the observation of symptoms that may be consistent with STI, bacterial vaginosis, thrush, or candida infection that was not determined during history-taking (in some cases diagnostic swabs were collected and analysed). The research team added these results and symptoms to the dataset when the participant's medical records were reviewed for the study.

If control participants were found to be suspected of PID during the chart review or due to follow-up clinical appointments after the samples were collected, the participant was reclassified as a PID case and moved into that group for the study, and vice versa for cases. This re-classification did not require further contact with the participant.

2.2.3. Questionnaire data

In order to explore potential risk factors for PID, participants were asked to complete a questionnaire about their gynaecological, obstetric, sexual activity, and sexual health history. This demographic and behavioural data provided a comparison to expected demographic and behavioural factors associated with PID in the existing literature; helping to validate the study design. Additionally, this data provided a comparison of the risk factors between the groups recruited. There currently is no standardised questionnaire available, but this was reviewed by all investigators and experienced colleagues.

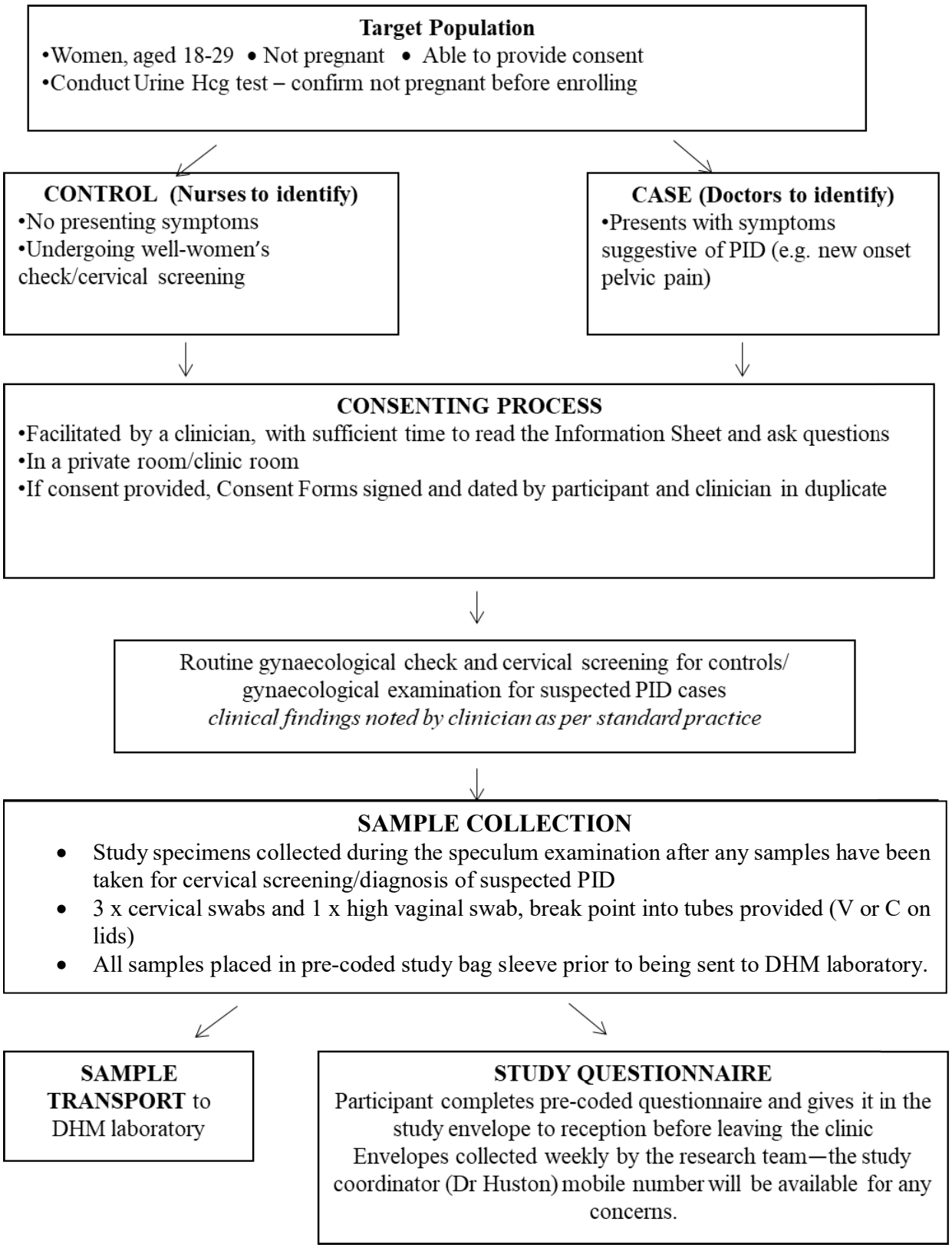


Figure 2.2. Flow chart outlining the recruitment protocol provided to clinical team members.

Doughlas Hanly Moir (DHM) is the pathology provider for family planning NSW.

2.2.4. Cairns Sexual Health Centre's recruitment of a comparison sexual health group

Women attending sexual health clinics generally have the same demographic and behavioural factors that correlate with increased risk of pelvic inflammatory disease. It is important to note that in this demographic sub-clinical PID is underreported as shown in a Western Australian population study [202]. Due to the uncertainty of the suitability of the controls group, but the risk of sub-clinical PID in a sexual health control group given the known underreporting of PID in these groups, this extra group was termed the test group for this study. The test group was prospectively recruited from women attending a sexual health clinic for routine sexual health investigations. These were women who would already be having a speculum examination for suspected *Chlamydia* or other STIs or other reasons. The women had a variety of specimens collected (Table 2.2) and analysed using the following samples and assays; blood, cervical swab into RNAProtect for human immune gene expression in the cervix and microbial community assays (Chapter 4), human serum to measure chlamydial or gonococcal antibody titres, vaginal swab into RNAProtect to analyse the microbial associations with PID (Chapter 3). This group were recruited under slightly distinct conditions and with a separate protocol, so some minor differences in samples and questionnaire should be noted during the interpretation of the results.

Table 2.2. Test group study tubes with storage buffer used and their purpose

Tube	Buffer	Purpose	Amount
Cervical	SPG[b]	Bacterial culture	500µL
Cervical	TE[c]	DNA extraction	500µL
Vaginal	RNAProtect[a]	DNA extraction	500µL
Vaginal	TE[c]	RNA extraction	500µL
Whole Blood	Pathology provider tubes	DNA, RNA and whole use	10mL
Sera			5mL

a: Qiagen Cat No, 76526, Germany

b: SPG is 10 mM sodium phosphate, 250 mM sucrose and 5 mM L-glutamate, chlamydia storage buffer

c: Invitrogen Cat No, 12090015, Australia

This site aimed to recruit 60 participants as a test group for the PID study. The participants provided all samples required for the study at the time of consent and recruitment. Participants were fully informed by clinical staff about the study and provided written consent before participating in the project. Participants were required to complete a questionnaire on sexual/gynaecological history, and samples were couriered to UTS. For the questionnaire, see appendix A. Similarly, there currently is no standardised questionnaire available, but this was reviewed by all investigators and experienced colleagues.

2.2.5. Statistical analysis

Participant demographic and behavioural data was graphed and analysed in GraphPad Prism version 8.00 [203]. This was used to calculate means, standard error of the mean (SEM) and p-values using independent non-parametric Mann-Whitney test [204] where data was assumed not to be distributed normally. Significance is when p-value <0.05 and is denoted by an '*'.

2.2.6. Ethics and biosafety

The study design was reviewed and approved by the Human Research Ethics Committees (HREC) at Family Planning NSW, Cairns (Far North QLD), and the University of Technology, Sydney (UTS). The following are the HREC approval numbers; UTS: 2015000621; FPNSW: R2016-07, and Far North QLD HREC: 2015000621. Biosafety approval was obtained from the UTS Institutional Biosafety Committee for the laboratory work on the human biospecimens and pathogens (UTS Institutional Biosafety Committee Approval number 2015-16-R-PC).

2.3. RESULTS

2.3.1. Participant demographic dataset are consistent between the groups

The study has successfully recruited 15 Cases, 31 Controls, and 13 Test group participants. Questionnaire data was received from a total of 59 with 50 fully complete (3 participants failed to provide the questionnaire) and six incomplete questionnaires (6 didn't fill out the back page). Biospecimens were successfully collected and received from 59 participants (resulting in 249 bio-specimens). The demographic, self-reported questionnaire, and medical record review datasets were compiled for each participant and subsequently analysed using the category of the groups to evaluate any differences in risk factors for PID that could confound or influence the interpretation of the study results.

There were no significant differences in mean age or age range between Control, Cases, and the Test group. To check for skew in the mean ages, the median was analysed and deemed to be 22, 24, and 23 for the case, control and test groups respectively. The median values indicated that there was no skew in the age distribution of women recruited for the three groups. Participants were asked what contraceptive methods they currently use and women in the Case group were more likely to use a hormonal IUD (p-value = 0.02) while those in the Control group were more likely to use a condom (p-value = 0.03). There was no other significant difference in the reported types of contraceptive used by women in the study.

Women were asked if they have had experienced bacterial vaginosis and/or thrush in the past three months with cases more likely to self-report a history of these than controls (p-value = 0.03). Furthermore, the cases more frequently self-reported bacterial vaginosis and/or thrush when compared to test group (p-value = 0.027). Interestingly, a higher proportion of women with PID self-reported using antibiotics three months before onset than those women in the control group (p-value = 0.02). Use of antibiotics in the past three months was also more frequent in women with PID when compared to the test group (p-value <0.01) (refer to Table 2.3).

Participants from Family Planning NSW were questioned about the number of sexual partners in the last three months and in the last 12 months. For the case and control categories, the numbers of reported sexual partners increased with time from the previous question (past three months). For example, in the past 12 months, 40% recorded 2-4 sexual partners, while in the past three months, 20% recorded 2-4 sexual partners (Table 2.3). Women from the test group were asked about the number of sexual partners in the past 12 months and their lifetime. 46% of the test group reported 2-4 sexual partners in the past 12 months, and 61.5% of women had >5 sexual partners in their lifetime. Cases with a diagnosis of an STI had more sexual partners in the past three months than compared to those who were STI negative (p-value = 0.09).

Table 2.3. Demographic factors of participants who have PID, control and test groups

	CASE	CONTROL	TEST	p-value	
Participants	n = 15	n = 31	n = 13	Control group compared to case group	Test group compared to case group
Age	**Mean Age (Range)**				
	22.9 (16–37)	24.1 (19 – 29)	23.7 (18–38)	0.133[a]	0.738[a]
Contraceptive	**n (%)**				
None	1 (6.7)	2 (6.5)	3 (23.1)	-	-
Implanon	3 (20)	1 (3.2)	2 (15.4)	0.06	-
Contraceptive Pill	3 (20)	8 (25.8)	4 (30.8)	0.67	-
Hormonal IUD	7 (46.7)	5 (16.1)	0	0.02	-
Copper IUD	0	0	0	-	-
Condom	0	10 (32.3)	1 (7.7)	0.03*	-
Vaginal Ring	0	1 (3.2)	0	-	-
Depot provera	1 (6.7)	0	0	-	-
Other	0	0	3 (23.1)	-	-
Withdrawal	0	2 (6.5)	ND	-	-
Mini-pill	0	2 (6.5)	ND	-	-
Total responses[h]	15/15	31/31	13/13	-	-
Vaginal Thrush/BV[c]	10 (66.7)	10 (32.3)	0	0.03*[g]	0.027*[g]
Total answers[h]	15/15	31/31	13/13		
Usage of antibiotics[d]	6 (40)	3 (9.7)	0	0.02*[g]	<0.01*[g]
Total responses[h]	15/15	31/31	13/13		
Previous STI's	**n (%)**				
Chlamydia	4 (26.7)	6 (19.4)	7 (53.8)	0.059[g]	0.157[g]
Gonorrhoea	1 (6.7)	0	4 (30.8)	0.164[g]	0.11[g]
Total responses[h]	15/15	30/31	9/13		
Fertility	**n (%)**				
Past pregnancies	5 (33.3)	9 (29)	4 (30.8)	0.07[g]	0.359[g]
Total responses[h]	15/15	31/31	11/13		

	CASE	CONTROL	TEST	p-value	
Sexual Partners[f]	**n (%) past 3 month's**				
0	1 (6.7)	2 (6.5)	[j]	-	-
1	9 (60)	19 (61.3)	[j]	-	-
2 – 4	3 (20)	3 (9.7)	[j]	-	-
> 5	2 (13.3)	1 (3.2)	[j]	-	-
Total responses[h]	15/15	25/31	[j]	-	-
	past 12 months				
0	1 (6.7)	0	[j]	0.19	-
1	7 (46.7)	17 (54.8)	[j]	0.25	-
2 – 4	6 (40)	5 (16.1)	[j]	0.16	-
5 – 7	1 (6.7)	3 (9.7)	[j]	0.62	-
> 7	0	4 (12.9)	[j]	0.66	-
Total responses[h]	15/15	26/31	[j]	-	-
Sexual Partners[k]	**Lifetime**				
1	[j]	[j]	-	-	-
2-5	[j]	[j]	1 (7.7)	-	-
>5	[j]	[j]	8 (61.5)	-	-
Total responses[h]	[j]	[j]	9/13	-	-
	past 12 months				
1	[j]	[j]	0	-	-
2-5	[j]	[j]	6 (46.2)	-	-
>5	[j]	[j]	3 (23.1)	-	-
Total responses[h]	[j]	[j]	9/13	-	-

a: independent samples Mann-Whitney U test with significance level at 0.5

b: participants were asked what current contraceptive they are using

c: participants were asked if they had been diagnosed with vaginal thrush/bacterial vaginosis in the past three months

d: participants were asked if they have used antibiotics for any reason within the last 3 months

e: rate of live birth is calculated by live births / number of pregnancies × 1000 Participants were asked out of the number of pregnancies they have had

f: participants were asked to fill in the number of sexual partners they have had in the past 3 and months, 20.1% of control participants left the question blank in case vs control

g: independent sample nonparametric binomial test for Yes/No answers where '*' indicated significance.

h: total responses is indicative of the total responses/total number recruited

i: the test group was not asked this question

j: questions were differently worded test group compared to case-control groups also denoted as 'ND' in table

k: sexual partner history of participants in the test group. Two questions were asked the history of partners in lifetime and history in the past 12 months

'-': indicates the inability to compute statistical analysis due to 'n'

2.3.2. Categorisation of PID aetiology

Medical record review, and review of diagnostic test results was conducted to confirm the allocation of participants into cases and to categorise the aetiology (Chapter 1, describes the differing aetiologies in detail). The three-group characteristics of PID are based on the pathogen associated (STI's and respiratory), recent instrumentation (e.g. IUD), or no recognised pathogen (idiopathic) [205-207].

A total of 14 women were recruited with PID and remained in the study as PID Cases after medical record review. Following a medical record review of 15 people with PID, one person was excluded from the case group as it had become clear from failure to respond to treatment and further examination that this patient had a different medical condition (therefore, PID cases = 14). No further analysis was conducted and they were excluded from the study as they could not be reliably considered a control either. Eight of these women had PID of an idiopathic nature, three had PID due to sexually transmitted infections (one from *Chlamydia,* one from *gonorrhoea* and one from *Mycoplasma*) and three had PID suspected related to the recent instrumentation (insertion of an intrauterine device) (Figure 2.1). Due to low numbers, no statistical significance was seen amongst aetiology of PID cases and demographic factors.

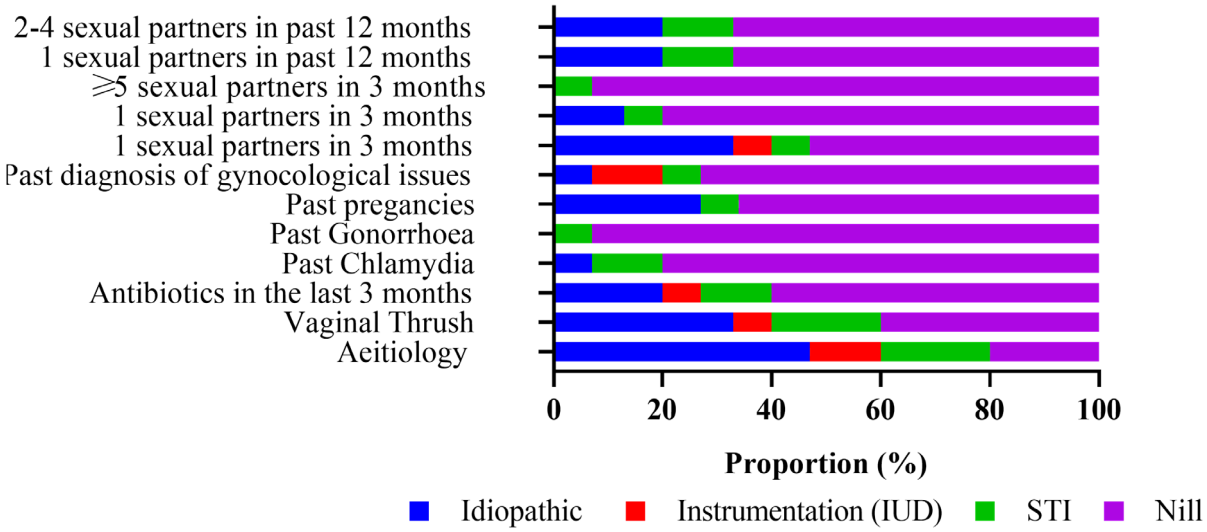


Figure 2.1. Aetiologies of PID cases and demographic factors. The stacked column graph displays the aetiologies of PID cases after medical chart review. There were 8 Idiopathic associated PID, 2 instrumentation (IUD) associated PID, 3 STI associated PID, one positive for each of the following; *Chlamydia trachomatis, Neisseria gonorrhoeae,* and *Mycoplasma genitalium.*

2.4. DISCUSSION

Overall, this study has achieved successful recruitment of women with PID that 14 out of 15 were confirmed to be PID cases and with a mix of aetiologies. Successful recruiting of participants and test groups have supported the procedure for a pilot project of this type. As discussed here, there are some differences in some demographic factors that would be expected to be associated with PID and some findings of interest. The

sample size target for this pilot study was not achieved, 45/60 participants recruited, this resulted into insight for recruitment in a future more extensive study.

2.4.1. Demographic differences between groups are not apparent, supporting no unintended differences in recruitment

Demographic and behavioural characteristics were compared between the groups to see if the PID characteristics are consistent with the previous literature which would validate that this pilot recruitment protocol is appropriate. Participant age was not significantly different between the groups, indicating there were no differences caused by the slightly different recruitment criteria used in the study for the groups. After an analysis of participant behaviour and demographic data that was collected using the self-reported questionnaire, several factors were similar.

However, there was a significant difference in the proportions of women using two of the contraceptive methods. These included women with PID were significantly more likely to use a hormonal IUD (p-value = 0.02), compared to the controls. Furthermore, women in the control group were significantly more likely to report usage of condoms as contraception (p-value = 0.03), compared to cases. This result is consistent with a prospective cohort study of 9000 women, those that had an IUD had a significantly higher chance (46%) of developing PID when compared to non-IUD (9%) (p-value = 0.005) [208]. Instrumentation such as IUD insertion, both in the presence or absence of STI, was also identified to correlate with the development of PID in a baboon animal model [209]. Additionally, a number of studies have shown that the chance of PID is higher after the

recent insertion of a hormonal IUD, specifically in one study within the first 20 days after insertion, PID incidence rate was 9.6% compared to women that had instrumentation insertion but had not developed PID [210-212]. However, these studies were only conducted in women with PID, rather than any women that uses an IUD. Overall, the factors that were different between the groups recruited in this study were those that would be expected to differ based on the known risk factors for PID.

Women with PID were more likely to self-report a history of bacterial vaginosis and/or thrush when compared to controls and those undergoing sexual health testing (Test group) (p-value = 0.03, p-value = 0.027; respectively). This is also consistent with the previous literature, as in a study of 545 women with suspect PID, irrelevant of the cause, bacteria associated with bacterial vaginosis were associated with recurrent PID, endometritis, and infertility with an adjusted risk ratio of 4.7 when compared to controls [167]. While there hasn't been a direct association of thrush (infection with the *Candida species,* most commonly *C. albicans*) with PID, it has been shown to form biofilms on IUD's and recent insertion of an IUD has been associated with PID [213]. The questionnaire tested in this pilot study framed this question by group thrush and BV together. However, it did not define the terms so that it may have been misinterpreted. Future studies should consider evaluating the wording of this question

It is well-known that antibiotics disrupt the normal flora in the human gut [214], with this in mind, I wondered if the use of antibiotics disrupts the vaginal flora, and if this could represent a risk for the development of PID? In this study, women with PID were more likely to have self-reported using antibiotics in the past three months when

compared to the control groups (p-value= 0.02, p-value <0.01; respectively). This was also found to be true regardless of STI status in a retrospective cohort study within the United Kingdom, where previous antibiotic prescription (regardless of STI status) increased the risk of PID [215]. Furthermore, in an *in vitro* study on two common antibiotics used to treat some STI's (doxycycline and azithromycin) and their effect on selected bacteria that are dominant in each community state type (CST); it was found that CST III (*L. iners*) and CST IV (*Gardnerella vaginalis*) were more resistant to these [216]. These CST's are found to be less protective against STI's discussed in more detail in Chapter 3. It is thus plausible that recent use of antibiotics could lead to a depletion of protective microbial flora and increase the risk of PID. Although given that this is a pilot study with a small sample size, designed to establish the protocols and parameters for a larger clinical study, this finding should be interpreted with caution.

Overall, there was no significant difference in the number of recent past sexual partners, a slight trend to higher numbers in cases and test group, albeit the sample size is low. It is also a well-known risk factor that a new sexual partner in the past 12 months increases the risk of PID (as discussed in Chapter 1). However, sexual activity and the number of sexual partners remains a mostly private subject, and the ability to recall or report this information has been shown to have a significant error rate [217]. Thus, it is essential not to entirely to rely on the self-reported sexual history of participants, but still use it as an indication for risk factors.

Missing questionnaire responses was a significant factor in terms of self-reported questionnaires. In this study, 15 Case participants were recruited, but only 14 completed

the questionnaire and one had partially completed the questionnaire. In the control group, two questionnaires were blank but returned and six were incomplete. In the test group, only one was incomplete. The response rate was 85%, which is higher than reported in many sexual health studies such as POPI were 40% of participants did not respond to the questionnaire for the study [218]. A recommendation for future studies based on this pilot study it might be useful to consider online or phone application based questionnaires.

2.4.2. Sexually transmitted infections and the increased risk of subclinical PID

In this study, a test group was recruited, consisting of women undergoing an STI check with or without symptoms. These women were more likely to have had an infection with *C. trachomatis* and/or *N. gonorrhoeae* (Table 2.1). This is crucial as studies have shown that within the time point of testing positive for *C. trachomatis* to coming back for treatment, the rate of acquiring PID is anywhere between 2% - 45%, where the most robust evidence suggests 10% [219-221]. Furthermore, the possibility of subclinical PID, where there was an uncomplicated infection by STI or cleared infection is high [222]. Currently, there is no knowledge of how to diagnose sub-clinical PID, and there is an opportunity to determine if some of the test group have a bio-marker profile consistent with PID cases in this study. Therefore, this group was included in the study. In the event of this possible analysis, the population and behavioural data of the test group would ideally be consistent with the cases. While *C. trachomatis* is the most commonly isolated pathogen from PID cases, *N. gonorrhoea* accounts for the most hospitalised cases [223, 224]. In a population study of Western Australia, it was identified that women that have at least one STI check are more likely to get a repeat STI, but that the more repeat

infections of *C. trachomatis* and *N. gonorrhoea,* the higher the adjusted incidence ratio for PID. The incidence ratio of two or more repeat infections with *N. gonorrhoea* is 2.8 times higher than that of *C. trachomatis* [202]. Hence, it is vital to include this cohort in this study as the majority of the time, idiopathic PID is diagnosed [55]. Furthermore, another recommendation for a future more extensive study based on this pilot study would be the recruiting all groups from a single state in order to ensure swift transport of samples.

2.4.3. Differing aetiologies associated with PID

It is well understood that PID is represented by different aetiologies; these include idiopathic, instrumentation (IUD) related PID (Chapter 1) and pathogen associated (STI's and respiratory pathogens *s*uch as. *Mycobacterium tuberculosis, Haemophilus influenza, Streptococcus pneumonia, Staphylococcus aureus)* [55, 225-227]. A medical chart review was conducted to identify potential false positives, ensure follow up appointments confirmed PID, and classify the cause of PID. In this study, 57% of the cases were classified as idiopathic (no known pathogen detected); in the PEACH study, 33% of cases had idiopathic PID [228]. However, the proportion of idiopathic PID varies depending on the setting; for example, a study of out-patient diagnosed PID cases in the United Kingdom found 70% idiopathic [228]. Here, eight out of the total 14 cases recruited were classified as idiopathic PID (Table 2.4). The complicated criteria and protocol for the clinical diagnosis of PID means that the true burden of PID is underestimated [229, 230]. Furthermore, subclinical PID cases could be missed as these are cases with the same microbiological and pathophysiological characteristics as acute symptomatic PID [231, 232]. On the day of diagnosis, the STI's were tested for in the

cases and 7 % had *C. trachomatis,* or *N. gonorrhoea,* or *M. genitalium.* This result differs to the PEACH study were positive diagnosis for *C. trachomatis* and/or *N. gonorrhoea* ranges from 13.5% to 14.8% but does not report *M. genitalium [43]*. Whereas, in the POPI cohort (England), *C. trachomatis* was detected in 5.4% of women with PID and 0.3% for *N. gonorrhoea* [218]. Hence, the association of PID with STIs differs amongst populations, differing recruitment settings (i.e. general practice, in-patient or outpatient (emergency department)). A vital point is that the small sample size in this pilot study may mean that the participants are not be reflective of the general characteristics of women presenting with PID.

2.4.4. Limitations of recruitment and design

The questionnaire provided did have its limitations in terms of the questions that were asked. One crucial question that was missed out upon was height and weight to calculate BMI, as one study showed that increased BMI was significantly correlated with bilateral tubal occlusion [233]. While this is not PID, it is a reproductive issue. Chronic pelvic pain (a possible complication of PID) was also significantly associated with BMI in another study [234]. The wording of questions within the questionnaire can influence the way a person may answer the questions. An example is the use of short questions that come across suddenly and do not give time for thought gathering [235]. Another question bias is known as starting time bias, instead of setting a specific time frame (e.g. from 1st January to 31st December), an ambiguous time frame was set (e.g. in the past 12 months) which will vary depending on the date of recruitment [236]. Possible bias is the use of a scale or bins (e.g. less than 1, 2-4, 4-5, >7) there is an overlap and forceful choosing (as

there was no none of the above), this was found to confuse people [237]. The use of wording is crucial as it has been shown that the use of uncommon words such as “assist” can cause confusion, it is therefore recommended to replace with the word “help” [237]. An issue that may have led to partial completion of questionnaires is that the questions flow through on two pages and it has been shown that people may forget (or not realise) to turn the page [237]. Future work based on this pilot study findings could be to conduct a study aimed specifically to improve the questionnaire tool used in collaboration with social scientists to improve the usability and completion rate of the questionnaire.

Another limitation of this study was the inability to perform a Nugent score on a vaginal smear. This can only be done on site and within a few hours of sample collection and is not part of routine practice in the majority of medical practices in Australia. It is known that a Nugent score can be used to identify bacterial vaginosis, which is associated with PID [227]. The recruitment protocol is not devoid of limitations, PID is difficult to diagnose, as described in chapter 1, and this led to the small recruitment number of cases. Additionally, on top of diagnosis, the recruitment process is laborious meaning it was not always possible for clinicians to recruit potential participants.

In this research, the number of cases recruited is small and more are required to assess the prevalence of idiopathic PID and to gain good representation of numbers within each of the aetiologies. Additionally, the inability to detect subclinical PID is challenging and raises the question about whether or not our controls and test groups are the correct group for comparison? The control group that was used from the same clinic had no difference in the demographic variables measured compared to cases. However,

differences occurred, such as a higher proportion of women reporting previous of vaginal thrush and/or bacterial vaginosis in cases compared to the controls. This leads me to the question of whether we need a group that is at risk of vaginal symptoms but has not been diagnosed with PID. Hence the objective of the test group, while the test group is more at risk of STI's, differences were found such has the absence of self-reported bacterial vaginosis and/or vaginal thrush. Albeit, the issue of the most appropriate control group is not unique to this study as many sexual health studies encounter these issues, especially in the case of a pilot study with small sample size. It is not possible to resolve the issue of which control group (Control or Test here) is more appropriate for a robust study design with the limited findings in this pilot study. The sample size for the study was targeted for a pilot study to inform a future power size calculation for a larger study. Experience in our group, and with published cervico-vaginal microbiome studies showed that significant differences could be detected with sample sizes of 50 or more [238]. There was no pre-existing data to predict the required sample size for the immune gene expression from PID, although other published studies [177, 178, 239] had sample sizes of 50-68 and did identify differences.

Whilst this study had limitations that are common through pilot clinical recruitment advantages were noted and can be considered for future studies. These include the close relationship between the research team and the clinical team that allowed for consistent involvement and recruitment. Another advantage is that specifically trained clinicians were allowed to recruit participants, after training, this allowed for consistent recruitment with an accuracy of diagnosis. Whilst this was a small pilot study, statistical analysis did

display some significance even though participant numbers were low. With this in mind, a more extensive study may lead to even greater differences between case and control.

2.4.5. Conclusion

Whilst the study did recruit controls, the target sample size for cases was not achieved. Recruitment of participants proved difficult resulting in limited numbers. Consequently, the results of analysis should be interpreted with caution, even though intended as a pilot study to guide a larger trial. No unexpected differences in the demographic and behavioural data collected was apparent, which supports that there was no recruitment bias evident based on the factors analysed (age, contraception use, previous pregnancies and previous infection with STI etc). This supports that the protocol for recruitment did not inadvertently introduce gross biases and thus is, in general, an appropriate protocol for a larger trial (with the improvements to the questionnaire considered). Furthermore, overall the PID risk factors we expect to see were identified in the study supporting the participants were consistent with PID, lending further support to the design. Interestingly, antibiotic use previous to the diagnosis of PID was observed to be more highly reported in the Cases compared to controls. This previous antibiotic usage may have altered the cervicovaginal microbiome and this observation should be as one of the objectives to evaluate in designing a larger trial informed by this pilot study. Overall, the analysis here supports that the data collected can be used in this study without the presence of confounding factors (such as medical practitioners and bias in case recruitment), although keeping in mind the limitation of the small sample size.

Chapter 3 - Profiling the microbes and microbial compositions within the vagina and cervix associated with pelvic inflammatory disease

3.1. INTRODUCTION

3.1.1. Understanding the cervicovaginal microbiome

The vaginal flora was first described in 1892, when gynaecologist Albert Doderlein reported that it was a low diversity flora dominated by bacillus type bacterial species (based on morphology) [240]. In the early 1900s, the concept that microbes uniquely inhabited specific tissues became commonly accepted [241] (e.g. skin, intestines, etc). A study of 375 women in 1938 expanded the known vaginal flora beyond *lactobacillus spp.* to include staphylococci, streptococci, bacterial coli (modern equivalent is *Escherichia coli*) and related organisms, yeasts and fungi, anaerobes, diphtheroid, spirochetes and acid-fast organisms [241]. It was later determined (~40 years later) that women with adverse vaginal symptoms were commonly found to have less or no *Lactobacillus spp.* [241].The absence of *Lactobacillus* dominant flora was commonly found in association with malodorous discharge, this discharge had a pH >4.5, and when viewed under a microscope had "clue cells." This discharge also had amines and metabolites of anaerobic bacteria; this condition was termed non-specific vaginitis [242].

Early culture-based studies associated members of the vaginal flora to vaginitis (vaginal inflammation). Previous studies only were able to culture *Gardnerella vaginalis* from those with non-specific vaginitis. However, another study was able to find that *G. vaginalis* was present in non-symptomatic healthy women and found that an increase in several anaerobic organisms associated with this condition, now called bacterial vaginosis [243]. It was also found that the most effective antibiotic for this condition was

metronidazole due to its established protozoacide and bactericidal activity against anaerobes [244]. Conversely, the more recent definition of vaginitis is the term referring to a vaginal itch which is a symptoms of parasite *Trichomonas vaginalis, or* "yeast" infection caused by *Candida vaginitis [22]*

3.1.2. Using Gram-stain to identify bacterial vaginosis – the Nugent score

In the 1990s, it was reported that the vaginal microflora is ever-changing and dynamic. A healthy flora was proposed to consist of at least three different bacterial species at any one time (usually *Lactobacillus*), but under physiological pressures, the ecosystem could shift towards a diverse species [245]. Due to the dynamic nature of the bacterial microflora, diagnosis of bacterial vaginosis by the presence or absence of specific species (i.e. ecosystem imbalance) became troublesome. The Nugent score was developed as a standardised test and uses a vaginal smear to diagnose bacterial vaginosis. The score is a system based on Gram stain of a vaginal wet-mount slide, and the slide is analysed using a scoring system (score system is outlined in Table 3.1). A score of zero indicates a smear dominated by Gram-positive rods (*Lactobacillus* morphotype), where a score of four indicates an increase of vaginal diversity and the presence of Gram-negative bacteria [246]. Figure 3.1 displays examples of wet mount vaginal smears of asymptomatic women with vaginal flora dominated by Gram-positive rods (Figure 3.1, A and B). An intermediate vaginal microbial flora with some Gram-positive rods some curved and gram variable rods and small amounts of Gram-negative's such as potentially *Gardnerella* (Figure 3.1C, D). Finally, vaginal smears from women with bacterial

vaginosis showing absence of Gram-positive rods and high abundance of presumptive *Gardnerella* and *Bacteroides spp*. (Gram-negative bacteria) (Figure 3.1E, F).

This scoring of vaginal smears in order to diagnose bacterial vaginosis correlated with the associated symptoms of bacterial vaginosis, such as pH, smell, and discomfort that women might report.

Table 3.1. Criteria for Nugent Score[a]

Score	*Lactobacillus* type bacteria (Gram positive morphotypes)	Gardnerella and Bacteroides spp. type bacteria	Curved and Gram variable rods
0	4 or more	0	0
1	3 or more	1 or more	1 or more
2	2 or more	2 or more	3 or more
3	1 or more	3 or more	
4	0	4 or more	

a: adapted from [246]

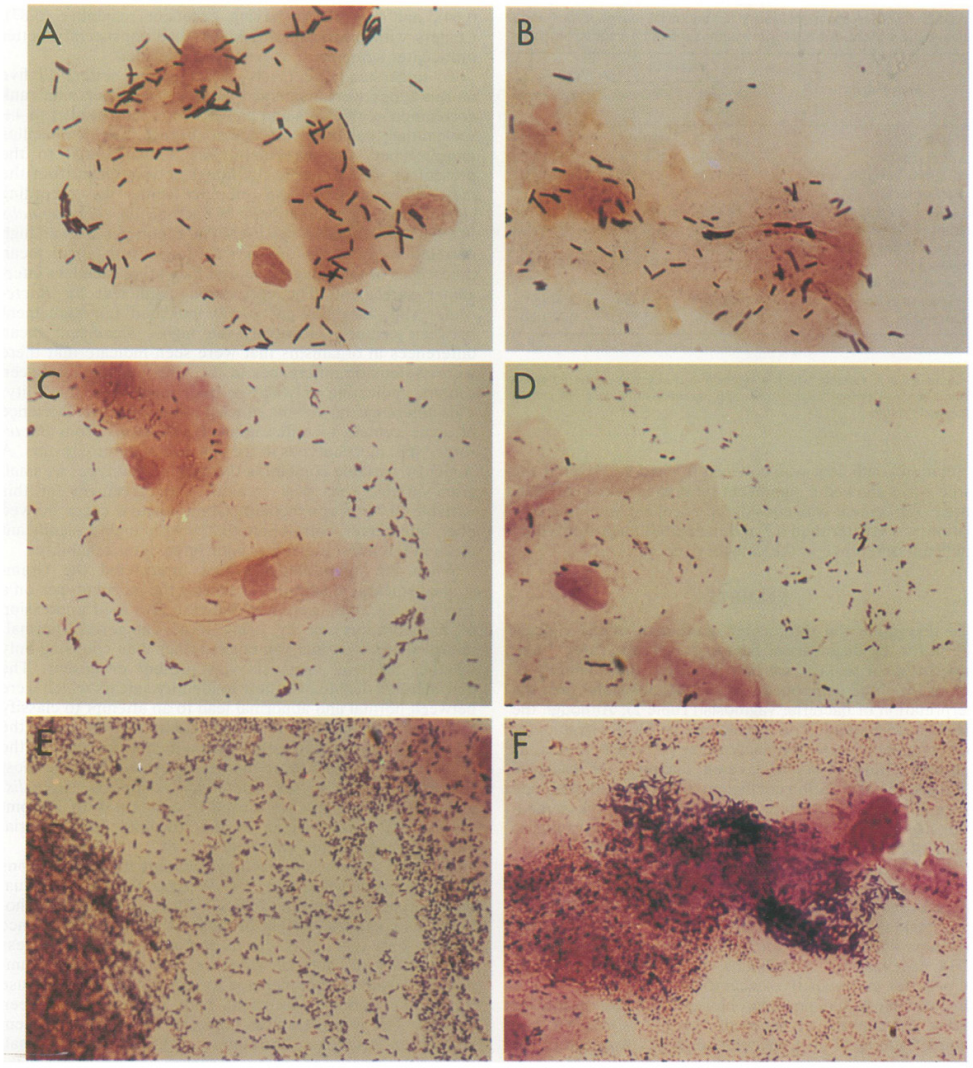


Figure 3.1. Image of representative vaginal smears that typify the Nugent score system. (A) and (B) A lactobacillus dominated smear is depicted (i.e. a score of zero). (C and D) An intermediate smear (i.e. a score of 1-3) is shown. (E and F) A Gram variable smear with a high prevalence of Gram-negative's. The figure has been directly reproduced from [246].

3.1.3. *Lactobacillus* as part of the vaginal flora

As the role of vaginal flora was beginning to be understood (1950-1990s), asymptomatic women's vaginal flora has been shown to contain up to 96 percent *Lactobacillus spp* that produce hydrogen peroxide (H2O2) [247]. In an *in vitro* study of *L. acidophilus,* it was found that the acid production by this species was able to inhibit the growth of *G. vaginalis;* bacterial vaginosis associated bacterium [248]. However, there was uncertainty on the exact species of *Lactobacillus* that predominated the vaginal flora due to the identification methods of the time (biochemical and morphological) as they don't distinguish the entire *Lactobacillus* genus well into species. However, with advancements in DNA technologies (*in situ* hybridisation), investigators were able to identify that what was thought to be '*L. acidophilus'*(based on the inaccurate biochemical tests of the time) actually consisted of two species, *L. crispatus* and *L. gasseri* [249, 250]. Furthermore, it was found that in a study of 319 women; using multiple probes to make whole-chromosomal detection assay (random primed DNA labelling kit, Boehringer mannheim, Indianapolis), that more than a third had *L. crispatus* detected as the dominant species, with fewer women found to have *L. jensenii* and *L. gasseri* as the dominant species [251].

Application of polymerase chain reaction (PCR) and gel electrophoresis enabled the identification of unculturable bacteria found to colonise the vaginal epithelium, mainly *L. iners.* This particular species can only be grown on specific media and was possibly overlooked as previous studies focused on *Lactobacillus* specific media (MRS) [252]. Application of first-generation DNA sequencing (Sanger sequencing) found that

healthy vaginal flora was dominated by *Lactobacillus species* when compared to women with vaginitis [241]. It was not until the development of next-generation sequencing that it was found that the vaginal microbiome is dominated by one of four *Lactobacillus species (L. crispatus, L. gasseri, L. iners or L. jensenii*) or had no dominant *Lactobacillus.* This was due to the ability of high through put analysis of multiple samples. Furthermore an improvement in the specificity and sensitivity of next-generation sequencing compared to the first generation allowed for resolution of organisms of the same genus but different species [162]. Each microbial composition was termed a different community state type (CST); from CST I to CST V, as outlined in Chapter 1.

3.1.4. Different cervicovaginal microbial community state types and disease

It is now well established that the microbiome is the first line of defence for the health of the female reproductive tract. In a longitudinal study of 3620 women with Nugent Gram stain confirmed bacterial vaginosis (score of ≥ 2), with/without a sexually transmitted infection (STI), it was found that there was an increased risk of gonococcal, trichomonal, and chlamydial infection compared to women with a Nugent score of 0 - 3 [253]. Furthermore, in another study, it was found that women with *C. trachomatis* and *N. gonorrhoea* infection were less likely to have a *Lactobacilli* dominant flora than women without these infections with an odds ratio of 3.4 and 4.1 respectively [254].

Interestingly, vaginal microbiomes dominated with *L. gasseri* (CST II), *L. iners* (CST III) or no dominant *Lactobacillus species* (CSTIV) have been associated with an increased risk of human papillomavirus (HPV) and cervical intraepithelial neoplasia

(abnormal cell growth that could lead to cervical cancer) at time of diagnosis (i.e. sample collection) [255]. Further, a cross-sectional study demonstrated that women who are currently infected with HPV had a greater diversity of microbes in their vaginal flora [256]. However, this study had a small sample size (n=70), used species-specific PCR for microbial flora analysis (this is a limitation because one selects in advanced what organism they are looking for), and that majority of women will have encountered HPV within their reproductive life. Furthermore, a study showed that the transmission of human immunodeficiency virus (HIV) was least likely in women with a community state type with a high abundance of either of two particular *Lactobacillus sp* (CST I (*L. cripsatus*) and CST II (*L. gasseri*)) [257]. This was also observed with culture based experiments [258] with *in vitro* HIV infection of cells with different *Lactoabcillus* present. The vaginal microbiota has been able to be classified into a small series categories (CST I – V), due to the overall relatively low abundance and low diversity of organisms in this niche. These categories have simplified experimental evaluations and interpretations.

While PID has not been linked to certain compositions of the vaginal flora or a specific CST, endometritis (inflammation in the endometrium [259]), a significant risk factor or component of the inflammation sometimes observed during or prior to PID, has been associated with microbiota compositions [61]. In a study of 117 women with clinically identified endometriosis, the most commonly isolated organism other than *N. gonorrhoea* and *C. trachomatis* were anaerobic organisms such as Gram-negative rods *Prevotella bivia* and *G. vaginalis* [260]. The question remains, does disease drive a

change in the vaginal flora, or does a change in vaginal flora cause an increase in the risk for the onset of disease.

Early investigations of women with upper genital tract inflammatory conditions such as salpingitis and endometritis have shown the presence of anaerobic bacteria through vaginal fluid collection, and association with a positive diagnosis of bacterial vaginosis [60, 261-263]. Hence, bacterial vaginosis could be associated with or a preluding factor to pelvic inflammatory disease. This was supported by the findings of a longitudinal study of 1140 women, which found that those that had bacterial vaginosis had increased likelihood of PID incidence with an adjusted odds ratio of 2.03 [264].

3.1.5. Microbial associations with pelvic inflammatory disease

In a sample of 541 women suffering from inflamed endometrium (endometritis), culture-based and polymerase chain reaction microbial profiling, the risk ratio was evaluated for PID (for women who ended up developing PID) in terms of the differing microbial agents detected. Other than STI's, bacteria that were associated with an increased incidence of chronic endometritis include *Ureaplasma urealyticum*, *Gardenerella vaginalis* and *Prevotella* (Table 3.2) [60]. While all of these anaerobic Gram-negative organisms were concatenated into one group (CST IV like), there still remains the possibility of only some of these (e.g. *Prevotella spp*) being associated with an increased incidence of PID (or even specific sub-strains or patho-variants).

Further studies of different organisms that are found in PID show that *Mycoplasma genitalium* was strongly associated with PID when compared to healthy

women using PCR detection in the vagina (p-value <0.01) [265]. In another study comparing women in hospitals for PID, and pregnant women admitted for delivery and miscarriage; it was found that a biovar of *Ureaplasma urealyticum* (T960) was more frequently identified in women with PID compared to other groups (p-value <0.0001) [266]. However, this study compared these two very different groups, and other species of *Ureaplasma* were not examined. The current consensus is that there is still a large amount of work to be done to understand whether and which anaerobic/pathogenic bacteria (other than STI's) detected in the upper genital tract of women with PID can be associated with the disease.

Table 3.2. Bacterial species found by culture and PCR in 831 women with endometritis[a].

Microorganism	Number of participants	No endometritis (%)	Chronic endometritis (%)	Acute endometritis (%)
Chlamydia trachomatis	26	2.1	7.3	25.6
Neisseria gonorrhoeae	34	4.2	8.1	33.8
Mycoplasma hominis	19	7.9	2.2	7.3
Ureaplasma urealyticum	19	6.6	6.7	7.3
H_2O_2-producing *Lactobacillus species*	21	10.6	8.9	1.2
Gardnerella vaginalis	81	31.1	31.1	30.5
Any anaerobic Gram negative rod	61	18.5	6.7	31.7

a: Table has been directly altered from [60].

The work in this chapter aimed to determine whether the composition of microbial communities in vaginal and cervical microbiota is significantly between Case-Control participants, and identify any new microbiota, or microbiota profiles, associated with PID profile the microbial community composition in women with PID, using a case-control prospective study design. Specifically, this work aimed to:

1. Analyse the cervicovaginal microbiota compositions in the Case-Control and Test groups.

2. Use quantitative PCR to determine if the abundance of particular microorganisms is associated with PID cases

3.2. MATERIALS AND METHODS

3.2.1. DNA extraction

Swab biospecimens (containing 1mL total volume) from the vagina and cervix were thawed to room temperature and vortexed (30 seconds) before removing the breakpoint swab. Mutanolysin (7000U/ml), lysozyme (100mg/ml), and lysostaphin (1mg/ml) were added to each sample to enhance bacterial cell lysis and incubated for 60 minutes at 37ºC. 50 µL Proteinase K solution (QIAGEN DNeasy Blood and Tissue Kit), 1.25 µl of RNase A solution (QIAGEN) and 55 µl of SDS (10%) were then added to each sample and incubated at 55°C for at least 60 minutes or until the lysate was visibly

translucent. This was to ensure degradation of cellular debris. Remaining bacterial cells were then disrupted through mechanical bead beating by placing the sample on a bead beater with three silica beads within each tube. Samples were then centrifuged (3 min at 10,000 × g) and the supernatant removed into new Eppendorf tube containing an equal volume of both buffer AL (QIAGEN DNeasy Blood and Tissue Kit, Cat No. 69504) and ethanol (96-100%) compared to the supernatant. The lysate was then placed in a mini-spin column that had a collection tube attached and centrifuged for 1 minute at 6000 × g. The flow-through was discarded, and the collection tube replaced. 500µL of AW1 was centrifuged in the mini-spin column and flow-through discarded. AW2 (500µL) was then centrifuged in the spin column for three minutes at 20 000 × g, and this was then repeated with fresh AW2. 100µL of ultra-pure water was then added to the spin column and incubated for five minutes at room temperature before being placed in a new sterile Eppendorf tube and centrifuged for one minute at 6000 × g to elute the purified DNA. The flow-through containing the DNA was aliquoted for safety and stored at -80˚C.

The above DNA extraction was then quantified using a Qubit dsDNA BR Assay Kit (ThermoFisher Scientific Cat No. Q32853). The Qubit assay was conducted by diluting the HS buffer with reagent (1:200) to form a working solution. 1µL of each sample of the product was placed in individual Qubit assay tubes and 199µL of working solution. Two qubit standards were prepared by mixing 190µL of working solution and 10µL of qubit standard one and standard two in each tube. The solutions were vortexed for 30 seconds before being quantified in the Qubit 2.0 fluorometer (ThermoFisher Scientific Cat No. Q32866). Due to the nature of the sample (vaginal and cervical) and

small amounts of DNA, the Qubit quantification included a concentration in ng/μL, and if the sample wasn't quantifiable, the sample was not proceeded to further analysis.

3.2.2. Quality control and assurance

A quality control and assurance process included the preparation and consistent use of a set of microbiota standards. These standards and aliquots were prepared on the same day. Aliquots of these standards were included in every sequencing analysis conducted as quality control for the performance of the different batches of sequencing analysis. These included four community state type standards representing cervicovaginal community state type I-IV. These internal controls were constructed by combining 2-9 participant swab samples (from a previous study with known community type) for each community state type and extracting the DNA as per section 2.4. Furthermore, DNA was extracted from the RNA storage buffer alone, extraction controls using all extraction reagents without extracting from an original buffer, and swab only controls, as previous studies show contamination can occur from these sources [267].

3.2.3. Preparation of 16S rRNA gene amplicons

A two-step PCR process was used as this has been shown to reduce PCR bias and chimera formation to prepare 16S rRNA gene amplicons from mixed DNA. This protocol includes the use of KAPA Hi-Fi (Millennium Science Australia Cat No ROC-07958935001), which was also shown to reduce PCR bias for Illumina sequencing [268].

The first PCR amplified the 16S rRNA gene V3-V4 region using primers that anneal to the 319F and 806R positions of the *Escherichia coli* 16S rRNA gene. These primers also contain a phaser, so that amplicons on the MiSeq will be at different positions during sequencing, and part of the Illumina TruSeq adaptor (Table3.3). The same combination of F and R primer was used on multiple samples where there are more samples than combinations (Table 3.4). The PCR tubes were arranged in a plate layout with eight columns and 11 rows for ease of work and samples randomly assigned a position in this layout. Samples were prepared in a PCR laminar flow cabinet with all equipment and surface sterilised with 10% bleach for 15 minutes to reduce environmental contamination. Equipment and surfaces were then cleaned from the bleach with ultrapure water and then placed under ultraviolet light for 20 minutes. Table 3.5. shows the PCR reaction composition; all reagents were placed on ice while mixing tubes. The PCR tubes containing the mixture were placed in an Evo Mastercyler PCR machine (Eppendorf, Germany) and PCR reaction conducted using the conditions described in Table 3.6

Table 3.3 Forward and Reverse primers for PCR1[a].

	Truseq p7 adaptor	**phaser**	**319F**
Forward phaser0	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		ACTCCTACGGGAGGCAGCAG
Forward phaser1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	T	ACTCCTACGGGAGGCAGCAG
Forward phaser2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GT	ACTCCTACGGGAGGCAGCAG
Forward phaser3a	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CGG	ACTCCTACGGGAGGCAGCAG
Reverse phaser0	ACACTCTTTCCCTACACGACGCTCTTCCGATCT		GGACTACTVGGGTWTCTAAT
Reverse phaser1	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	A	GGACTACTVGGGTATCTAAT
Reverse phaser2a	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TA	GGACTACTAGGGTATCTAAT
Reverse phaser3	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTT	GGACTACTVGGGTATCTAAT

Table 3.4. Possible combinations of F and R primers for PCR1

	Reverse phaser0	**Reverse phaser1**	**Reverse phaser2a**	**Reverse phaser3**
Forward phaser0	F0 + R0	F0 + R1	F0 + R2	F0 + R3
Forward phaser1	F1 + R0	F1 + R1	F1 + R2	F1 + R3
Forward phaser2	F2 + R0	F2 + R1	F2 + R2	F2 + R3
Forward phaser3	F3a + R0	F3a + R1	F3a + R2	F3a + R3

a: Gaps shown below are for illustration purposes only

Table 3.5. PCR 1 reaction set up

Component	1 x reaction	Conc.
2 x KAPA hot start HiFi ready mix	10 µl	1 ×
10µM F primer	1 µl	0.5 µM
10µM R primer	1 µl	0.5 µM
Template DNA	x	1 ng
water	(20 – [12 + ×]) µl	
Total	20 µl	

Table 3.6. PCR 1 cycling conditions

95°C	2 mins
20 cycles of	
95°C	15 sec
55°C	15 sec
72°C	30 sec
Followed by	
72°C	2 min
4°C	hold

After the first PCR, AxyPrep MAG-PCR Clean-up Kit, (Fisher Biotech, Cat No. MAG-PCR-NM-5) was used to remove excess primers and primer dimers. This was conducted in a biological safety cabinet, where all equipment and surfaces were sterilised with 10% bleach for 15 minutes, followed by a 20 minute UV cycle. A 0.7 x volume of beads compared to the PCR volume, was used to remove the majority of DNA < 200bp, which included primer dimers. The sample was then incubated at room temperature for five minutes, followed by magnetic excitation for three minutes on a magnetic plate. The supernatant was removed, and 185µL of 85% ethanol added, all while still on the magnetic plate. After 30 seconds, the ethanol was removed and tubes placed in a centrifuge (30 seconds, 10000 × g). The tubes were then allowed to air dry (in a tube rack) for five minutes to completely rid of ethanol as it can impact on the quality of sequence results. 200µL of water was then added to each tube to resuspend the beads. This mixture was then incubated for five minutes at room temperature followed by magnetic excitation for three minutes on a magnetic plate. The liquid supernatant was then removed and used in the next PCR.

The second PCR enriches for products from PCR 1 that have the Truseq p7 and p5 adaptors. Sample barcodes were added at this stage; differently, barcoded enrichment primers were combined to give a unique combination of barcodes per sample (Table 3.7), Combinations are shown in Appendix B. Enrichment primers were aliquoted into single-use tubes, to avoid cross-contamination of primer stocks with different barcodes. PCR reactions were set up according to Table 3.8, in a biological safety cabinet were equipment was sterilised with 10% bleach, wiped off with Ultra-pure water and then placed under a 20-minute UV cycle. After which the PCR reaction was conducted

according to the conditions outlined in Table 3.9. The magnetic bead clean method, as described above, was used to clean the PCR product. Final product was resuspended in 60µl of ultra-pure water. This product was placed a new tube and quantified by Qubit dsDNA BR Assay Kit (ThermoFisher Scientific Cat No. Q32853).

Table 3.7. Primer sequences for enrichment primers

Enrichment_i7	CAAGCAGAAGACGGCATACGAGAT(8nt_barcode)GTGACT GGAGTTCAGACGTG
Enrichment_i5	AATGATACGGCGACCACCGAGATCT(8nt_barcode)ACACT CTTTCCCTACACGA

Table 3.8. Enrichment PCR reaction set up

Reagent	reaction	Conc.
2X Kapa Hi-Fi ready mix	10 µl	1 x
10µM Enrichment_i7	0.5 µl	0.5 µM
10µM Enrichment i5	0.5 µl	0.5 µM
Template DNA	9 µl	
Total	20 µl	

Table 3.9. Enrichment PCR cycling conditions

95°C	2 mins
10 cycles of	
95°C	15 sec
60°C	15 sec
72°C	30 sec
Followed by	
72°C	2 min
4°C	hold

3.2.4. 16S rRNA gene amplicons library preparation

The pooled samples for sequencing were prepared by combining equal amounts of DNA per sample (ng). The pooled samples were purified by 1.8 × total volume of magnetic beads (AxyPrep MAG PCR Clean-up Kit, 50 mL Cat No. MAG-PCR-NM-50) in order to remove non-specific PCR product. The pooled sample was assessed by Bioanalyser HS-DNA chip (Agilent High Sensitivity DNA Kit, Cat No. 5067-46, Germany) for concentration and 16S amplicon size (neat, 1:10, 1:100). The dilution that was selected for calculation of concentration is one that had the correct size in an appropriate resulting concentration under 10 nM.

For a working DNA stock, the sequencer requires 20 pM. The selected pool (1:10) was diluted to 4nM and then combined with 0.2 M of NaOH. This was vortexed and heated for five minutes at 30˚C. After heating, 990 µL of HT1 buffer (Illumina V3 – V4 600 cycle sequencing kit) was added. This formed the last 20 pM working solution.

The final library was prepared by adding 270 µL of working stock DNA, 60 µL of PhiX (control library) and 270 µL of cold HT1 buffer. This library was then placed in the MiSeq v3 600 cycle cartridge and loaded into the Illumina MiSeq sequencer with reagents. 16S amplicons were sequenced using the Illumina Mi-Seq with a V3 600 cycle full flow-cell. The Illumina platforms allow for paired-end sequencing, which allows for fragments to be sequenced from both the forward and reverse position (5 prime end and 3 prime end). This facilitates a more significant and more precise reading distribution and the capacity to identify variations of 16S rRNA fragments [269]

3.2.5. Analysis of sequences using Qiime 2 and taxonomic identification of samples

Demultiplexed sequences were retrieved and imported to the University of Technology Sydney servers [270]. A summary of the data was generated and based on this; the deblur method was used deconvolute algorithms used to filter the reads [271]. This included trimming the reads and adjusting each read to the same length (i.e. same number of base pairs based on quality plot Figure 3.2) (Appendix B displays the threshold used in the codes), the read pair merging was conducted using qiime2 q2-vsearch plug in protocol [272]. Then a feature table was generated, allowing for the correct export format to R-studio. This produced a format of number of features per sample and a taxonomic

table (using deblur [271]). A phylogenetic tree was created using (via q2-phylogeny in Qiime2 [273]) and the unrooted tree exported to R-studio. Using the representative sequences produced from Qimme2, the software: Species-level Taxon Identification of rDNA Reads using Usearch Pipeline Strategy (STIRRUPS) [274] was used to classify each feature (i.e. OTU) to the species level based on the vaginal 16S rDNA reference database [274] using an identity threshold of 97%. This produced a taxonomic table with relative abundances to be imported to R-studio for analysis and figure generation. It is important to note that STIRRUPS is only trained for V1-V3 and the only vaginal 16S data base to date. Hence only one of the reads from the read pairs was used.

3.2.6. Analysis of taxonomy and figure generation using R studio

The package phyloseq [275] was used in R studio [276] to import the feature table, taxonomic table, unrooted tree, metadata table, and sequences. These were then merged and a collapsed to species rank, and a summary of the number of reads per sample conducted. Samples were pruned that had less than 2000 reads. This resulted in samples being removed if they have less than 2000 reads. Reads were then rarefied at an even depth, which normalizes samples and ensures they have the same number of total counts (also 2000, due to the lowest coverage that was deemed reasonable compared to negative controls). A distance matrix was created using the Jensen-Shannon divergence method (via phyloseq package version 1.8 [275]), this matrix was the clustered in a hierarchal structure based on Ward's method (via stats package version 3.4 [276]). This produced a cluster dendrogram to allow for visualization. A cluster dendrogram linkage analysis heat map was produced using the top 20 most relatively abundant species and annotated using

the package complex heat maps[277]. The code used in the R environment can be found in Appendix B.

3.2.7. Quantification of selected bacteria using quantitative real-time PCR

Validation of the 16S rRNA gene amplicon sequencing results was conducted using quantitative PCR (qPCR). Primers targeting select bacterial species (Table 3.10) 16S rRNA gene regions were used as previously described [278, 279]. After extensive research in the literature and to my knowledge, no primers are designed to differentiate the different species within the genus *Prevotella,* hence the targeting of the entire genus annotated by *Prevotella spp.* The MgPa gene was targeted for quantifying *Mycoplasma genitalium,* as previously described [280]. Table 3.10. shows the primers and amplicon size for each target. The quantification protocol used EVAgreen (JOMAR life research Cat No. 31000, USA), a fluorescent dye optimised for PCR products of sample size < 500bp, as consistent with those planned in this study [281]. Standard curves were generated to determine absolute copy numbers and were constructed from synthetic DNA made by Integrated DNA Technologies (Singapore). The synthetic DNA was a double-stranded fragment (gBlocks® Gene Fragments, IDT, Singapore) of the target amplicon with an extra 15-20 bases according to the target sequences. Table 3.11 shows the synthetic DNA sequences from 5' to 3' for each bacterial species targeted. This DNA was in a supplied concentration of 10ng/µL. This was diluted to 0.6ng/µL as the first standard reaction followed by a 1 in 10 dilution series to make eight standard reactions.

Table 3.10. Bacterial genus/species-specific primers used for quantitative PCR assays

PCR bacterial targets (Ref)	Forward primer (5' – 3')	Reverse primer (5' - 3')	Amplicon size
L. crispatus [277]	AACTAACAGATTTACTTCGGTAATGA	AGCTGATCATGCGATCTGC	145 base pairs
L. iners [277]	AGTCTGCCTTGAAGATCGG	CTTTTAAACAGTTGATAGGCATCATC	166 base pairs
Atopoboium vaginae [277]	TAGGTCAGGAGTTAAATCTG	TCATGGCCCAGAAGACCGCC	155 base pairs
Gardnerella vaginallis [277]	GCGGGCTAGAGTGCA	ACCCGTGGAATGGGCC	206 base pairs
Prevotella species [278]	CCAGCCAAGTAGCGTGCA	TGGACCTTCCGTATTACCGC	151 base pairs
Mycoplasma genitalium [279]	GAGAAATACCTTGATGGTCAGCAA	GTTAATATCATATAAAGCTCTACCGTTGTTATC	77 base pairs

Table 3.11. The synthetic DNA used to construct gene fragments for standard curve construction

PCR bacterial targets	Synthetic DNA for standard curve[a]
L. crispatus	TGCTATACATGCAAGTCGAGCGAGCGGAACTAACAGATTTACTTCGGTAATGACGTTAGGAAAGCGAGCGG CGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAATAC CGGATAAGAAAGCAGATCGCATGATCAGCTTTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCC
L. iners	CTATACTGCAGTCGAGCGAGTCTGCCTTGAAGATCGGAGTGCTTGCACTCTGTGAAACAAGATACAGGCTA GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAGAGATCGGGATAACACCTGGAAACAGATGCT AATACCGGATAACAACAGATGATGCCTATCAACTGTTTAAAAGATGGTTCTGCTATCACTCTTGGATGGACC TGCGGTGCATTA
Atopoboium vaginae	CAAGCGTTATCCGGATTCATTGGGCGTAAAGCGCGCGTAGGCGGTCTGTTAGGTCAGGAGTTAAATCTGGG GGCTCAACCCCTATCCGCTCCTGATACCGGCAGGCTTGAGTCTGGTAGGGGAAGATGGAATTCCAAGTGTA GCGGTGAAATGCGCAGATATTTGGAAGAACACCGGTGGCGAAGGCGGTCTTCTGGGCCATGACTGACGCTG AGGCG
Gardnerella vaginalis	GTCTGGTGTGAAAGCCCATCGCTTAACGGTGGGTTTGCGCCGGGTACGGGCGGGCTAGAGTGCAGTAGGGG AGACTGGAATTCTCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCT CTGGGCTGTTACTGACGCTGAGAAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG CCGTAAACGGTGGACGCTGGATGTGGGGCCCATTCCACGGGTTCTGTGTCGGAGCTAACGCGTTAAG
Prevotella species	TGAGGAATATTGGTCAATGGACGGAAGTCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTT GTAAACTGCTTTTGTATGGGGATAAAGTTAGGGACGTGTCCCTATTTGCAGGTACCATACGAATAAGGACC GGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAAGGTCCAGGCGTTATCCGGATTTATTGGGTTTA
Mycoplasma genitalium	TCAAGTATCTCAATGCTGTTGAGAAATACCTTGATGGTCAGCAAAACTTTGCAATCAGAAGGTATGATAAC AACGGTAGAGCTTTATATGATATTAACTTAGCAAAAATGGAAAACCCA

a: although these are double-stranded only 5'-3' is shown.

The qPCR was conducted on the Qiagen Rotor-GeneQ-5plex platform (Cat No./ID: 9001580) in 100 well rings prepared on the Qiagen QIAgility (Cat No. 9001904). The PCR reaction and conditions were in accordance with the previous studies, with the modification of dye and polymerase master mix (Amplitaq Gold 360 Master mix, Thermofisher Cat No. 4398881) as described in Table 3.12. [278, 279]. The reaction cycling conditions for the qPCR are outlined in Table 3.13.

Table 3.12. qPCR reaction combination

Reagents	**Volume**	**Conc**
Amplitaq Gold 360	12.5 µL	1 ×
Forward primer	1 µL	0.5 µM
Reverse primer	1 µL	0.5 µM
DNA	5 µL	
Water	0.5 µL	
EVA green	5 µL	1 ×
Total	25µL	

Table 3.13. qPCR cycling conditions

95°C	10 mins
Followed by 40 cycles of	
95°C	15 sec
60°C	60 sec

3.2.8. Statistical analysis

Participant bio-specimen qPCR data was graphed and analysed in GraphPad Prism version 8.00 [203]. This was used to calculate means, standard error of the mean (SEM) and p-values using independent non-parametric Mann-Whitney test [204] where data was assumed not to be distributed normally. Where differences were observed between groups to a p-value of less than 0.05, this was considered to be significant.

3.3. RESULTS

3.3.1. DNA yield for every sample indicated successful extractions

DNA was extracted from 51 cervical and 51 vaginal samples. The DNA yield was recorded for each sample and biological site (Table 3.14). It is important to note that chapter 2 accounts for 13 test group participants, but the following results account for 6 test group participants. The variability is due to recruitment samples (n=3) not being collected along with demographic data and the remaining five were analysed after completion of the thesis. Comparing biological locations, independent of sample types, the cervical samples had an average yield of 1.65 ng/µl (0.03 – 12.9), while the vaginal samples had an average yield of 28.23 ng/ul (0.18 – 600). This vaginal sample DNA extraction yields were significantly higher than the cervical samples with a p-value < 0.001 (Wilcoxon signed-ranked paired test). Using the Qubit dsDNA BR Assay Kit samples were excluded from further analysis if the yield determined was not appropriate (less than 0.1 ng/µl).

Table 3.14. Concentration of DNA per sample

	Participant	DNA concentration (ng/µl)[a]		Mean (SD)	
		Cervix	Vagina	Cervix	Vagina
Test Group	19	1.33	0.50	1.21 (1.08)	205.08 (279.33)
	20	0.46	600		
	21	0.27	21.0		
	22	0.50	7.04		
	23	1.21	600		
	24	3.46	1.94		
Case	200	0.72	1.04	2.20 (3.39)	4.79 (4.93)
	201	0.83	1.07		
	202	0.14	0.19		
	203	0.15	5.14		
	204	0.03	1.17		
	205	0.63	0.36		
	206	0.85	1.34		
	207	2.04	17.6		
	210	5.68	8.92		
	216	0.33	3.07		
	217	1.12	8.16		
	218	0.75	4.72		
	219	12.9	11.6		
	220	4.57	2.72		
Control	400	0.82	7.56	1.49 (1.98)	4.6 (5.18)
	401	0.53	0.55		
	402	0.74	1.09		
	404	1.12	2.71		
	405	6.28	12.2		
	408	0.13	3.30		
	409	0.79	0.27		
	410	1.11	2.94		
	411	0.08	0.36		
	412	0.80	1.03		
	413	0.15	0.18		
	414	1.00	0.97		
	415	0.18	5.40		
	416	0.78	0.95		
	417	1.46	0.88		

	Participant	DNA concentration (ng/µl)[a]		Mean (SD)	
		Cervix	Vagina	Cervix	Vagina
	418	0.13	11.8		
	419	0.19	13.9		
	420	3.18	12.2		
	428	0.58	0.61		
	429	0.46	0.88		
	430	0.82	0.20		
	431	2.04	0.99		
	432	2.24	2.18		
	433	2.16	1.31		
	434	0.94	1.74		
	209	0.08	3.04		
	211	1.04	4.52		
	212	9.76	18.6		
	213	3.96	14.1		
	214	1.03	11.4		
	215	1.70	4.76		
Experimental Controls	Community state type 1	600		600 (0)	
	Community state type 2	600			
	Community state type 3	600			
	Community state type 4	600			
	Extraction control 1	0.50		0.5 (0)	
	Extraction control 2	0.50			
	Extraction control 3	0.50			
	Extraction control 4	0.50			
	Extraction control 5	0.50			
	RNA buffer	0.50			
	SWAB only	0.50			

a: using Qubit with standards to determine DNA concentration

3.3.2. Quality control allowed for adequate sequencing data

All samples with appropriate yield after extractions alongside controls were sent to a sequencing center (Ramaciotti Center for Genomics, University of NSW, Sydney),

with demultiplexed trimmed data returned (a similar protocol for amplification was used as seen in [282]). Quality control as outlined in the materials and methods, was conducted, including trimming low-quality regions resulting in 190bp reads with a quality score above 20 (example quality plot is shown in Figure 3.2). This resulted in 1183 features with an average of 8518 reads per sample. Five cervical (204, 216, 415, 432 and 209) and one vaginal sample (019) did not have adequate sequence coverage and were not included in the analysis.

Control samples (Table 3.15) had lower reads, with an average of 8,096 (2,696 - 13,910) reads, the RNAlater buffer had 2,000 reads, and swab only control had 11,560 reads. The positive control representative samples had an average of 104,692 (85,388 - 123,090) reads per community state type control (Table 3.15). Based on these controls and their average assigned reads, filtering methods were used to remove the background noise found in the buffers and extraction controls. Due to challenge of PID genital samples (large amounts of mucus, RNases, DNAses), the balance between removing background noise and samples is a complicated one. Hence the rarefication only selected to remove the contaminants in the RNA buffer solution. Whilst this may seem to include some negative controls, the advantage of classifying samples based on dominance means these are likely to be removed from the analysis (and they were filtered out). Rarefication resulted in 450 representative sequences per sample. Appendix B displays the resulting files from the qiime2 pipeline, including the full feature table and full taxonomic tables.

Table 3.15. Read depth per control samples

	Control	Read depth
Negative controls	Extraction control 1	7,854
	Extraction control 2	13,910
	Extraction control 3	2,696
	Extraction control 4	5,902
	Extraction control 5	10,116
	RNA buffer	2,000
	SWAB only	11,560
Positive controls	Community state type 1	99,886
	Community state type 2	85,388
	Community state type 3	110,404
	Community state type 4	123,090

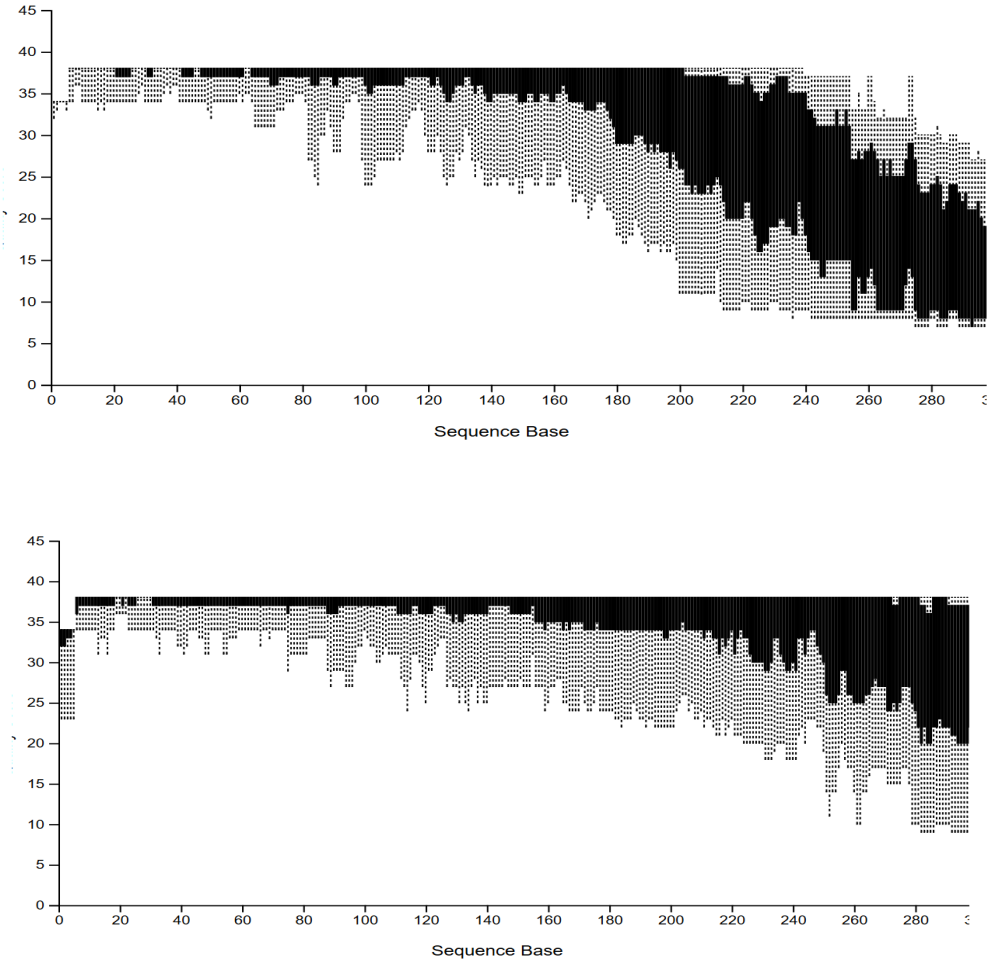


Figure 3.2. Forward and reverse reads quality plot generated by Qiime2. Panel A shows the forward reads and panel B shows the reverse reads. The x-axis represents the number of bases pairs, and the y-axis is a quality score, where above 20 is recommended. The plots are of all samples. The black sections is the mean and grey are the standard deviation**.**

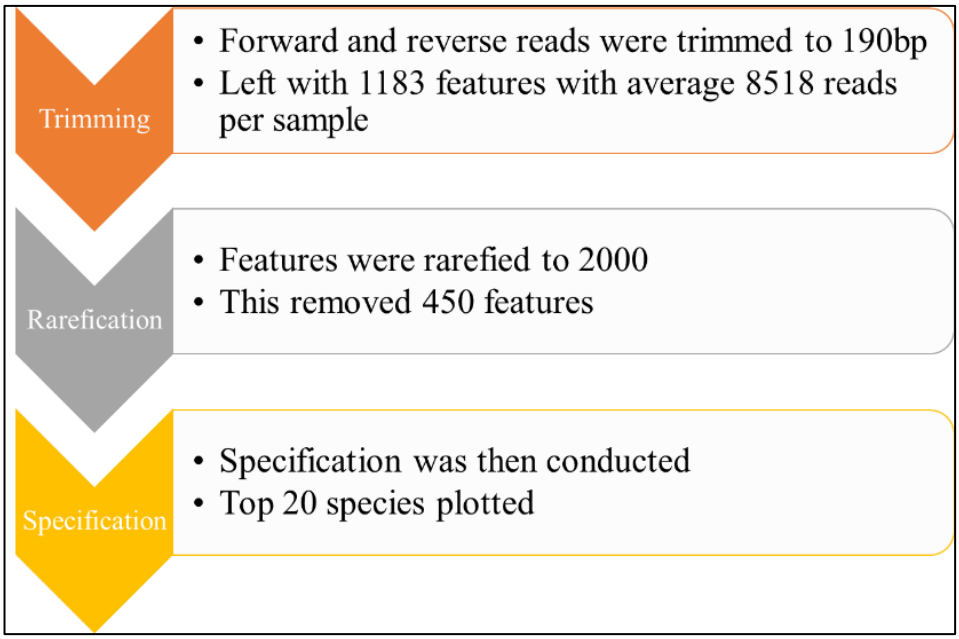


Figure 3.3. Data quality control. Trimming, rarefication, and specification were used in order to generate a refined sequence set that was then used to construct a cluster dendrogram plotted into a heat map. It is important to note, only the forward read (V3 region, 190bp) was used due to the STIRRUPs data base being trained for V1-V3.

3.3.3. Relative abundance of microbial species in the cervicovaginal microbiome was different across groups, but consistent in each individual between the cervix and vagina

The most abundant species was concordant between the cervical and vaginal samples within each individual (Table 3.16). The resultant read depth (sequencing coverage) can be seen in Table 3.16 with a mean read depth of 102,920 and 145,534 for cervix and vagina, respectively.

Table 3.16. Read depth of the most abundant species detected within each participant

	Participant	Read depth		Most abundant species[a]
		Cervix	Vagina	
Test group	19	182,410	12,384	*Lachnospiraceae. BVAB1*
	20	169,242	290,186	*L. iners*
	21	145,928	239,078	*G. vaginalis*
	22	97,520	155,010	*L. crispatus*
	23	268,254	262,110	*S. amnii*
	24	149,734	231,758	*L. BVAB1*
Case	200	134,320	258,538	*L. iners*
	201	41,276	146,160	*G. vaginalis*
	202	140,054	165,756	*L. iners*
	203	110,284	149,184	*G. vaginalis*
	204	5,906	125,410	*L. crispatus*
	205	124,744	93,298	*L. iners*
	206	56,774	134,246	*L. crispatus*
	207	111,122	153,588	*L. iners*
	210	97,952	129,464	*L. iners*
	216	10,498	173,928	*L. iners*
	217	75,164	152,396	*L. iners*
	218	84,842	161,316	*L. iners*
	219	93,576	116,122	*G. vaginalis*
	220	134,262	210,644	*G. vaginalis*
Control	400	115,998	129,314	*L. crispatus*
	401	92,506	185,814	*L. crispatus*
	402	160,988	65,084	*L. crispatus*
	404	111,894	116,208	*L. crispatus*
	405	131,478	144,072	*L. iners*
	408	58,178	180,098	*G. vaginalis*
	409	104,694	103,558	*L. iners*
	410	81,404	96,852	*L. iners*
	411	109,266	148,388	*L. crispatus*
	412	85,456	188,426	*G. vaginalis*
	413	78,874	121,732	*L. crispatus*
	414	136,002	95,470	*L. crispatus*
	415	9,168	160,168	*L. iners*
	416	41,216	110,072	*L. iners*
	417	69,144	103,328	*L. iners*
	418	20,898	115,110	*L. crispatus*
	419	71,176	157,442	*L. crispatus*
	420	176,386	136,258	*L. iners*
	428	72,720	157,654	*L. iners*
	429	53,860	90,868	*G. vaginalis*

Participant	Read depth		Most abundant species[a]
	Cervix	Vagina	
430	132,286	24,614	*G. vaginalis*
431	126,074	185,414	*L. crispatus*
432	9,984	126,052	*L. iners*
433	133,260	140,818	*L. iners*
434	165,066	68,794	*L. crispatus*
209	19,498	124,882	*L. crispatus*
211	207,438	152,770	*L. iners*
212	112,044	158,660	*L. iners*
213	98,364	136,884	*L. crispatus*
214	102,480	157,748	*G. vaginalis*
215	127,262	179,090	*L. crispatus*
Average	102,920	145,534	
SD	53,625	53,482	

a: Most abundant species was the same for cervix and vagina. In every single participant

The relative abundance per participant of the top 30 most abundant species (defined by most prevalent species that was most relative abundance per participant by total read counts) across both sites is shown in Figure 3.4. The most relatively abundant species was *Lactobacillus crispatus*, followed by *Lactobacillus iners.* The stacked bar graph (Figure 3.4) shows the top 30 species and groups the others into the 'other' categories, while the lowest relative abundance of value (30th most abundant species) is *Mycoplasma homins*.

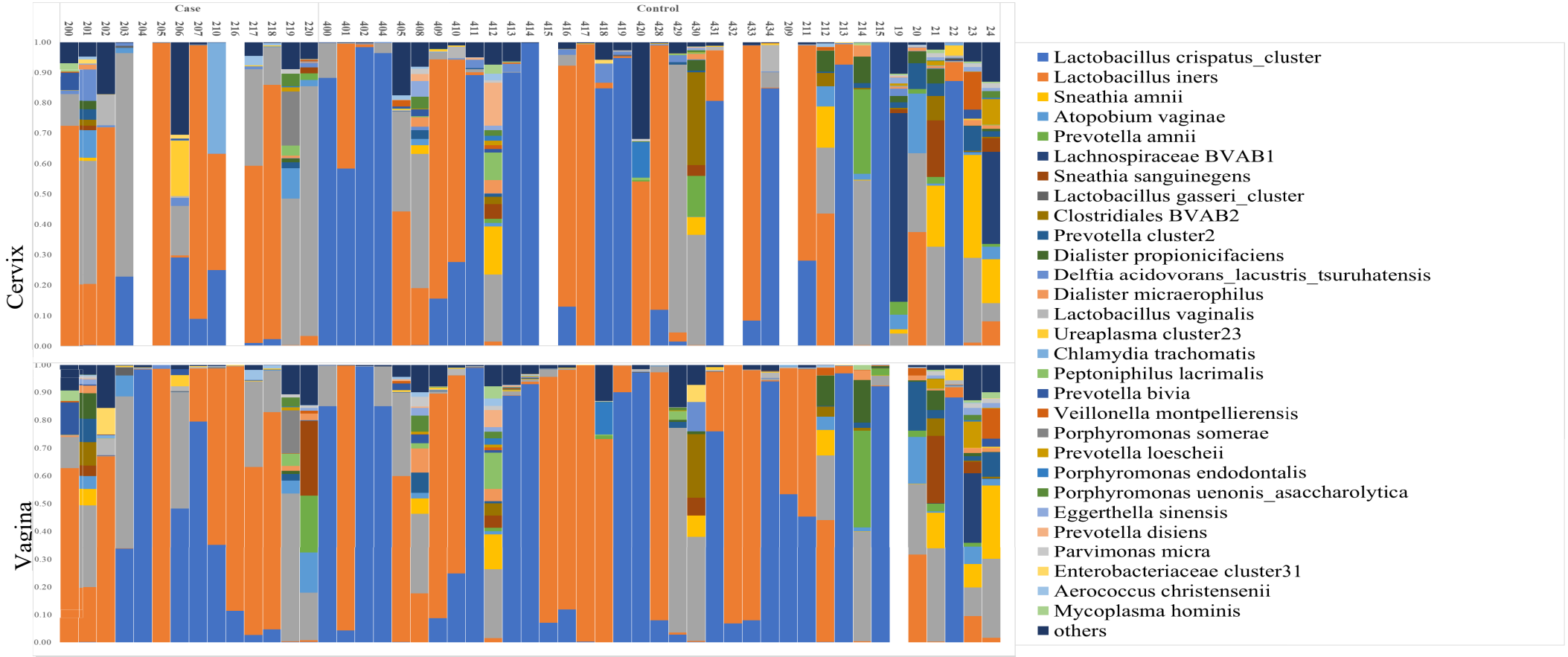


Figure 3.4. Stacked bar graph showing relative abundance for the top 30 species. The x-axis displays each individual participant with the left y-axis showing the two sampling sites. The left y-axis displays the relative abundance score from 0 to 1. The coloured legend at the right shows the colour code used to distinguishing of the different species from most abundant (top left) to least abundant (bottom right). Top is sample from cervix bottom is from vagina

The relative abundance of the top 5 species and *Prevotella species* (all *Prevotella species* were combined into one group) were compared per groups using the Mann-Whitney pairwise analysis. Table 3.17 displays the median, range, and mean, relative abundance of each of the taxonomic profile per group. Table 3.17 displays the p-values comparing the relative abundance of selected high abundant species in each of the participant groups. *L. crispatus* was significantly more abundant in controls compared to cases and test group. *S. amnii* was significantly more abundant in the test group when compared to case and control groups. *A. vaginae* was also significantly more abundant in the test group when compared to case and controls. Finally, the *Prevotella* genus was significantly more abundant in the test group compared to case and controls.

Table 3.17. Relative abundance of top 5 species and *Prevotella spp*. in each group

Taxa/Microbial community profile	**Control (n=31)** relative abundance (min, max), mean	**Cases (n=14)** relative abundance and distribution, mean	**Test group (n=6)** relative abundance and distribution, mean	**p-value[a]**		
				Case/Control	**Case/Control**	**Case/Control**
L. crispatus	(0-10169), 116.3	(0-1753), 13.5	(0-4562), 37.3	0.018	0.018	0.018
L. iners	(0-7606), 80.1	(0-8431), 126.4	(0-3632), 39.0	0.398	0.398	0.398
G. vaginalis	(0-2598), 12.9	(0-6162), 34.4	(0-2674), 42.3	0.067	0.067	0.067
S. amnii	(0-563), 5.4	(0-19) , 0.1	(0-3618), 110.0	0.395	0.395	0.395
A. vaginae	(0-290), 1.8	(0-412), 7.1	(0-1974), 51.2	0.162	0.162	0.162
Prevotella spp.	(0-1313), 1.4	(0 -538), 1.2	(0-651), 8.7	0.558	0.558	0.558

a: Man-Whitney pariwise comparison test, significance is where p-value <0.05

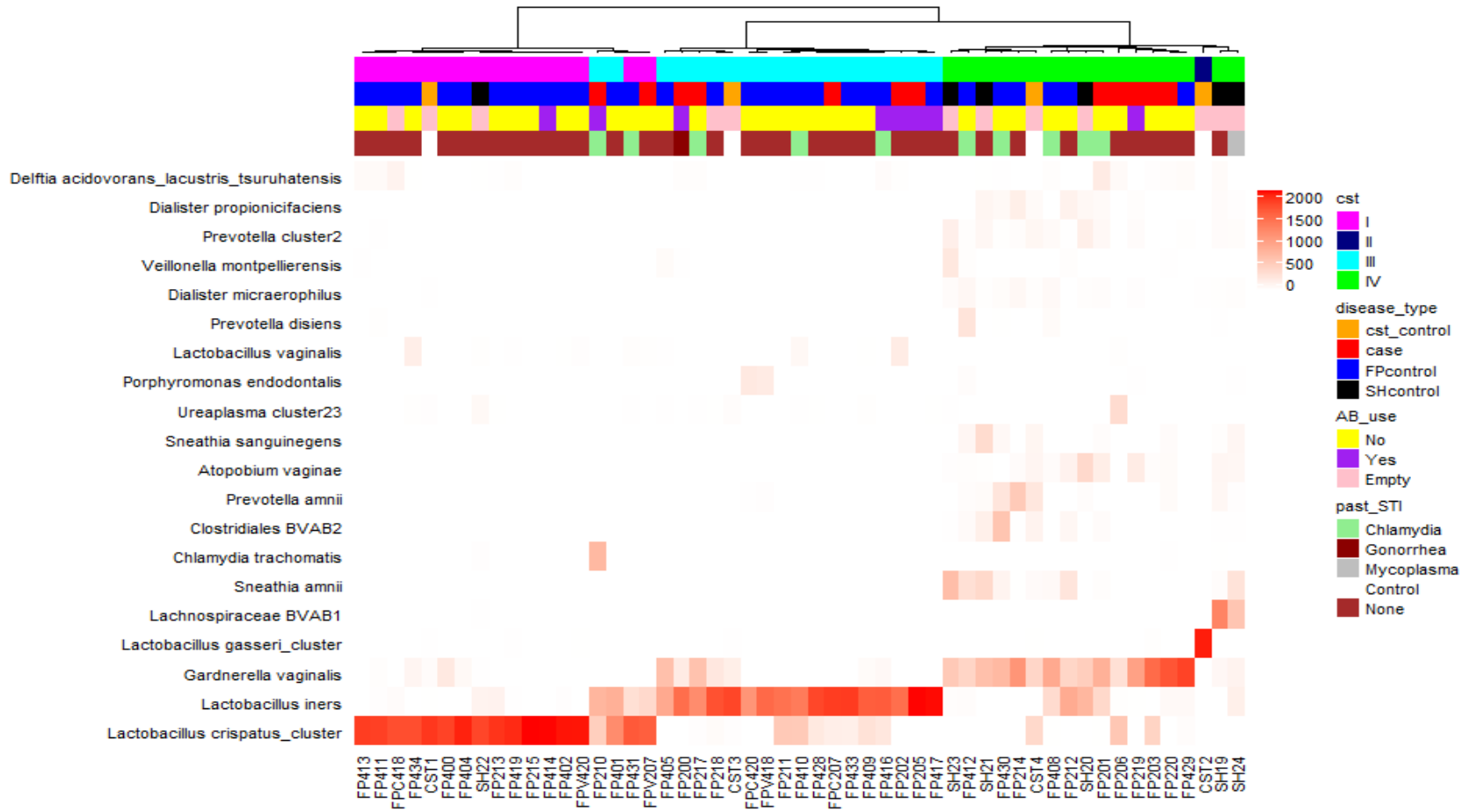


Figure 3.5. Cluster linkage dendrogram heat map of top 20 most abundant species in participants. The relative abundance scale (on top right) shows reads from 0 to 2000 reads (samples were rarefied to 2000 reads), and colour scale is white to red (red is highest relative abundance and white lowest). The heat map is arranged by a column cluster linkage dendrogram followed by annotation with corresponding legends on the top and right respectively. The annotations label samples by community state type (Pink = CST I, Navy = CST II, light blue = CST III, Green = CST IV); if they are a case (red), control (FPcontrol = Blue) or internal experimental control (cst_control =organe), test group (SHcontrol = black), their past sexually transmitted infections (Chlamydia = light green, gonorrhoea = dark brown, Mycoplasma = grey, control = white, none = light brown) and past antibiotic use (AB), no = yellow, yes = purple, empty = pink. vaginal samples reads were merged by addition of total read counts.

Whilst the most abundant species are likely to have significant functional impacts on the ecology and related disease outcomes, the vaginal microflora have also been classified into clusters termed the community state types (CST) (as reviewed in Chapter1). From the 14 cases mapped; 50% were found to have a microbiota composition consistent with CST III, 29% CST IV, and 21% CST I (Table 3.19). The controls were found to have a broader distribution, with 38% CST I, 32% CST III, and 30% CST IV. No samples were found to have a microbiota composition consistent with CST II. The test group was mostly clustered into CST IV, 80% of participants that were successfully amplified and sequenced, while only 20% were found to have compositions consistent with CST I.

Table 3.18. Proportions of microbial compositions of community state type

Dominated	**Community state type**	**Case % (n)**	**Control % (n)**	**Test Group % (n)**
L. crispatus	I	21 % (2)	38 % (12)	17% (1)
L. gasseri	II	0 % (0)	0% (0)	0% (0)
L. iners	III	50 % (7)	32 % (10)	0% (0)
No dominant species	IV	29 % (5)	30 % (9)	83% (5)

3.3.4. Quantitative PCR analysis demonstrates that the cases had lower levels of *L. crispatus*

The microbiota analysis using 16S rRNA gene amplicon sequencing provides only relative abundance based on the performance of that particular PCR on that particular sample. Therefore, species-specific qPCR was used to quantify the absolute abundance of a selected number of species of interest. It is important to note that not every participant had the specific organism detectable in their samples (note, all PID (n=14) and control samples were tested (n=31) and only cervical samples tested). As shown in Figure 3.6, panel A, *L. crispatus* was found in higher abundance in controls compared to cases (p-value <0.0001). It was also found that *A. vaginae* and *Prevotella spp* were in higher copies in the cases compared to controls (Figure 3.6, panel C and F, p-values = 0.0002 and 0.0239, respectively). Figure 3.7. shows the bacterial gene copy number by differing PID cause (idiopathic (n=9), instrumentation (IUD) (n=2), STI (n=3)). While there was no significant difference across bacterial copy number and PID cause, interestingly, two species stand out. *L. iners* is in higher copies in those with idiopathic and STI associated PID than instrumentation (IUD) associated PID. Furthermore, *Prevotella spp.*in Figure 3.7 are in higher copies in STI associated PID than the other two categories of PID (note, due to low numbers this was not able to be statistically analysed).

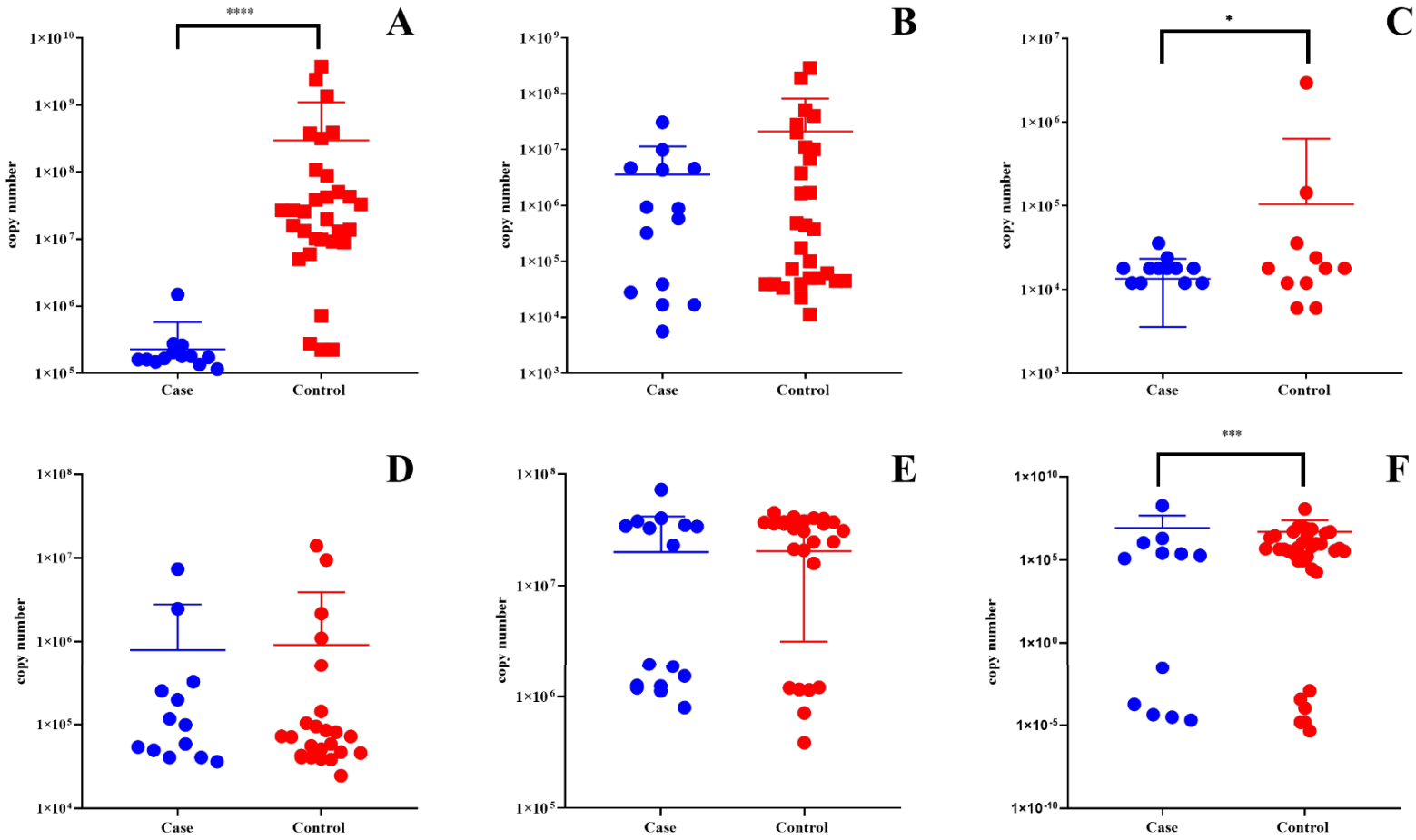


Figure 3.6 Quantitative real-time PCR to establish quantities of selected bacterial species. Cases are indicated by blue dots, and Controls are indicated by red dots for all panels. The y-axis is measured in copy number (of target gene) per sample using absolute abundance determined with a standard curve. The plots shown are scatter plots with the mean and standard deviation shown indicated by the lines. Panel A shows the results for *L. crispatus.* Panel B shows the results for *L.iners*. Panel C shows the results for *A. vaginae.* Panel D shows the result for *G. vaginalis*. Panel E shows the result for *M. genitalium.* Finally Panel F shows the result for *Prevotella spp.* Significance was calculated using non-parametric Man-Whitney test. ****: p-value <0.0001, ***: p-value = 0.0002, *: p-value = 0.0239

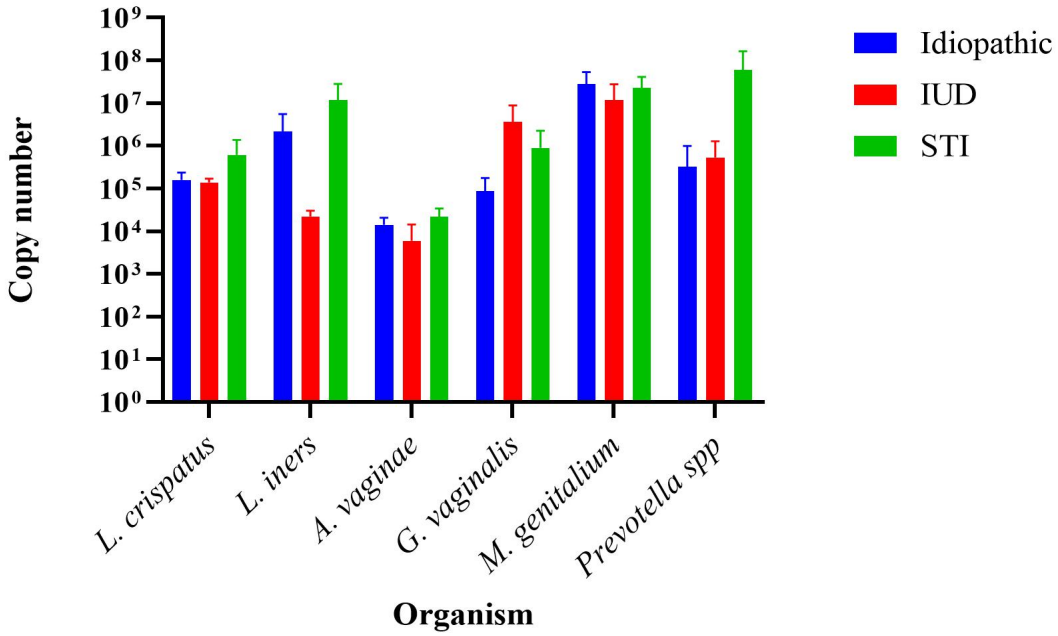


Figure 3.7. Quantified bacterial chromosome copies according to PID aetiology. Based on the results of the quantitative species specific PCR in Figure 3.6, the bacterial gene copy number is displayed by category of PID (Idiopathic, instrumentation (IUD) or STI). The x-axis is the organisms category and y-axis the copy number found at the cervix. The bars represent the mean with the error bars being standard error of mean. The PID cause are coloured; blue for idiopathic, red for Instrumentation(IUD caused), green for STI caused.

3.4. DISCUSSION

There are many studies on PID using Nugent score and bacterial species-specific PCR or culture methodologies; however, this is the first PID study to our knowledge that uses 16S rRNA gene amplicon sequencing analysis complemented by species-specific quantitative PCR analysis. This is advantageous as it allows for absolute abundance to be

calculated for specific organisms. Overall, whilst a pilot study, the results suggest that women experiencing PID were more likely to have a cervicovaginal microbiome community state types consistent with CST III (dominated by *L. iners*). This was also validated by species specific PCR where women with PID had a lower abundance of *L. crispatus* and a higher abundance of *A. vaginae* and *Prevotella spp.* The other organisms of interest were *L. iners, G. vaginalis* and *M. genitalium* and they were not shown to be significantly different between case – control groups

3.4.1. PID cases were found to have a CST III and IV vaginal microbiome

A study of salpingitis (an inflammatory condition, that may also lead to PID) elucidated several organisms that were associated with the condition, including *Prevotella spp*, *Peptrostreptococcus*, *Streptococcus pyogenes,* and *Leptotricha* spp [283]. All these genera can be found in high relative abundance in CST IV [162]. This raises the question as to whether a disruption of the vaginal flora is one factor that leads to the onset of PID or has occurred as a consequence of PID. We have only taken a single sample so we cannot determine the relative timeframes of PID onset, and any microbiota composition changes. Bacterial vaginosis (BV) is associated with an absence of *Lactobacilli* and presence of Gram negative microbes in the vagina (some consider this to be dysbiosis of the microbial flora) and BV has also been associated with endometritis, salpingitis, and oviduct damage [60, 61, 261, 284, 285]. However, it has been shown that BV is not a specific causative agent of PID [227]. Table 3.22 shows that the majority of PID cases in this study had cervicovaginal microbial community compositions that were lacking in obligate aerobic acid-producing organisms, which raises the question of

whether certain BV-associated bacterial and/or strains or absence of dominant *Lactobacilli* have a role in the onset of PID?

In this study, the Gram-positive anaerobe *A. vaginae* was present in significantly higher abundance in PID cases when compared to controls (Figure 3.6). While this bacterium has been associated with endometritis and tubal factor infertility, it was not investigated alone [286, 287]. In a study, that grouped together *S. sanguinegens, S. amnionii, A. vaginae* and *L. BVAB1*, they found that women that were positive for all these bacterial species (using PCR) had an adjusted risk ratio of 3.9 (95% CI, 1.9 – 8.2) for recurrent PID when compared to women who tested negative for these bacteria [167]. This brings up the relevance of the test group who can be classified as at a high risk of developing PID. This group is classified at high risk for developing PID, being at a sexual health clinic implies highly sexually active young people which also fits the risk criteria for PID (Chapter 1). In a clinical research study [202], it has been found that people who have more than one positive (detected *C. trachomatis* or *N. gonorrhoea*) STI check are at increased risk of PID, and that being a sexual health clinic where patients are at high risk for recurrent positive STI results raises the probability of pre-PID or sub-clinical PID recruitment. In this test group, there were one participant whose cervicovaginal microbiota was consistent with CST I and five participants had a microbiota composition consistent with CST IV (typically dominated by anaerobes and BV-associated bacterium).

Interestingly, the *Prevotella spp* qPCR (unable to distinguish between different species within the genus) displayed significantly higher copy numbers in PID cases

compared to control (Figure 3.6). This particular genus has been associated with PID using culture-dependent methods, where it was proposed that *P. bivia* associated with BV caused PID [66, 288, 289]. However, there were 10 (10/31) control and 5 (5/14) case participants clustering to CST IV (no dominant *Lactobacillus*); leads me to the question of whether a shift in the concentration of a single strain or substrain of BV-associated organism is more associated with PID, rather than the CST? Adding to this speculation, could the absence of *L. crispatus* be correlated with the expansion of BV-related species (*A. vaginae, Prevotella spp, G. vaginalis, etc.*), whereby one of these species is capable of colonising and extending PID influences, and in effect, this is affected by host conditions, chance or a number of specific organisms that can induce PID, but only in susceptible hosts?

Due to storage buffer used, *N. gonorrhoeae* and *M. genitalium* would have most likely not survived storage as it is not the recommended buffer to store these organisms (STI classified PID). For future studies, one collection sample should include 10-15% glycerol as the swab storage buffer [290]. Uncertainty lies on whether more STI caused PID cases will be recruited; however, if this is the case, attempted isolation, storage and DNA extraction will be conducted on all recruited to conduct whole-genome sequencing. It is vital to consider the different storage condition for different STI's, especially the fastidious organisms. Whilst the original hypothesis was that women with PID will be lacking *Lactobacillus spp,* the results suggest that women with PID have less abundant *Lactobacillus spp.* Hence, resulting in a future hypothesis to test, that women with PID have no abundant *Lactobacillus spp* or have a microbiome consistent with CST III.

3.4.2. Limitations

Using 16s rRNA gene sequencing to elucidate the vaginal flora has its limitations. Firstly we chose to amplify the variable region V3 – V4 due to important vaginal bacterial species (*C. trachomatis, G. vaginalis,* and *Bifidobacterium bifidum*) being more clearly differentiated by sequencing this region compared to V1 – V3 [291]. Full length 16S rRNA gene amplicons combined may have been able to resolve more bacterial species but would be more costly. Future studies should consider nanopore technology which will be able to resolve full-length 16S rRNA gene and can be quantitative as published in the following reference [292]. There is the issue of different bacteria having varying copies of the 16S rRNA gene; hence, sequencing could favour one bacterial species compared to another, also the PCR conditions and primer sequences may preferentially amplify genes from certain bacteria. An important drawback is that 16S data is not objective, the data examined is compositional, and when one species abundance increases or falls, the relative abundance of other species shifts [293]. To provide quantitative data, a PCR was used for selected bacterial species, and as can be seen, significant differences in abundance was detected. As 16S is only a taxonomic classification and does not provide genomic information that could allow for functional classification of microbial composition or specific genetic traits of the species and strains present (as would be demonstrated by metagenomics) the functional impacts of these differences remains unknown. Furthermore, 16S rRNA gene amplicon sequencing doesn't take into account the presence of a virome or fungi/yeast, which may also have importance for PID.

Specification of the amplified V3-V4 region was carried out with STIRRUPS which is the only current classification software trained on a vaginal 16S rRNA gene database. However, the database contains only V1-V3 reads encompassing only the forward read from the amplicons used here. As this is a limitation, we used our positive controls (community state types) to provide a comparison with a known composition of species.

While less DNA yield was collected from the cervical swab (five cervical samples were filtered out due to low quality reads from low DNA yield), it is still comparable with the vaginal swabs and other studies, such as that as described by Virtanen *et al.* [294]. Cervical swabs are uncomfortable, and thus less time is spent swabbing the surface to reduce pain to the participant. Moreover, these swabs are collected in addition to standard clinical procedures just for this study; hence, pain and discomfort is already high. In addition, there may be reduced proliferation of bacterial populations or more inhibitors on the cervical surface that may need a specific swabbing process. The DNA extraction protocol has been optimised for vaginal swabs, consequently biasing vaginal swabs over cervical swabs. It also has been shown that cervical bacterial loads are lower when compared to vaginal site [279]. While different mucosal sites in the gut have been shown to have different bacteria [295], it has been challenging to work with the cervical site, whether it be the endocervix or ectocervical epithelium due to the high chance of contamination through sample collection due to anatomical proximity.

The question addressing past sexual partners (Chapter 2) is another limitation, as it was different between the FPNSW clinics and Cairns clinic. Furthermore, geographic

locations may influence the type of people that are used in this study. Cairns is in the tropics, whereby the environment is humid and parasite's such as *Trichomonas vaginalis* are more prevalent [296]. This parasite also thought to be associated PID, was not tested for, and is likely to be rare in the Penrith (Sydney) and Newcastle (NSW) clinics. Hence, geo-environmental differences may impact the results. Furthermore, there may be a difference in the population that attend these clinics. As the Penrith clinic is within an area proximal to high density multicultural population compared to Newcastle and Cairns [297].

3.4.3. Conclusion

In summary, with limitations accounted for, the cervicovaginal microbial composition was found to be variable across women regardless of disease status. Overall the recruitment protocol and specimen collection and processing methods worked and produced usable quality data that further supports that the pilot study has demonstrated the feasibility of the protocol. In spite of the study only being a pilot sample size, quantitative PCR confirmed that a higher cervical bacterial load of *A. vaginae* and *Prevotella spp.* was associated with PID. However, the ecological functionality of different organisms need to be taken into account in the future with a larger cohort. This includes the ability to understand the role of different *Lactobacillus* species and how they interact with the host and surrounding bacterium. Furthermore, the development of species-specific *Prevotella* PCR targets may shed light on the role of the different species within the *Prevotella* genus. Similarly, a strain or sub-strain specific PCR, or metagenomics approach, could also enlighten the association of the bacterium tested

above in PID. With the possibility of antibiotics altering the vaginal microbiome that could lead to disease (as previous antibiotic use was found to correlate in the demographic data in Chapter 2), more needs to be done to understand the relationship of disease, vaginal microbiome, and factors that influence the flora within the vagina. The interaction between the cervicovaginal microbiome and reproductive tract disease is still yet to be fully understood. Currently, the treatment for PID does not consider the protective vaginal flora that may help against acquiring PID or could be important to prevent further complications.

Chapter 4 - Cervical host gene expression associated with pelvic inflammatory disease

4.1. INTRODUCTION

4.1.1. The immune system in the female genital tract

The innate and adaptive immune response are vital in protection against pathogens. These two arms of protection are present in the body and at mucosal surfaces can have unique features, such as the respiratory system [298] which has the ability to contain mucosal markers, secretory immunoglobulins (Igs) and tissue-resident innate lymphocytes (common to all mucosal surfaces) [299]. The adaptive immune system is responsible for the identification of infections and the response to antigens using T and B cells (white blood cells). This adaptive response includes the generation of antibodies and the ability to recognise re-infection. The endogenous innate immune mechanism is a basic reaction to pathogen detection. An example of a reaction to pathogen detection is the release of antimicrobial peptides (AMPs) from the mucosal membrane [300].

While the female reproductive tract shares similar immunological defence's with other mucosal systems, it has unique features that are only found in the reproductive tract. This includes the ability of a foetus not to be detected by the host defence mechanisms. Furthermore, the composition of the cervicovaginal microbiome, sex hormones (especially the menstrual cycle), and the host defence systems all interact in a dynamic and complex system, as shown in Figure 4.1 [301]. The female reproductive tract can be divided into the lower reproductive tract containing the vagina and ectocervix made up of multiple layers of squamous epithelia (with parts having a monolayer) in tight junctions allowing for molecule secretion and uptake [302]. In contrast, the upper tract consists of

the uterus and fallopian tubes made up of a layer of columnar epithelial cells that are tightly bound [302]. It is this transition zone around the endocervix, where cells change from squamous to columnar that is thought to be a significant effector and inductor site to the immune system [302].

The innate immune system secrets a large number of antimicrobial peptides from the cervix. These antimicrobial peptides include the secretion of secretory leukocyte protease inhibitor (SLPI), lysozyme, lactoferrin, elafin, and cathelicidin from neutrophils and epithelial cells [303]. In addition, the innate system produces interferons (INFs) which has been shown to play a part in defence against Human Immune deficiency Virus (HIV) [304].

Toll-like receptors (TLRs) 1-9, which are in high abundance in the female genital tract, dendritic cells and macrophages, have also been identified to monitor mucous membranes for pathogen antigens [305-307]. Neutrophils and natural killer cells, components of the innate immune system, are also found throughout the reproductive tract. Both of these are influenced throughout the menses cycle and found in higher concentrations in the upper genital tract [308-310]. The majority of these studies have emerged from analysis of the pathogenesis of HIV, or some studies that monitored the stages of pregnancy from coitus to foetus implantation. Hence, there are many gaps in knowledge about other gynaecological conditions such as PID and associated immune responses.

The adaptive immune system also has unique features within the female reproductive tract. The endometrium contains organised lymphoid aggregates surrounded by T cells [311]. While the ability of local humoral immunity to be activated remains unclear, there has been the detection of immunoglobulin (Ig) type A in the reproductive tract [312], with the presence of weak signals of IgA and IgG after infection of *N. gonorrhoeae, C. trachomatis* and HIV-1 [313]. The majority of T cells and B cells are found amongst the cells of the endocervix when compared to the ectocervical and vaginal cells [314].

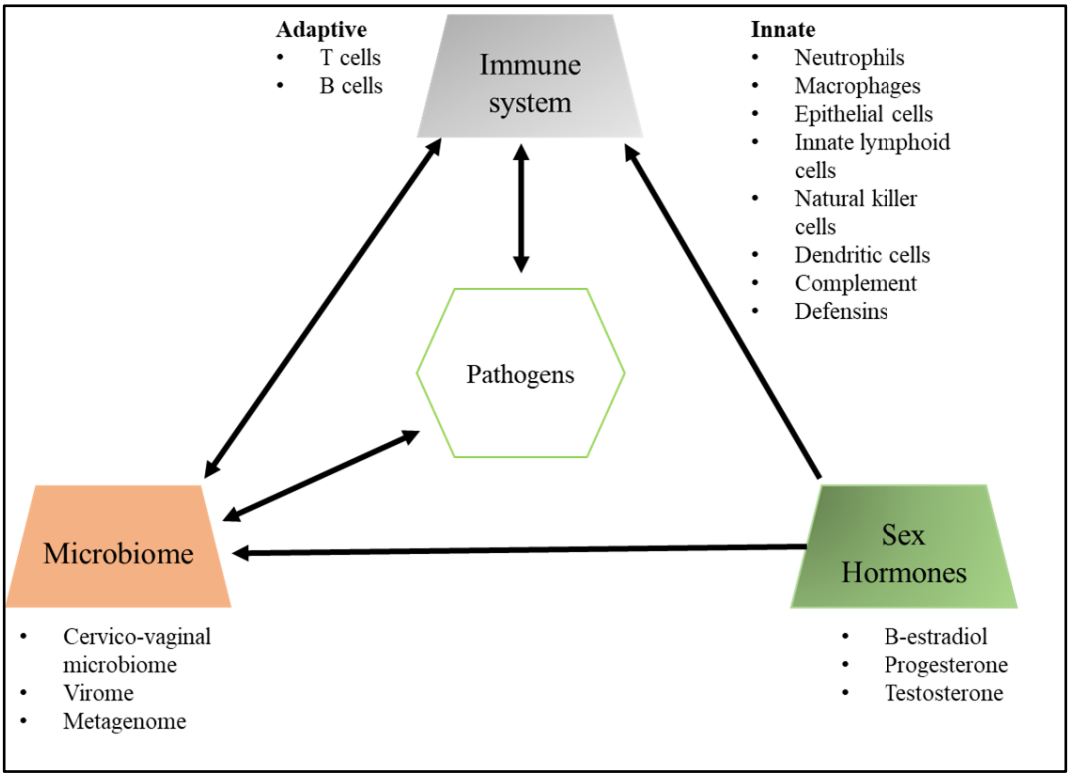


Figure 4.1. The unique female reproductive tract interactions with host defence, microbiome and sex hormones. Adapted from [5]. This entails that the vaginal ecosystem which includes, the microbiome, the virome, and the metagenome interacts

with the immune system based on the organism that reside amongst the cells. In turn the host response can influence the constituents of the ecosystem. This ecosystem also fluctuates due to sex hormones, an example is where diversity in the microbiome is shown to decrease in pregnancy. Sex hormones as described above can also affect the host response, whereas in pregnancy the foetus is protected from the host response. Finally, pathogens affect the immune system by creating a host response, in turning effecting the pathogen by providing host defence. The microbiome affects pathogens as it can outcompete the growth niche of the pathogen. However, an opportunistic pathogen can arise from the cervicovaginal ecosystem.

4.1.2. Pelvic Inflammatory disease as an immune condition

Pelvic inflammatory disease (PID) is an inflammatory condition of the female genital tract, initially classified by histological correlates of inflammation as defined by white cervical discharge, blood cells in the epithelial tissues, and elevated white blood cells [315-317]. Historically, laparoscopy and histology of endometrial biopsies were used to diagnose the endometritis, salpingitis, and PID. In the early 1940s, culdoscopy was used and visualisation of the fallopian tubes and ovaries were used to identify signs of inflammation (swelling, redness etc) [318]. In the 1960s laparoscopy was used to diagnose PID, this process involved visualisation of the entire reproductive tract, to identify signs of inflammation [9]. Furthermore, endometritis (inflammation of the endometrium) is thought to be the first step to PID and not entirely a different condition [319]. Hence, inflammation is the defining feature of PID.

More recently, the Centre for Disease Control (CDC, USA), as part of their diagnostic guidelines for the definitive confirmation of PID, use standard inflammation markers such as a fever of >38.3 ºC [320]. The CDC further suggest that a definitive diagnosis of PID includes elevated erythrocyte sedimentation rate (> 15 mm/hr), elevated C- reactive protein and elevated white blood cell count (>10,000 cells/mL) [320, 321]. These are standard markers of inflammatory response for the majority of human illnesses and are conducted through a blood test. In Australia, it is not routine to collect blood for suspected PID cases (diagnostic methods discussed in Chapter 1).

4.1.3. Gene expression during PID

The gene expression profile from an immunological and host response perspective has not been the focus of many previous investigations in PID. To date, there have only been a few studies conducted on the host response of women with PID. In a study of three different cohorts, two cohorts had women with confirmed PID and the last cohort had asymptomatic women at high risk of an STI, whole blood was collected to use in micro-array profiling of mRNA [177]. This research focused on STI induced PID, where PID was characterised as "when sexually transmitted pathogens transfer from the cervix to the uterus and oviducts, resulting in endometritis and salpingitis [177]" overlooking that the majority of PID is idiopathic with no established pathogen. This research identified that there were 21 genes listed in Table 4.1 that can be used to identify STI induced PID, where blood is collected.

Table 4.1. Whole blood gene microarray used to classify STI induced PID

	Gene[a]	Function[b]	Adjusted p-value	p-value	Fold change
Upregulated	AIM2	Tumorigenic reversion and regulates cell proliferation	1.31E-05	1.32E-08	2.06
	DSC2	Interactions of plague proteins and mediating cell-cell adhesion	1.65E-05	3.02E-08	2.3
	SIGLEC5	Mediates sialic-acid dependent binding	1.65E-05	2.81E-08	1.97
	CASP1	Protease involved variety of inflammatory processes	1.87E-05	6.99E-08	1.58
	CD58	Ligand of the T-lymphocyte CD2	1.87E-05	5.34E-08	1.53
	GBP2	Hydrolyses GTP to GMP	1.87E-05	7.90E-08	1.47
	RNF19B	ligase with a role in the cytotoxic effects of natural killer (NK) cells	1.87E-05	8.15E-08	1.44
	LIN7A	Establishes and maintains distribution of membranr channels and receptors	4.47E-05	5.87E-07	1.6
	ADM	Preprohormone involved in vasodilation and hypotension	4.83E-05	7.22E-07	2.48
	NAMPT	Intermediate protein in the biosynthesis of NAD	5.85E-05	1.08E-06	2.09
	LOC440731	Unknown function	6.76E-05	1.48E-06	1.61
	CYB5R4	Endoplasmic reticulum stress response	6.88E-05	1.56E-06	1.46
	PLAUR	Receptor for urokinase plasminogen activator	9.56E-05	2.93E-06	1.56
	FAS	Regulation of programmed cell death	0.00022	1.30E-05	1.39
	LYSMD2	Unknown function	0.000698	8.78E-05	1.4
	RPS15A	Structure of ribosome	0.009756	0.0032	1.35
Down-regulated	FAIM3	Protects cells from FAS-, TNF alpha- and FADD-induced apoptosis	1.87E-05	8.15E-08	−1.89
	CD79A	Initiation of the signal transduction cascade	3.33E-05	3.29E-07	−1.96
	TCL1A	Enhances cell proliferation and survival, stabilises mitochondrial membrane potential	6.67E-05	1.41E-06	−1.85
	EEF1D	Subunit of the elongation factor-1 complex	0.000134	5.55E-06	−1.54
	TSPAN3	Regulates the proliferation and migration of oligodendrocytes	0.001352	0.00025	−1.35

a: reproduced from [177], b: functions retrieved from GeneCards suite[322]

In another study by the same group, two cohorts were recruited and biospecimens were analysed using whole blood microarray analysis. The first cohort were women with PID and the second cohort were women with asymptomatic *C. trachomatis* infection [239]. The aim was to determine whether there is a difference in host response between women with STI and no PID and those with STI and PID. It was determined that there was an overexpression of myeloid cell and IFN genes with suppression of T cells in women with STI induced PID [239]. The host response was found to be similar in women with STI induced PID compared to those with an STI infection without PID [239]. The top differentially expressed pathways without displaying genes involved (US patent application 15/304,836) included downregulation in neutrophil and monocyte signalling and upregulation in T cell signalling, protein synthesis and mitochondrial respiration. However, this was conducted using whole blood which is not a common biological specimen to collect in most general practice settings during a suspected PID consultation. This research group also conducted endometrial pipelle biopsy (a painful process) and tested for two inflammatory genes (IFNβ and CXCL-1) using *in situ* hybridisation staining showing that women with STI induced PID had the mRNA gene transcripts detected within the endometrial tissues [239].

In a cross-sectional study of adolescent females presenting in the emergency department for abdominal pain and diagnosed with PID, a blood sample was collected for analysis [178]. A microarray was used on extracted RNA from blood cells to determine significant genes that are differentially expressed in PID compared to the asymptomatic controls. Only nine controls and nine PID cases were compared and 170 genes were detected as being differentially expressed. While the authors [178] don't list the 170

differentially expressed genes (patent application is pending), they provide the names of the dysregulated (up and down regulated) pathways using network analysis. Those of interest were genes involved in cytokine signalling, immune cells signalling, transcription factors and those involved with anion exchange [178]. Once again, this study utilised whole blood, and this study only concentrated on inpatient setting and not outpatient, where the majority of Australian women get treated for PID. The study may be limited by their definition of PID, as although in the introduction they state that PID is thought to be polymicrobial, the authors later acknowledge the focus within their study definition "the diagnosis of PID constitutes a sexually transmitted infection" [178].

4.1.4. Knowledge gaps in the host response in PID

The three studies above indicate that at least in blood cells and potentially the endometrium that there is likely to be a PID specific gene expression profile. However, as they are all a relatively small samples size, and only one study has looked at the reproductive tract, there remains a knowledge gap on what the immune response or even more broad gene expression profile at the cervix is in women with PID. This represents important new knowledge and also an opportunity for the potential to develop a new diagnostic based on gene expression profiles. Hence, this chapter sought to address these knowledge gaps, by examining gene expression at the cervix in women with PID compared to controls. Limitations in the current knowledge we would like to address include, the inclusion of a more conventional bio-specimen collection consistent with PID diagnostic samples already collected (e. g. cervical swabs). Furthermore, these studies have not included idiopathic PID.

This study aimed to determine whether cervical expression of host immune genes are significantly different in women experiencing PID and aimed to profile the expression of a selection of immune genes in women with PID, using a case-control study design (the same women as in Chapter 2). Specifically, this work aimed to:

1. Profile the cervical innate and adaptive gene expression of the cases and controls to test for factors significantly associated with PID.

2. Analyse the cervical innate and adaptive gene expression profile of Cases by the type of PID (e.g. idiopathic, STI, instrumentation (IUD)).

3. Analyse the cervical innate and adaptive gene expression profile of women in Case and Control groups and by microbial community state types detected in the microbiome analysis.

4.2. MATERIALS AND METHODS

4.2.1. RNA extraction

Swab biospecimens from the cervix (collected method in Chapter 2.2.2) were thawed to room temperature and vortexed (30 seconds) before removing the breakpoint swab. Using the Qiagen RNeasy Plus mini kit (Qiagen, Cat No. 74134, Sydney) manufacturer's instructions were followed to extract total RNA. Before prepping the lysate, 20 µL of two millimolar of 1,4-Dithiothreitol (DTT) (Sigma Cat No. 10197777001, Sydney) was added to 1 mL of RLT buffer in order to denature endogenous ribonuclease (RNase) within the lysate. The cervical sample was then placed on dry ice for five minutes and then thawed to degrade the mucous that is typical in cervical samples. The sample was then divided into two tubes contain 500 µL each, where 350 µL of the RLT buffer containing DTT was added and vortexed for 30 seconds. The lysate was then transferred to a gDNA elimination column and centrifuged for 30 seconds at 8000 × g. 70% ethanol (Sigma Cat No. E7023, Sydney) was added to the lysate to precipitate the RNA. The extraction of the RNA was commenced with 700 µL of the lysate centrifuged in the RNeasy column for 15 seconds at 8000 × g, where this was repeated until all the lysate has been used. After the flow-through was discarded, the RNA that was bound to the column is centrifuged with 700 µL of RW1 buffer for 15 seconds at 8000 × g. The RNA that was bound to the column was centrifuged with 500 µL of RPE (wash) buffer for 15 seconds at 8000 × g; this was then repeated for two minutes at 8000 × g. The RNA was eluted using 50 µL of ultrapure RNase and DNase free water (Invitrogen Cat No. 10977023, Sydney), where RNA and water was incubated for 2 minutes at 25°C, followed by centrifugation at 10000 × g for one minute.

Quality control and assurance process included the preparation and consistent use of internal standards and negative controls. These controls included triplicate no template controls, cDNA synthesis control (reverse transcription control), Human genomic DNA contamination control (if detectable sample was not used) and a PCR amplification control. Two genomic DNA removal procedures were conducted per sample and one sample was analysed per day to eliminate cross-sample contamination.

4.2.2. Complementary DNA (cDNA) synthesis

The total RNA concentration was measured using a Nanodrop One Micro-UV/Vis spectrophotometer (Thermo scientific Cat No. 269-342200, Wilmington, USA) before cDNA synthesis. 70 ng of RNA was added to the cDNA reaction. The RT^2 first strand kit (Qiagen Cat No. 330404, Sydney) was used for the synthesis which included another genomic DNA elimination step. This included adding two µL of buffer GE, ultrapure water and RNA to total 10 µL which was then incubated at 42°C for five minutes followed by a rapid ice-cooling for one minute. The reverse transcription mix was prepared by adding four µL of buffer BC3, one µL of internal control P2, two µL of RE3 reverse transcriptase mix, three µL of ultrapure water and the genomic DNA elimination mix containing the RNA sample. The mixture was incubated at 42°C for 15 minutes and then 95°C for five minutes, followed by addition of 91 µL of ultrapure water.

4.2.3. Gene expression analysis using RT-qPCR

In order to evaluate the participant cDNA for relative expression of 84 genes compared to the five housekeeping genes (Table 4.2), Qiagen's RT2 Profiler Array for Human Inner and Adaptive Immune Reactions (100 well-preloaded rings) (Qiagen Cat No. 330231, configuration PAHS-052ZR-24, Australia) was used. 102 µL of cDNA was mixed with 1150 µL of RT^2 SYBR green ROX fast master mix (Qiagen Cat No. 330623, Australia) and 1048 µL of RNase free water (Invitrogen Cat No. 10977023, Sydney). The Qiagen QIAgility (Cat No. 9001904, Germany) was used to load samples in the preloaded wells of the profiler array. The profiler arrays were then sealed with sealing film, and real-time PCR run on the Rotorgene Q MDx 5plex HRM (Qiagen, Germany) under the conditions outlined in Table 4.3, where fluorescence was acquired.

Table 4.2. Genes used in RT-qPCR array

Position	UniGene	GenBank	Symbol	Description
A01	Hs.507080	NM_001639	APCS	Amyloid P component, serum
A02	Hs.529053	NM_000064	C3	Complement component 3
A03	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
A04	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2
A05	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
A06	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4
A07	Hs.450802	NM_000579	CCR5	Chemokine (C-C motif) receptor 5
A08	Hs.46468	NM_004367	CCR6	Chemokine (C-C motif) receptor 6
A09	Hs.113222	NM_005201	CCR8	Chemokine (C-C motif) receptor 8
A10	Hs.163867	NM_000591	CD14	CD14 molecule
A11	Hs.631659	NM_000616	CD4	CD4 molecule
A12	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5
B01	Hs.592244	NM_000074	CD40LG	CD40 ligand
B02	Hs.838	NM_005191	CD80	CD80 molecule
B03	Hs.171182	NM_006889	CD86	CD86 molecule

B04	Hs.85258	NM_001768	CD8A	CD8a molecule
B05	Hs.709456	NM_000567	CRP	C-reactive protein, pentraxin-related
B06	Hs.1349	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
B07	Hs.632586	NM_001565	CXCL10	Chemokine (C-X-C motif) ligand 10
B08	Hs.198252	NM_001504	CXCR3	Chemokine (C-X-C motif) receptor 3
B09	Hs.190622	NM_014314	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
B10	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
B11	Hs.247700	NM_014009	FOXP3	Forkhead box P3
B12	Hs.524134	NM_002051	GATA3	GATA binding protein 3
C01	Hs.181244	NM_002116	HLA-A	Major histocompatibility complex, class I, A
C02	Hs.650174	NM_005516	HLA-E	Major histocompatibility complex, class I, E
C03	Hs.643447	NM_000201	ICAM1	Intercellular adhesion molecule 1
C04	Hs.37026	NM_024013	IFNA1	Interferon, alpha 1
C05	Hs.529400	NM_000629	IFNAR1	Interferon (alpha, beta and omega) receptor 1
C06	Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast
C07	Hs.856	NM_000619	IFNG	Interferon, gamma
C08	Hs.520414	NM_000416	IFNGR1	Interferon gamma receptor 1
C09	Hs.193717	NM_000572	IL10	Interleukin 10
C10	Hs.845	NM_002188	IL13	Interleukin 13
C11	Hs.41724	NM_002190	IL17A	Interleukin 17A
C12	Hs.83077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)
D01	Hs.1722	NM_000575	IL1A	Interleukin 1, alpha
D02	Hs.126256	NM_000576	IL1B	Interleukin 1, beta
D03	Hs.701982	NM_000877	IL1R1	Interleukin 1 receptor, type I
D04	Hs.89679	NM_000586	IL2	Interleukin 2
D05	Hs.98309	NM_016584	IL23A	Interleukin 23, alpha subunit p19
D06	Hs.73917	NM_000589	IL4	Interleukin 4
D07	Hs.2247	NM_000879	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
D08	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
D09	Hs.624	NM_000584	IL8	Interleukin 8
D10	Hs.522819	NM_001569	IRAK1	Interleukin-1 receptor-associated kinase 1

D11	Hs.75254	NM_001571	IRF3	Interferon regulatory factor 3
D12	Hs.166120	NM_001572	IRF7	Interferon regulatory factor 7
E01	Hs.172631	NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)
E02	Hs.656213	NM_004972	JAK2	Janus kinase 2
E03	Hs.660766	NM_015364	LY96	Lymphocyte antigen 96
E04	Hs.524579	NM_000239	LYZ	Lysozyme
E05	Hs.431850	NM_002745	MAPK1	Mitogen-activated protein kinase 1
E06	Hs.138211	NM_002750	MAPK8	Mitogen-activated protein kinase 8
E07	Hs.499674	NM_000242	MBL2	Mannose-binding lectin (protein C) 2, soluble
E08	Hs.458272	NM_000250	MPO	Myeloperoxidase
E09	Hs.517307	NM_002462	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
E10	Hs.82116	NM_002468	MYD88	Myeloid differentiation primary response gene (88)
E11	Hs.654408	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
E12	Hs.81328	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
F01	Hs.159483	NM_183395	NLRP3	NLR family, pyrin domain containing 3
F02	Hs.405153	NM_006092	NOD1	Nucleotide-binding oligomerisation domain containing 1
F03	Hs.592072	NM_022162	NOD2	Nucleotide-binding oligomerisation domain containing 2
F04	Hs.73958	NM_000448	RAG1	Recombination activating gene 1
F05	Hs.256022	NM_005060	RORC	RAR-related orphan receptor C
F06	Hs.591607	NM_000578	SLC11A1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
F07	Hs.642990	NM_007315	STAT1	Signal transducer and activator of transcription 1, 91kDa
F08	Hs.463059	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)

F09	Hs.80642	NM_003151	STAT4	Signal transducer and activator of transcription 4
F10	Hs.524518	NM_003153	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced
F11	Hs.272409	NM_013351	TBX21	T-box 21
F12	Hs.29344	NM_182919	TICAM1	Toll-like receptor adaptor molecule 1
G01	Hs.654532	NM_003263	TLR1	Toll-like receptor 1
G02	Hs.519033	NM_003264	TLR2	Toll-like receptor 2
G03	Hs.657724	NM_003265	TLR3	Toll-like receptor 3
G04	Hs.174312	NM_138554	TLR4	Toll-like receptor 4
G05	Hs.604542	NM_003268	TLR5	Toll-like receptor 5
G06	Hs.662185	NM_006068	TLR6	Toll-like receptor 6
G07	Hs.659215	NM_016562	TLR7	Toll-like receptor 7
G08	Hs.660543	NM_138636	TLR8	Toll-like receptor 8
G09	Hs.87968	NM_017442	TLR9	Toll-like receptor 9
G10	Hs.241570	NM_000594	TNF	Tumour necrosis factor
G11	Hs.591983	NM_004620	TRAF6	TNF receptor-associated factor 6
G12	Hs.75516	NM_003331	TYK2	Tyrosine kinase 2
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Table 4.3. PCR cycling conditions

95°C	10 mins
40 cycles of	

95°C	15 sec
60°C	30 sec

4.2.4. Statistical analysis

The comparative cycle threshold (C_T) method was used to determine differential expression data [323]. Firstly, a threshold was determined based on the negative controls and positive controls; where the C_T for the positive controls was 19 (according to manufacturer's instructions) and negative controls not registering a C_T. The threshold was determined to be 0.15 (y-axis, fluorescence reading) based on the controls. The geometric mean was calculated for the five housekeeping genes; Actin Beta (ACTB), Beta-2-microglobulin (B2M), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and Ribosomal protein, large, P0 (RPLP0), which was used to calculate the difference in C_T (ΔC_T) for each tested gene. Finally, fold change was calculated using the following formula:

$$fold\ change = 2^{-\Delta\Delta Ct} \text{ or in other words}$$

$$fold\ change = 2^{-(Control\ \Delta Ct - Case\ \Delta Ct)}$$

$$if, fold\ change < 1$$

$$fold\ change = \frac{-1}{2^{-\Delta\Delta Ct}}$$

Log_2(fold change) was then calculated where if log_2(fold change)> 1, it was determined to be significant and was validated by a Students t-test comparing individual cases ΔC_T to control ΔC_T. If the t-test resulted in a p-value of greater than 0.05, then the

fold change was not classified as significant. In other words, both fold change and t-test have to be in agreeance with each other to deem the gene expression significantly different between the cases and controls. Multivariate Pearson's correlation was conducted comparing the ΔC_T of genes with either CST (regardless of case or control status) and PID type resulting in three values (R, R^2 and p-value). This R-value can range from -1 to 1, where the closer to 1 the stronger the correlation if negative R then it is a negative correlation and *vice versa* for positive R-value. Strength of correlation is indicated by an R^2 value ranging from 0 to 1, whereby the closer to 1, the stronger the correlation. Significance is then determined by a two-tailed p-value, where significance is p-value <0.05.

Using the fold change value and p-value of case compared to control, pathways were analysed through the use of ingenuity pathway analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). This software uses machine learning and compares to 5700 000 human genetic data bases and generates multi-level regulatory networks that may explain the gene expression changes exhibited in PID cases compared to controls, with predicted p-values, furthermore, this determines the most significantly associated pathways with the disease and significance is indicated when p-value <0.05.

4.2.5. Analysis of ΔC_T and figure generation using R studio

A normalised table was generated using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA). The package phyloseq [275] was used in R studio [276]to import the normalised table and metadata table. A distance matrix was created using the pairwise divergence method, this matrix was the clustered in a hierarchal structure based on 'Median' method. This produced a cluster dendrogram to allow for visualisation. A heat map was produced and annotated using package complex heat maps [277]. Appendix C lists the code used in R studio to create a cluster linkage dendrogram with a heat map.

4.3. RESULTS

4.3.1. RNA extraction from cervical swabs was successful using this pilot protocol

RNA was extracted from 54 cervical swabs and the yield was recorded for each sample before cDNA synthesis (Table 4.4). Comparing the mean RNA concentration of the various groups, the test group had a mean of 10.48 ng/µL; the case's had a mean of 6.23 ng/µL and controls had a mean of 5.21 ng/µL. Thus swabs collected under this study protocol were successfully used to yield suitable RNA for this approach.

Table 4.4. RNA concentration per sample in ng/µL

	Participant	RNA concentration (ng/µl)[a]	Mean (SD)
Test Group	1	19.4	10.48 (7.39) n=6
	7	17.6	
	9	11.2	
	10	2.2	
	18	1.9	
	34	10.6	
Case	200	15.9	6.23 (4.65) n=16[b]
	201	2.1	
	202	4.1	
	203	11.8	
	204	3.0	
	205	2.1	
	206	2.2	
	207	3.1	
	210	4.6	
	216	8.14	
	217	3.9	
	218	3.0	
	219	12.5	
	220	8.3	
	221	2.2	
	224	12.8	
Control	400	8.8	5.21 (4.37) n=32
	401	2.2	
	402	1.9	
	404	2.1	
	405	2.8	
	408	2.9	
	409	8.1	
	410	2.0	
	411	2.1	
	412	9.5	
	413	13.7	
	414	0.8	
	415	1.2	
	416	2.2	
	417	8.5	
	418	2.9	
	419	2.8	
	420	4.6	
	421	2.1	
	428	2.1	

	429	2.0	
	430	3.3	
	431	5.5	
	432	7.5	
	433	9.3	
	434	20.2	
	209	3.61	
	211	4.6	
	212	8.14	
	213	3.9	
	214	3.0	
	215	12.5	

a: RNA concentration recorded using Nanodrop One Micro-UV/Vis spectrophotometer.

b: it is important to note that recruitment is still going and these new cases (n=2) have still yet to be placed under medical review

4.3.2. Normaliser genes allowed for intra-sample comparison

Five normaliser genes (housekeeping genes) were used for comparative analysis; the mean cycle threshold for individual normaliser genes for each of the different groups is shown in Table 4.5. The cycle threshold for theses conserved genes were compared between groups. Significance was found when comparing B2M gene in the test group to case and controls (Table 4.6). No significance between case and control groups for all normalising genes allowed for intra-sample comparison. Upon a more profound examination of the test group findings for these normalisers, it was determined the data was not of high quality and so they were excluded from multivariate and pathway analysis. Furthermore, this was due to test group due having low numbers (n=6/13, as 7 participants didn't have a swab to extract RNA from), secondly due to low gene signals detected ('n' in Table 4.11), and finally, the difference in housekeeper signal may have skewed the result.

Table 4.5. Housekeeping gene mean cycle threshold between study groups

Housekeeping gene	Function	Case (SD)	Control (SD)	Test Group (SD)
ACTB	cytoskeleton	19.59 (14.90)	15.89 (15.07)	14.36 (19.76)
B2M	presenting antigens to the immune system	21.23 (14.90)	20.53 (14.87)	33.20 (1.84)
GAPDH	RNA transport, DNA replication and apoptosis	24.21 (13.01)	24.22 (13.21)	12.67 (17.39)
HPRT1	generation of purine nucleotides	7.81 (13.52)	13.34 (17.52)	7.4 (10.20)
RPLP0	structural component of the ribosome	16.48 (14.36)	21.28 (12.92)	10.36 (15.19)

SD= standard deviation

Table 4.6. Analysis of housekeeping genes cycle threshold on the groups

Housekeeping gene	p-value[a] of housekeeping Ct		
	Case vs Control	Case vs Test group	Control vs Test group
ACTB	0.4156	0.8216	0.8584
B2M	0.8651	0.0354*	0.0083*
GAPDH	0.5844	0.3090	0.2062
HPRT1	0.3139	0.9571	0.6991
RPLP0	0.1447	0.4641	0.2146

a: p-value was calculated using non-parametric Mann-Whitney

4.3.3. Intrasample heterogeneity in relative expression was evident

Differences in relative expression of genes were apparent amongst all samples. Figure 4.2 displays a cluster linkage dendrogram of participants, with genes tested and annotated with participant metadata. Using log normalised ΔC_t of detectable genes on a scale of -4 to +4 variability was displayed amongst groups, microbiota community state types, aetiology of PID, and current STI status.

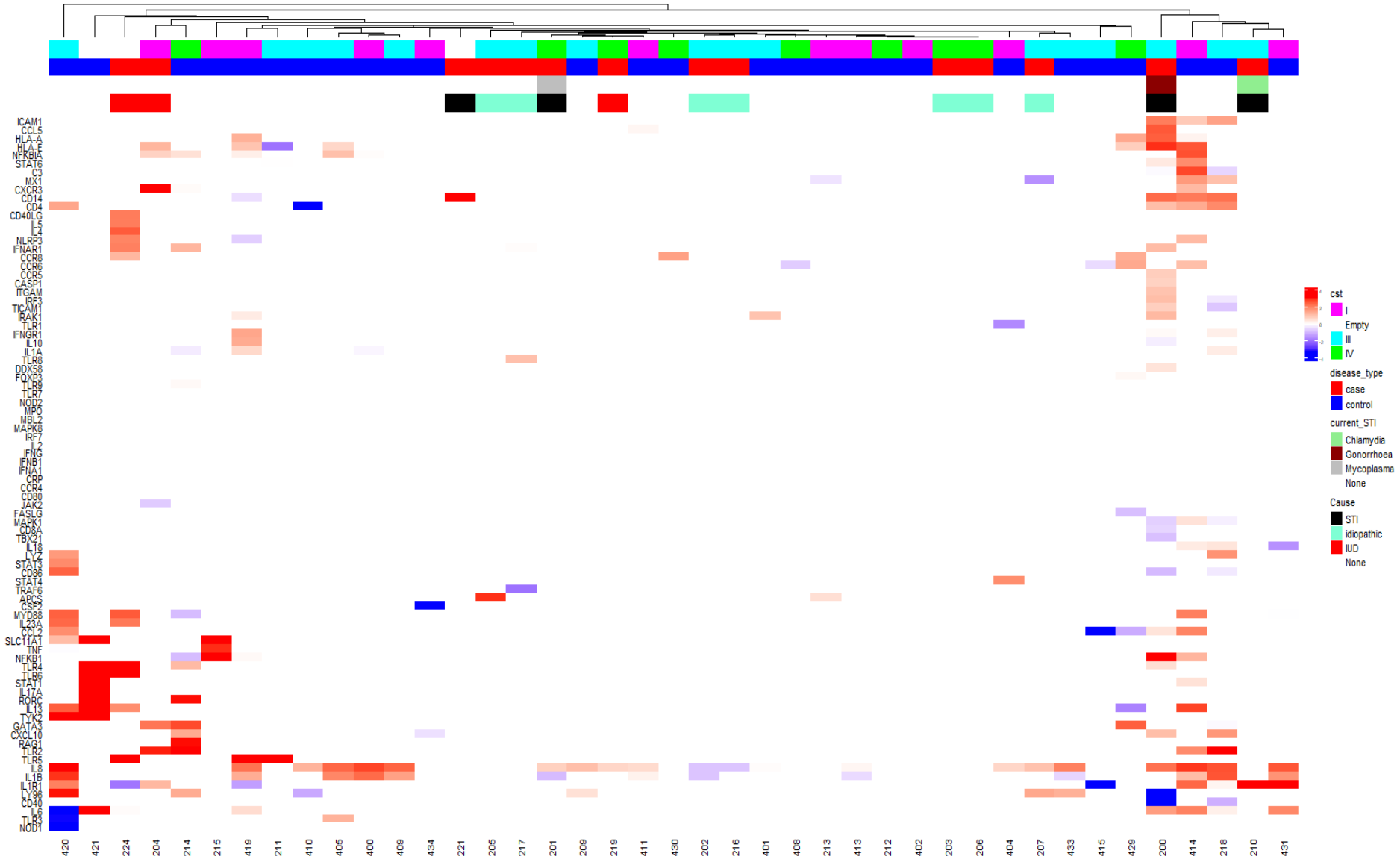

ICAM1
CCL5
HLA-A
HLA-F
NFKBIA
STAT6
C3
MX1
CXCR3
CD14
CD4
CD40LG
IL5
IL4
NLRP3
IFNAR1
CCR8
CCR6
CCR5
CASP1
ITGAM
IRF3
TICAM1
IRAK1
TLR1
IFNGR1
IL10
IL1A
TLR8
DDX58
FOXP3
TLR9
TLR7
NOD2
MPO
MBL2
MAPK8
IRF7
IL2
IFNG
IFNB1
IFNA1
CRP
CCR4
CD80
JAK2
FASLG
MAPK1
CD8A
TBX21
IL18
LYZ
STAT3
CD86
STAT4
TRAF6
APCS
CSF2
MYD88
IL23A
CCL2
SLC11A1
TNF
NFKB1
TLR4
TLR6
STAT1
IL17A
RORC
IL13
TYK2
GATA3
CXCL10
RAG1
TLR2
TLR5
IL8
IL1B
IL1R1
LY96
CD40
IL6
TLR3
NOD1
420
421
224
204
214
215
419
211
410
405
400
409
434
221
205
217
201
209
219
411
430
202
216
401
408
213
413
212
402
203
206
404
207
433
415
429
200
414
218
210
431
cst
I
Empty
III
IV
disease_type
case
control
current_STI
Chlamydia
Gonorrhoea
Mycoplasma
None
Cause
STI
idiopathic
IUD
None

Figure 4.2. Cluster dendrogram heat map of RT-qPCR analysis of immune gene expression in cervical samples. The scale (on top right) shows a colour grade from -4 to +4 , and colour scale is blue to white to red (red is upregulated, white no change and blue is downregulation, all compared to house keeping genes). The heat map is arranged by a column cluster linkage dendrogram followed by annotation with corresponding legends on the top and right respectively. The annotations label samples by community state type; if they are a case, control or internal experimental control, their current sexually transmitted infections and if they are a case what is the presumed aetiology of their PID. (CST colour scales; Pink = CST I, white = Empty (not analysed), green =III, Blue =IV; disease type; red = case, blue = control, current STI; green = *C. trachomatis*, brown= *N. gonorrhoeae*, Grey = *M. genitalium*, white= none; PID cause; black = STI, aqua = idiopathic, red = IUD, white = controls

4.3.4. Lysozyme gene was expressed higher in cases then controls

Lysozyme was found to be significantly more highly expressed in cases than the controls. Figure 4.3 is a box and whisker plot displaying the minimum, first quartile, mean, third quartile and maximum of the ΔC_t; using a case-control comparison. Case's significantly had a higher expression of lysozyme when compared to controls (p-value 0.04, Students t-test. The resulting fold change is significant at 3.73.

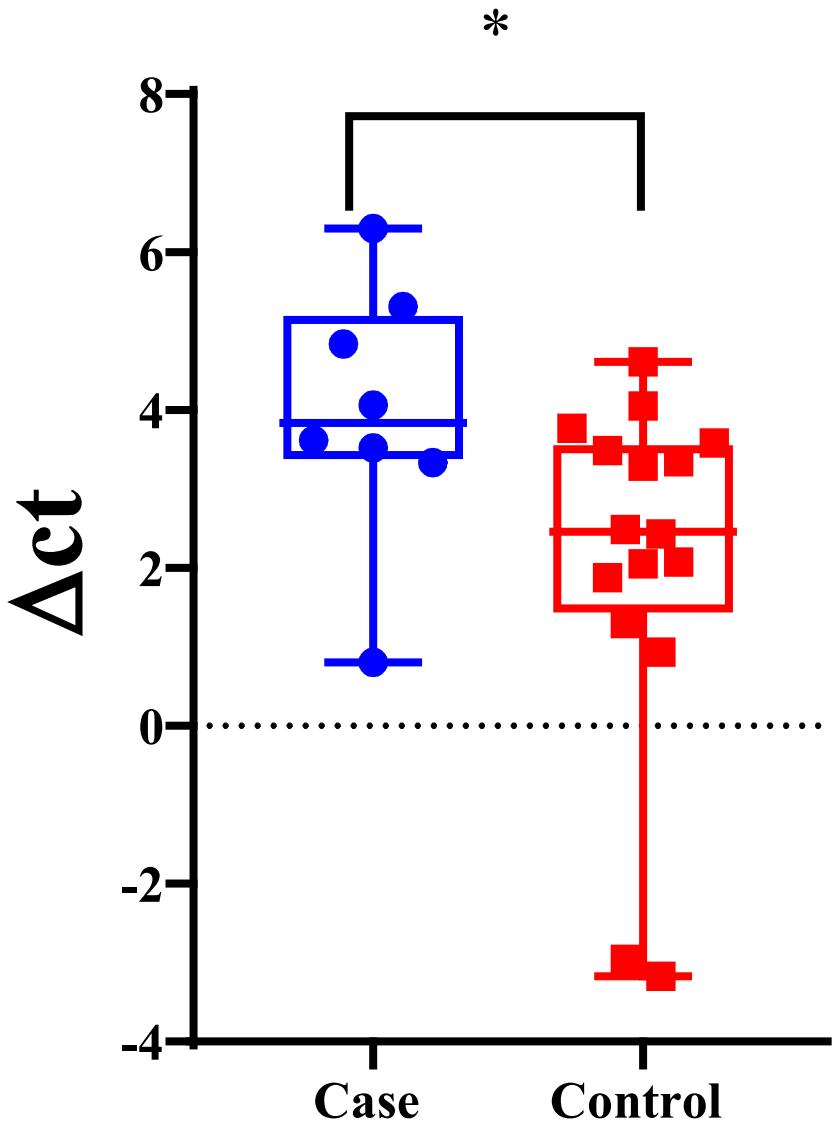


Figure 4.3. Lysozyme Cycle threshold change in case and control participants. Box and whisker plot showing the minimum, first quartile, mean, third quartile and maximum. The cases are in blue and controls in red (x-axis). This is comparing the gene Ct compared to the geometric mean of the housekeeping genes (y-axis). * p-value 0.04. (n=8 in Case and n=16 in Controls)

4.1.1. Gene expression variability was associated with differing PID type

Relative difference in cycle threshold (ΔC_t) was used to compare the difference in expression between the different types of PID (Idiopathic, instrumentation (IUD) and STI caused). Figure 4.4. shows the genes expressed when grouping all Case participants together based on the PID aetiology. Figure 4.4, A shows the ΔC_t of those women classified as idiopathic PID, Figure 4.4, B are women who were classified as PID associated by instrumentation (IUD) and Figure 4.4, C are women who had STI associated PID. Fewer genes were detected in women who had PID due to IUD. Performing a multivariate Pearson's correlation on all genes compared to PID (all types) resulted in negative correlation with STI caused PID (Table 4.8). These genes are CCR5 and RORC, with a strong negative correlation (R^2=0.94, p-value= 0.03) shown in RORC, a gene responsible for coding a protein that interplays in DNA transcription. In other words, a woman is less likely to have STI caused PID as the expression of these genes increases. This is also shown in Figure 4.4, where the ΔC_t of aforementioned genes is seen in higher in idiopathic (Figure 4.4, panel A) and IUD (Figure 4.4, panel B) caused PID but not STI (Figure 4.4, panel C) associated PID.

Table 4.7. Genes significantly correlated with PID type

Gene	Multivariate Pearson's correlation on PID types		
	R (95% CI)	R^2	p-value (two-tailed)
CCR5	-0.82 (-0.98 to -0.03)	0.68	0.04
RORC	-0.97 (-0.99 to -0.09)	0.94	0.03

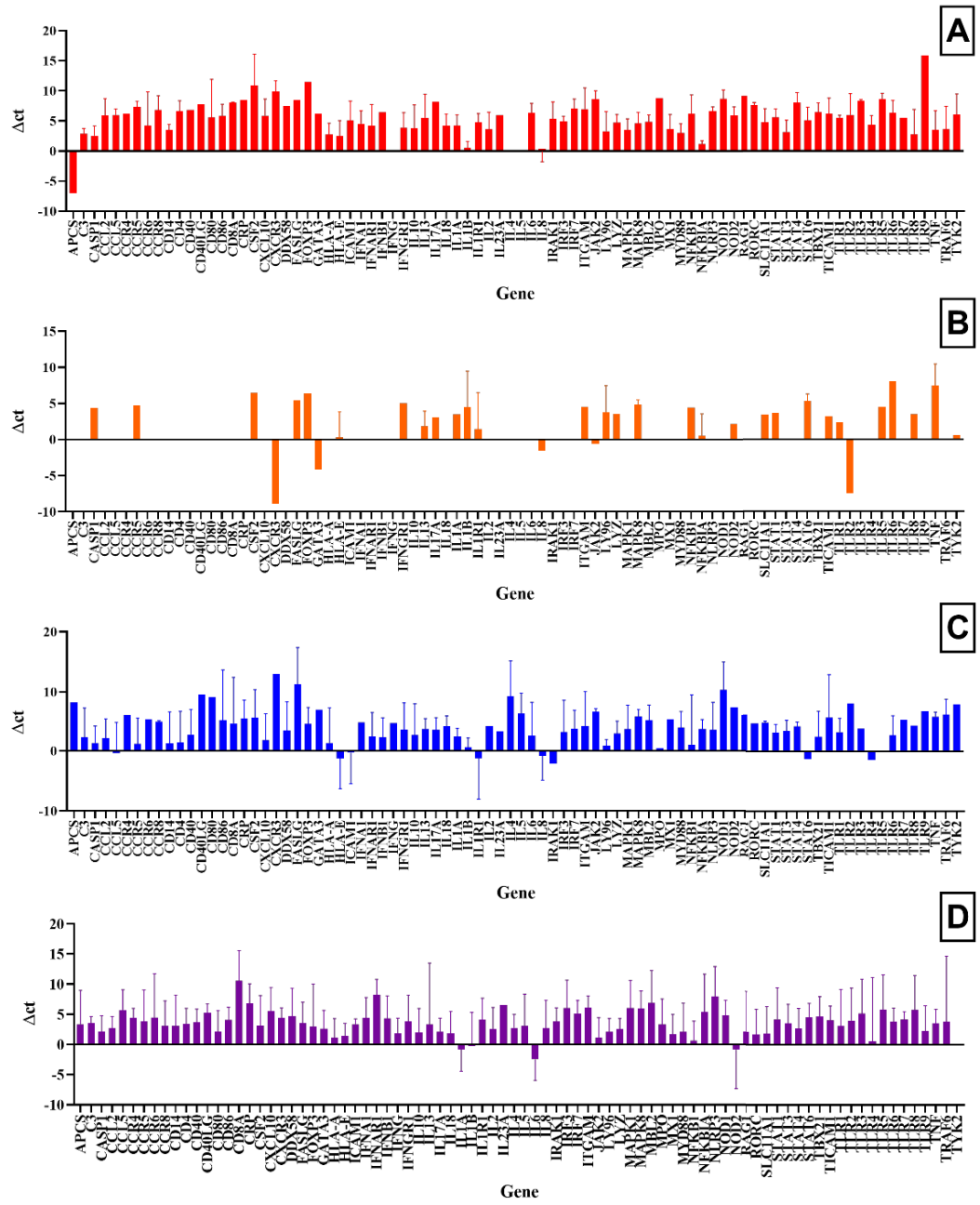


Figure 4.4. Relative cycle threshold expression with differing PID causes. ΔC_t is measured using the formula cycle threshold of sample – cycle threshold of the geometric mean of 5 housekeeping genes, and the mean of all participants in that specific group is shown with SD. The ΔC_t is on the y-axis and genes on the x-axis. Panel A is idiopathic

PID (n=7), panel B is STI associated PID (n=3) and Panel C is instrumentation (IUD) associated PID (n=2). Panel D is the Controls (n=28).

4.3.5. Gene expression variability was correlated with differing CST's

In brief, community state types (CST) (discussed in Chapter 1 and Chapter 3) are categorised from CST I to V, whereby I, II, III and V are dominated by a single *Lactobacillus* species and CST IV has no dominant *Lactobacillus*; instead anaerobic Gram-variable organisms. Regardless of disease state, CST was compared to relative expression. Figure 4.5, A shows the ΔC_t in CST I (*L. crispatus* dominated), Figure 4.5, B displays relative expression of women with CST III (*L. iners* dominated) and Figure 4.5, C is the relative expression of women with CST IV (No dominant *Lactobacillus*). There was no significant difference between gene expression and CST classification, regardless of disease status, visually looking at Figure 4.5 shows consistent expression of genes.

Figure 4.6 shows the ΔC_t in PID cases separated into the different CSTs, Figure 4.6, A shows the ΔC_t in CST I (*L. crispatus* dominated), Figure 4.6, B displays relative expression of women with CST III (*L. iners* dominated) and Figure 4.6, C is the relative expression of women with CST IV (No dominant *Lactobacillus*). Whilst no significance

was calculated, due to small sample size. Visually fewer genes are expressed in women with PID and CST I.

Conducting a multivariate analysis using Pearson's correlation on ΔC_t of genes and CST's resulted in seven positively correlated genes. This was calculated for all participants regardless of case-control status. The genes found to be positively correlated with CST are CD40LG, IL10, IL13, IL18, MYD88, NFKBIA and TLR5 (Table 4.9). Even with p-value defined as significant the relation was not strong, with the highest R^2=0.58 (CD40LG). Table 4.9 displays which CST the gene was positively correlated with, in other words, the increase in ΔC_t was more likely to be correlated with the CST listed. It is important to note this analysis was done on ΔC_t compared to contraception use and age, with no analysis showing no correlation at all with R= 0.

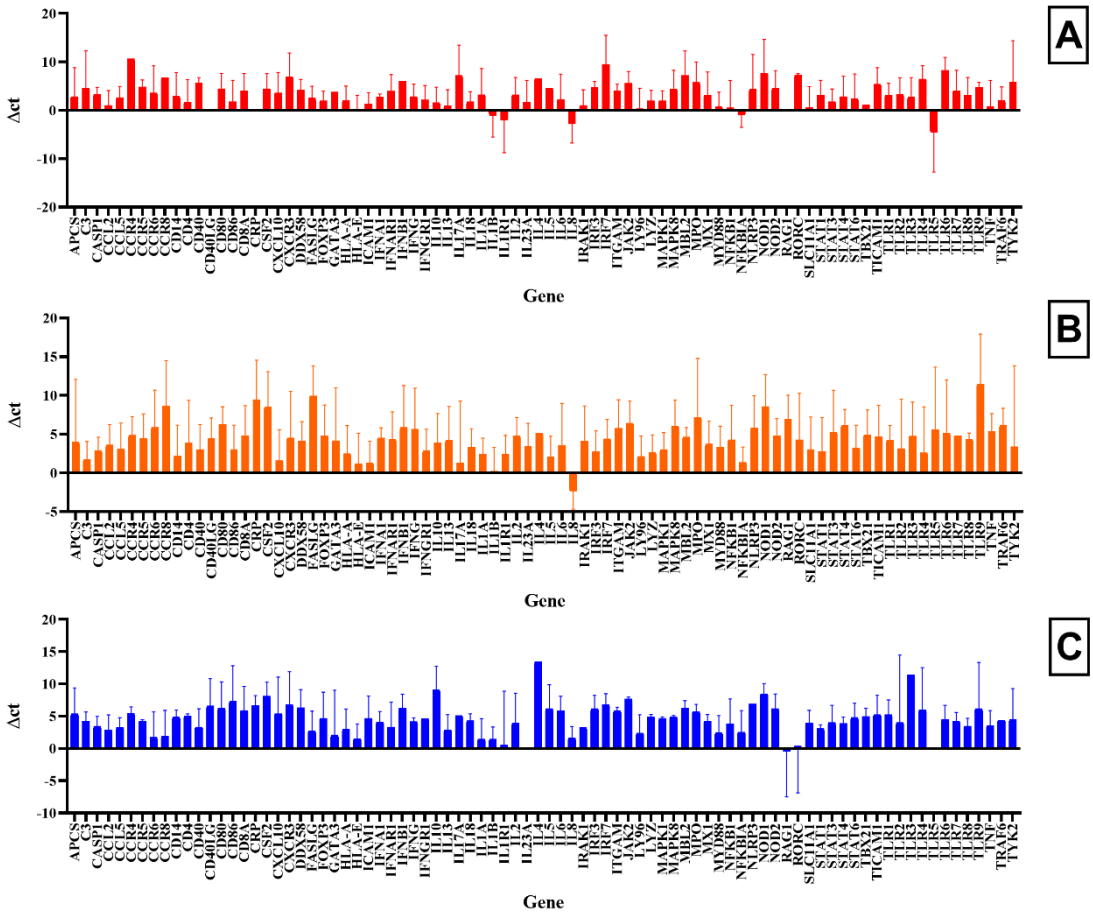


Figure 4.5. Relative cycle threshold expression with differing CST. ΔC_t is measured using the formula cycle threshold of sample – cycle threshold of geometric mean of 5 housekeeping genes. The ΔC_t is on the y-axis and genes on the x-axis. The bars represent mean ΔC_t and SD, for all participants. The plots in all panels are regardless of disease state. Panel A is CST I, panel B is CST III and Panel C is CST IV.

Table 4.8. Genes significantly correlated with CST (All participants)

Gene	Multivariate Pearson's correlation on CST			
	R (95% CI)	R^2	p-value (two tailed)	Correlated with
CD40LG	0.77 (0.21-0.95)	0.58	0.016	IV
IL10	0.51 (0.02-0.80)	0.26	0.044	IV
IL13	0.35 (0.04-0.61)	0.12	0.029	III
IL18	0.43 (0.01-0.72)	0.18	0.044	IV
MYD88	0.45 (0.09-0.70)	0.2	0.017	III
NFKBIA	0.43 (0.02-0.71)	0.18	0.042	IV
TLR5	0.69 (0.04-0.93)	0.47	0.041	III

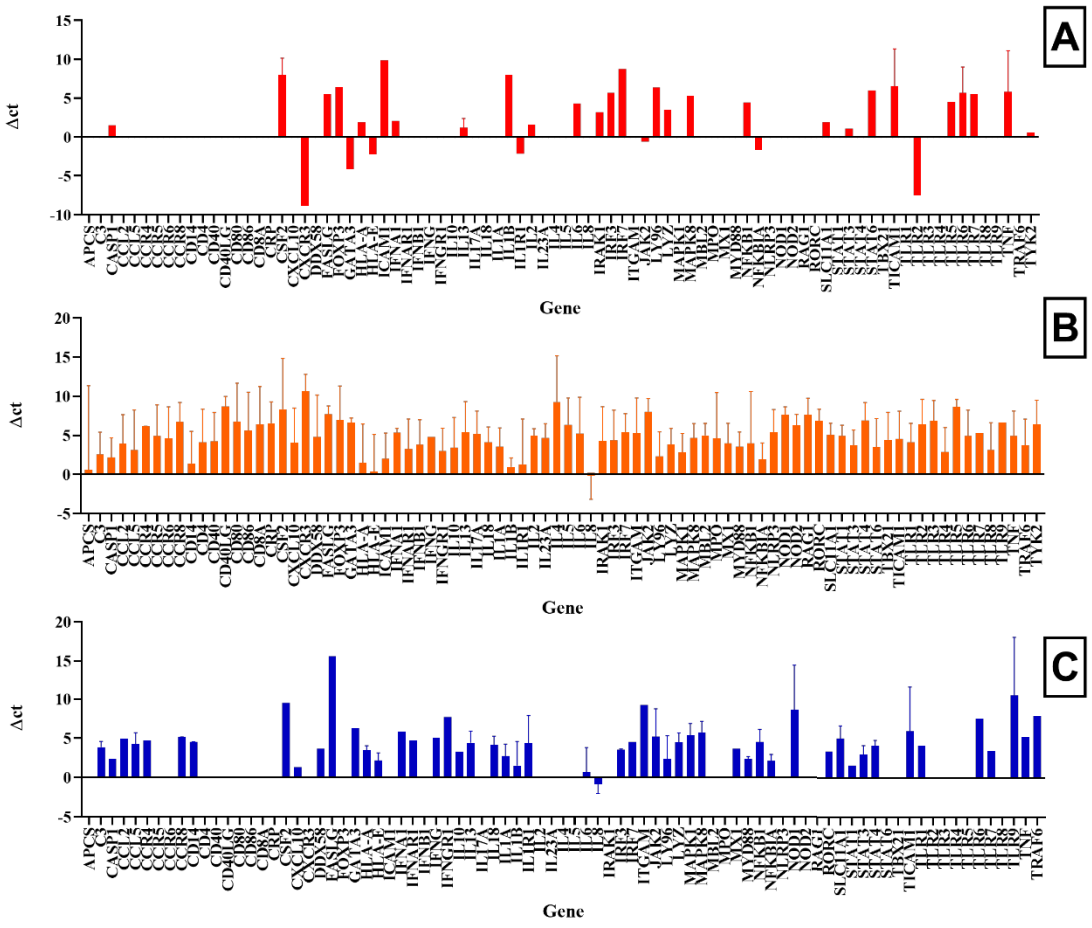


Figure 4.6. Relative cycle threshold expression of cases and CST. ΔC_t is measured using the formula cycle threshold of sample – cycle threshold of geometric mean of 5 housekeeping genes. The ΔC_t is on the y-axis and genes on the x-axis. The bars represent mean ΔC_t and SD for all women with PID. The plots in all panels are regardless of disease state. Panel A is CST I (n = 2), panel B is CST III (n=8) and Panel C is CST IV (n=4).

4.3.6. Comparative analysis of immune genes for case vs control suggested that expression of lysozyme is higher in women with PID.

The comparative C_t method was used for comparison between case and control with mean ΔC_t for each group shown in Table 4.11. Out of 84 innate and adaptive genes, it was found that 35 genes had a significant $\log_2$ fold change in cases compared to controls (Table 4.9). Table 4.10 displays 59 genes that had a significant $\log_2$ fold change in cases compared to the test group. Upon validation, with a Students t-test, it was found that the lysozyme (LYZ) had a significant $\log_2$ fold change and also a significant p-value using a Student's T-test. LYZ had a $\log_2$ fold change of 1.99 or fold change of 3.73 with a significant p-value of 0.04 (Table 4.11). It is important to note that only the case's compared to the two groups were statistically tested as the purpose of this is to determine if a unique disease signature is found in the cases, regardless of what it is compared to.

Table 4.9. Genes with significant[a] log_2 fold change case compared to control

Gene	Log_2Fold change
CCL2	1.94
CD4	1.02
CD80	1.52
CD86	3.42
CD8A	2.35
CSF2	1.55
CXCR3	1.25
FASLG	4.49
FOXP3	3.29
ICAM1	1.81
IFNA1	1.27
IFNGR1	2.09
IL13	1.89
IL17A	1.30
IL18	1.70
IL1A	1.25
IL1B	2.29
IL8	2.00
IRAK1	1.41
LY96	1.98
LYZ	1.90
MAPK1	1.04
NFKB1	2.26
RAG1	8.55
RORC	4.73
SLC11A1	2.98
STAT1	2.04
STAT4	2.37
STAT6	1.41
TLR3	2.84
TLR5	1.91
TLR7	1.52
TLR9	5.48
TNF	3.00
TYK2	1.63

a: significance is when log_2 fold change >1, or <-1

Table 4.10 Genes with significant[a] log_2 fold change case compared to test group

Gene	Log_2Fold change
APCS	4.49
C3	-12.83
CASP1	-10.51
CCL5	-2.44
CCR4	-1.43
CCR6	-5.99
CD14	-3.51
CD4	3.40
CD80	11.03
CSF2	1.58
FASLG	5.69
FOXP3	1.24
GATA3	-4.06
ICAM1	-3.49
IFNA1	3.23
IFNAR1	1.92
IFNB1	3.09
IFNG	-3.10
IFNGR1	1.16
IL10	-5.94
IL18	-2.50
IL1A	2.85
IL1B	-4.02
IL1R1	-9.21
IL2	-3.42
IL23A	-9.01
IL4	-9.82
IL5	-6.85
IL6	-4.42
IL8	-3.15
IRAK1	-8.54
IRF3	-4.90
IRF7	4.52
LY96	1.95
MAPK1	3.25
MAPK8	5.74
MBL2	6.03
MPO	9.90
NFKB1	-6.11
NFKBIA	6.03
NLRP3	-5.21
NOD1	8.23
NOD2	4.29

RAG1	10.73
RORC	9.93
SLC11A1	4.66
STAT1	5.16
STAT6	1.90
TICAM1	1.67
TLR1	1.30
TLR2	-2.81
TLR3	1.58
TLR4	-1.08
TLR5	1.87
TLR6	4.27
TLR7	-1.73
TLR9	7.04
TRAF6	3.23
TYK2	3.68

a: significance is when $\log_2$ fold change >1, or <-1, while the test group wasn't included in multivariate or pathway analysis, I have chosen to display fold changes and p-value (Table 4.11) for visual purposes and proof of analysis.

Table 4.11. Analysis of ΔC_t between case and controls for all genes

Gene	Function[322]	Case (n)[b]	Control (n)[b]	Test group (n)[b]	p-value[a]	
					Case vs Control	Case vs Test group
APCS	Amyloid P component, serum	0.6±10.8 (2)	5.2±4.6 (6)	3.9±0 (1)	-	-
C3	Complement component 3	2.7±2.5 (5)	3.4±5.6 (10)	-15.5±0 (1)	0.80	-
CASP1	Caspase 1	2.4±2.0 (8)	3.5±1.1 (15)	-12.9±0 (1)	0.09	-
CCL2	Chemotactic for monocytes and basophils	4.1±3.4 (6)	2.1±2.6 (18)	-4.6±7.6 (2)	0.16	-
CCL5	Chemoattractant for blood monocytes, memory T helper cells and eosinophils	3.5±4.2 (7)	2.7±1.9 (16)	-5.9±4.6 (4)	0.56	0.40
CCR4	Receptor for MIP-1, RANTES, TARC and MCP-1	6.2±0.02 (2)	5.7±3.4 (5)	-7.6±2.4 (3)	-	-
CCR5	Receptor expressed by T cells and macrophages	4.9±3.6 (6)	4.4±1.6 (10)	-5.6±5.1 (4)	0.68	
CCR6	B-lineage maturation and antigen-driven B-cell differentiation	4.6±4.0 (3)	3.9±5.2 (11)	-10.6±0 (1)	0.81	-
CCR8	Allows positioning of activated T cells within the antigenic challenge sites and specialised areas of lymphoid tissues	4.3±3.9 (5)	4.5±7.2 (8)	-3.7±9.1 (2)	0.97	-
CD14	Co-receptor for the detection of bacterial lipopolysaccharide (LPS)	0.75±5.6 (7)	3.1±4.2 (16)	-4.3±4.6 (4)	0.27	0.31
CD4	Membrane glycoprotein of T lymphocytes that interacts with major histocompatibility complex class II antigens and initiates or augments the early phase of T-cell activation	4.1±4.3 (4)	3.1±5.1 (12)	-0.7±0 (2)	0.73	-

CD40	A receptor essential for mediating a variety of immune and inflammatory responses	4.2±3.7 (3)	3.4±2.6 (7)	0	0.69	-
CD40LG	Expressed on the surface of T cells, regulates B cell function	4.5±7.3 (3)	3.7±2.1 (6)	0	0.82	-
CD80	T-cell proliferation and cytokine production	6.8±4.9 (3)	5.2±1.5 (7)	4.3±0 (1)	0.45	-
CD86	Expressed by antigen-presenting cells, and it is the ligand for two proteins at the cell surface of T cells	5.6±4.9 (4)	2.2±3.5 (9)	0	0.18	-
CD8A	Found on most cytotoxic T lymphocytes it mediates cell-cell interactions	6.4±4.8 (4)	4.1±2.1 (6)	0	0.31	-
CRP	Involved in several host defence related functions	6.6±2.7 (3)	10.6±5.0 (3)	0	0.29	-
CSF2	Cytokine that controls the production, differentiation, and function of granulocytes and macrophages	8.4±5.0 (8)	6.8±3.2 (10)	-6.8±7.7 (3)	0.43	0.70
CXCL10	Antimicrobial chemokine	3.3±3.6 (6)	3.1±5.0 (9)	0	0.94	-
CXCR3	A G protein-coupled receptor with selectivity for three chemokines	6.8±8.9 (5)	5.5±3.9 (15)	0	0.66	-
DDX58	Involved in viral double-stranded (ds) RNA recognition and the regulation of immune response	4.5±4.4 (4)	4.4±1.7 (15)	0	0.90	-
FASLG	Inducer of apoptosis	9.1±4.5 (4)	4.6±4.7 (9)	-3.4±2.5 (2)	0.13	-
FOXP3	Transcriptional regulator	6.8±3.6 (4)	3.5± 3.5 (11)	-5.6±2.4 (2)	0.13	-
GATA3	Regulator of T-cell development and role in endothelial cell biology	3.2±4.8 (5)	3.0±7.1 (8)	-7.3±1.6 (2)	0.94	-
HLA-A	Major histocompatibility complex	2.2±3.4 (8)	2.6±3.0 (16)	0	0.76	-

HLA-E	Major histocompatibility complex	0.9±3.5 (10)	1.1±3.2 (17)	0	0.91	-
ICAM1	Surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system	3.2±4.0 (7)	1.4±2.1 (13)	-6.7±8.3 (2)	0.19	-
IFNA1	Produced by macrophages and has anti-viral activity	4.6±1.8 (4)	3.4±0.7 (5)	-1.4±1.8 (2)	0.19	-
IFNAR1	Interferon (alpha, beta and omega) receptor 1	2.6±4.1 (8)	4.4±3.4 (12)	-0.7±0 (1)	0.30	-
IFNB1	A cytokine released as part of the innate immune response to pathogens	3.8±3.2 (3)	8.3±7.8 (3)	-0.7±0 (1)	0.13	-
IFNG	cytokine that is a member of the type II interferon class	4.8±0 (1)	4.3±3.7 (9)	-7.9±6.8 (2)	-	-
IFNGR1	Interferon gamma receptor 1	4.0±3.0 (7)	1.9±2.4 (12)	-2.8±0 (1)	0.11	-
IL10	Cytokine produced primarily by monocytes and to a lesser extent by lymphocytes, pleiotropic effects in immunoregulation and inflammation	3.4±3.9 (6)	3.8±4.4 (10)	-9.4±7.1 (3)	0.85	0.14
IL13	Immunoregulatory cytokine produced primarily by activated Th2 cells	3.9±3.9 (13)	2.0±4.0 (25)	-3.8±3.8 (5)	0.17	0.97
IL17A	Proinflammatory cytokine produced by activated T cells, regulates the activities of NF-kappaB and mitogen-activated protein kinases	4.6±2.6 (4)	3.3±10.1 (4)	-4.9±1.9 (4)	0.81	0.86
IL18	Proinflammatory cytokine that augments natural killer cell activity in spleen cells and stimulates interferon gamma production in T-helper type I cells	3.8±2.1 (9)	2.1±2.3 (13)	-6.3±3.9 (3)	0.09	0.17
IL1A	Cytokine is a pleiotropic cytokine involved in various immune responses	3.1±1.8 (7)	1.9±3.6 (10)	-0.3±0 (1)	0.42	-
IL1B	Cytokine is produced by activated macrophages as a proprotein	1.4±2.6 (9)	-0.9±3.6 (18)	-5.4±7.3 (4)	0.10	0.16
IL1R1	Receptor for interleukin-1 alpha, interleukin-1 beta, and interleukin-1 receptor antagonist	1.8±4.6 (10)	0.2±5.1 (13)	-11.0±0 (2)	0.44	-

IL2	Cytokine that is important for the proliferation of T and B lymphocytes	3.9±2.1 (3)	4.1±3.6 (9)	-7.3±5.2 (4)	0.91	0.34
IL23A	Activates the Jak-Stat signalling cascade, stimulates memory T-cells and promotes the production of proinflammatory cytokines	1.8±5.2 (3)	2.6±3.6 (11)	-10.8±0 (2)	0.76	-
IL4	A pleiotropic cytokine produced by activated T cells	4.4±9.4 (3)	6.6±0 (1)	-14.3±0 (2)	-	-
IL5	Cytokine plays a major role in the regulation of eosinophil formation, maturation, recruitment and survival	2.9±6.4 (3)	2.8±2.2 (3)	-9.8±0 (2)	0.96	-
IL6	Cytokine with a wide variety of biological functions	4.1±4.1 (8)	3.1±5.3 (22)	-8.5±4.4 (3)	0.63	0.15
IL8	Chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells	-0.5±2.4 (9)	-2.5±3.5 (20)	-2.7±8.3 (4)	0.13	0.30
IRAK1	A serine/threonine-protein kinase that plays a critical role in initiating the innate immune response against foreign pathogens	4.1±4.0 (6)	2.7±4.6 (5)	-12.7±0 (2)	0.60	-
IRF3	Key transcriptional regulator of type I interferon (IFN)-dependent immune responses	4.3±3.0 (8)	3.8±2.2 (9)	-9.2±0.4 (3)	0.70	0.20
IRF7	Has a role in transcriptional activation of virus-inducible cellular genes, including interferon beta chain genes	6.1±2.3 (7)	6.0±4.6 (8)	-1.5±0 (1)	0.98	-
ITGAM	Implicated in various adhesive interactions of monocytes, macrophages and granulocytes	6.1±3.9 (9)	5.1±2.2 (12)	-5.6±5.3 (5)	0.50	0.85
JAK2	Non-receptor tyrosine kinase involved in various processes such as cell growth, development, differentiation or histone modifications	6.8±3.3 (8)	6.1±1.9 (12)	-6.3±9.8 (4)	0.59	0.91
LY96	Associates with toll-like receptor 4 on the cell surface and confers responsiveness to lipopolysaccyaride (LPS)	3.1±3.2 (11)	1.2±3.3 (19)	-1.2±1.5 (2)	0.12	-
LYZ	Lysozyme	4.0±1.6 (8)	2.1±2.2 (16)	-3.5±0 (1)	0.04	-
MAPK1	Acts as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes	3.6±2.6 (8)	2.5±1.8 (14)	-0.3±0 (1)	0.27	-

MAPK8	Kinase is activated by various cell stimuli and mediates immediate-early gene expression in response to cell stimuli	5.0±1.5 (9)	6.0±4.6 (8)	0.8±6.3 (2)	0.52	-
MBL2	Calcium-dependent lectin involved in innate immune defence.	5.0±1.5 (5)	5.9±2.9 (5)	1.0±5.5 (2)	0.55	-
MPO	A haem protein that constitutes the major component of neutrophil azurophilic granules	4.6±5.9 (2)	6.9±5.4 (7)	5.3±0 (1)	-	-
MX1	A guanosine triphosphate (GTP)-metabolizing protein that participates in the cellular anti-viral response	4.0±2.3 (6)	3.3±4.2 (10)	0	0.73	-
MYD88	A cytosolic adapter protein that plays a central role in the innate and adaptive immune response	2.3±3.3 (9)	1.7±3.3 (19)	0	0.63	-
NFKB1	Activated NFKB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions	4.3±5.3 (9)	2.1±4.7 (16)	-10.5±4.4 (3)	0.28	0.10
NFKBIA	Inhibits the activity of dimeric NF-kappa-B/REL	1.6±1.8 (9)	0.7±3.2 (14)	4.4±0 (1)	0.43	-
NLRP3	Interacts with the apoptosis-associated speck-like protein PYCARD/ASC	3.9±4.5 (6)	5.4±6.3 (7)	-9.1±10.3 (3)	0.64	0.31
NOD1	Plays a role in innate immunity by acting as a pattern-recognition receptor (PRR) that binds bacterial peptidoglycans and initiates inflammation	7.8±4.5 (6)	7.9±5.0 (14)	0.4±0 (1)	0.95	-
NOD2	Primarily expressed in the peripheral blood leukocytes	5.5±2.2 (5)	4.9±2.4 (8)	-1.2±0.1 (2)	0.67	-
RAG1	Involved in the activation of immunoglobulin V-D-J recombination	7.7±2.1 (2)	7.7±6.4 (3)	3.1±0 (1)	-	-
RORC	A DNA-binding transcription factor and is a regulator of cellular differentiation, immunity, peripheral circadian rhythm as well as lipid, steroid, xenobiotics and glucose metabolism	6.9±1.5 (4)	2.1±6.7 (8)	3.1±0 (1)	0.20	-

SLC11A1	Functions as divalent transition metal (iron and manganese) transporter involved in iron metabolism and host resistance to certain pathogens	4.6±1.8 (8)	1.7±2.5 (19)	0.02±0 (1)	0.07	-
STAT1	Responds to cytokines and growth factors	4.0±1.9 (8)	1.9±4.5 (9)	1.3±0 (1)	0.25	-
STAT3	A regulator of the inflammatory response by regulating differentiation of naive CD4(+) T-cells into T-helper Th17 or regulatory T-cells (Treg)	3.2±1.8 (8)	4.2±5.3 (14)	0	0.62	-
STAT4	Essential for mediating responses to IL12 in lymphocytes and regulating the differentiation of T helper cells	5.9±2.2 (5)	3.5±3.2 (11)	0	0.15	-
STAT6	Has a role in exerting IL4 mediated biological responses	4.1±3.0 (6)	2.7±3.4 (9)	-2.2±3.8 (2)	0.42	-
TBX21	A transcription factor which initiates Th1 lineage development from naive Th precursor cells	4.4±3.5 (4)	4.6±2.3 (5)	-5.0±0 (1)	0.94	-
TICAM1	An adaptor protein containing a Toll/interleukin-1 receptor (TIR) homology domain, which is an intracellular signalling domain that mediates protein-protein interactions	5.3±3.8 (11)	4.7±3.3 (18)	-3.6±2.4 (4)	0.64	0.43
TLR1	Participates in the innate immune response to microbial agents	3.6±2.0 (7)	4.0±2.4 (13)	-2.2±2.9 (2)	0.64	-
TLR2	Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins and other microbial cell wall components	3.7±5.9 (7)	4.0±5.4 (5)	-6.5±4.7 (4)	0.84	0.44
TLR3	Activated by double-stranded RNA, a sign of viral infection	6.8±2.6 (3)	5.2±5.7 (14)	-5.3±3.3 (3)	0.43	0.55
TLR4	Cooperates with LY96 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide (LPS)	-0.1±7.2 (5)	0.5±10.6 (5)	-1±0 (1)	0.11	-
TLR5	Participates in recognition of small molecular motifs named pathogen-associated molecular pattern (PAMPs)	2.5±9.8 (4)	5.8±5.7 (16)	-0.6±1.1 (2)	0.79	-
TLR6	Participates in the innate immune response to Gram-positive bacteria and fungi	3.8±5.7 (8)	3.9±2.2 (5)	0.5±3.0 (2)	0.41	-

TLR7	Activated by single-stranded RNA	5.4±0.1 (2)	4.2±1.3 (7)	-7.1±0 (1)	-	-
TLR8	Predominantly expressed in lung and peripheral blood leukocytes and is a component of innate and adaptive immunity.	3.2±3.0 (5)	5.8±5.7 (6)	0	0.47	-
TLR9	Activated by unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides	11.3±6.5 (2)	2.3±4.2 (11)	-4.2±0 (2)	-	-
TNF	Cytokine secreted by macrophages involved in the regulation of a wide spectrum of biological processes	5.3±2.9 (7)	3.6±2.3 (3)	0	0.12	-
TRAF6	Plays a role in dendritic cells (DCs) maturation and/or activation	4.4±3.4 (6)	3.8±10.9 (12)	-1.2±0 (1)	0.70	-
TYK2	Component of both the type I and type III interferon signalling pathways	5.4±3.6 (6)	4.0±5.4 (5)	-1.8±0.7 (2)	0.73	-

a: p-value determined using Student's T-Test , where "-"symbol is present, 'n' was to low for a statistical test

b: result shown is mean ± Standard deviation (SD) with total number of samples analysed from the two groups was 14 cases and 32 controls and

c test group, but the data here for each gene depends on if it was detected at all in that participant,t while the test group wasn't included in multivariate or pathway analysis, I have chosen to display fold changes and p-values for visual purposes and proof of analysis

4.3.7. Pathway analysis reveals bacterial detection and cell signalling pathway overrepresented in PID.

Pathway analysis was conducted on the 84 tested genes looking for significant fold change differences between the cases and controls. This identified several different pathways and the one with the smallest p-value was cell signalling. Interestingly, the inflammatory response was the 4th most significant cellular function. It was preceded by 'cell-to-cell signalling and interaction' as the 3rd and 'cell death and survival' as the 2nd most significant functions (Figure 4.7). Based on the 84 genes tested, the analysis resulted in several canonical pathways that are overrepresented in PID. Figure 4.8 displays the most significant canonical pathways in PID and their interactions with each other. These pathways included: role of pattern recognition receptors in recognition of bacteria and viruses (p-value = 2.31×10^{-44}), neuroinflammation signalling pathway (p-value = 2.15×10^{-41}), systemic lupus erythematosus in B cell signalling pathway (p-value = 9.53×10^{-37}), communication between innate and adaptive immune cells (p-value = 1.86×10^{-34}) and TREM1 signalling (modulates the immune response stimulated by pathogen recognition receptors) (p-value = 2.87×10^{-34}).

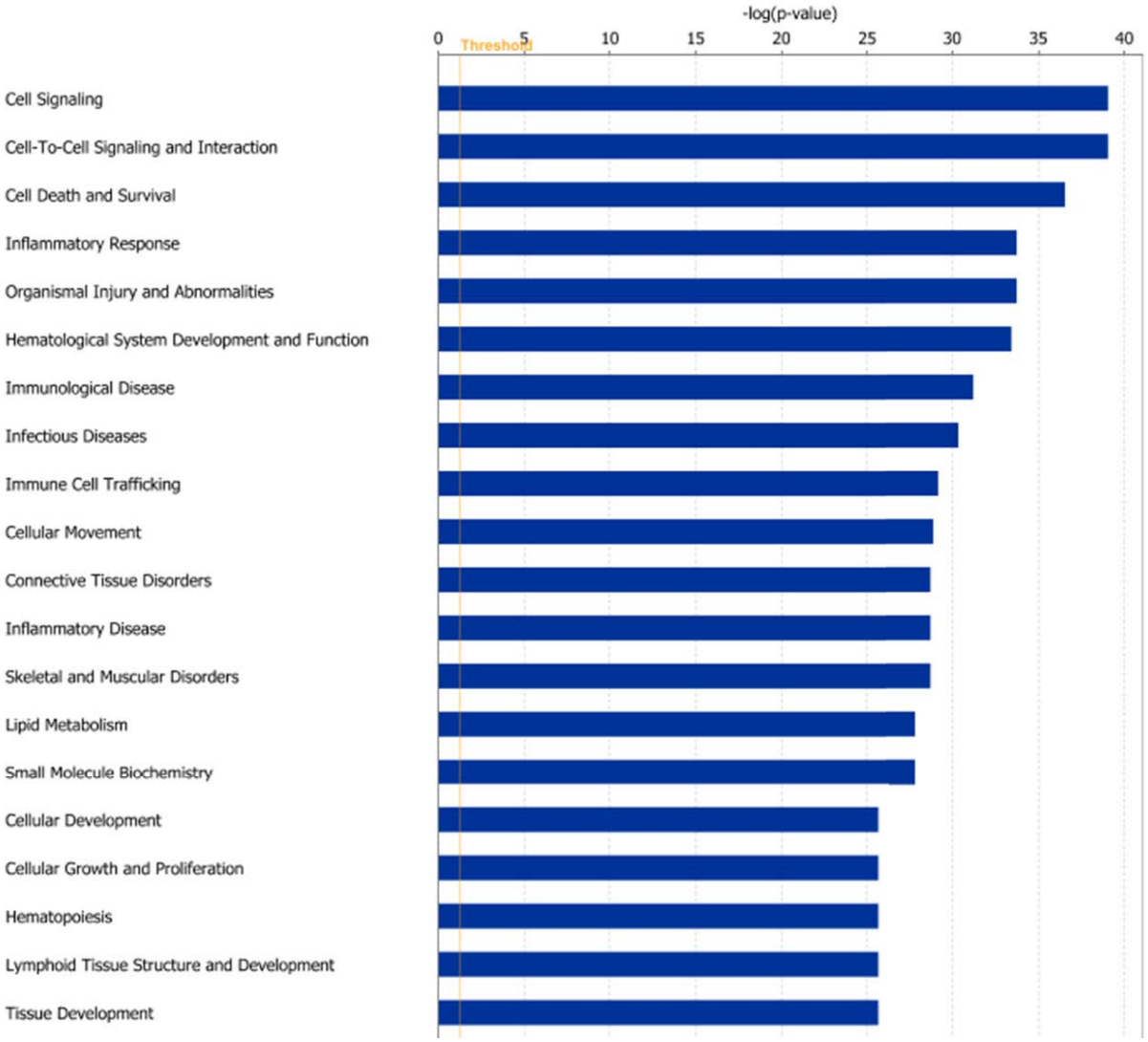


Figure 4.7. Top 20 significant cellular functions that were present at higher gene levels in Cases. Based on the fold change and p-value of 84 genes in cases compared to controls ($\Delta\Delta C_t$), analysis displayed the top significant cellular functions in PID compared to controls as a bar chart, where -log(p-value) is displayed on the x-axis. The orange line is the threshold for significance.

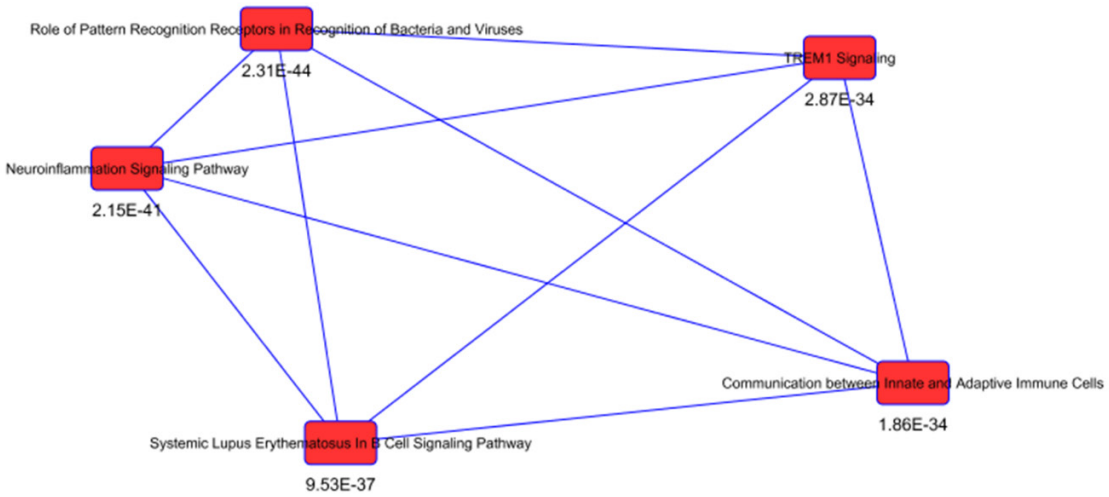


Figure 4.8. Top 5 pathways detected to be significantly different and with simplified interconnectivity. Based on the fold change and p-value of 84 genes in cases compared to controls, analysis displayed the top 5 significant pathways in PID compared to controls. The most significant was the pattern recognition receptors in recognition of bacteria and viruses with a p-value of 2.31×10^{-44}. The figure shows the top 5 pathways in the red boxes with p-value below each red box. The blue line shows how each pathway is interconnected with the other pathways displayed.

4.4. DISCUSSION

RNA was successfully extracted from 16 cases and 32 controls. With test group excluded due to the samples not having adequate quality, PID cases were dived into 3 aetiologies (idiopathic, instrumentation (IUD), and STI) and with the minimal numbers, the ability to detect differences between the host responses of cases compared to controls was limited. However, the protocol for recruitment and extraction of RNA that could be analysed from these samples is confirmed to be successful (at least for samples from FPNSW where the storage timeframes were relatively short). The only gene found to have significantly different expression levels was *lyz,* that was found in PID cases at a much higher level than controls. No significance was established between variations in contraceptive use or age. However, using multivariate analysis negative correlation between expression of two genes and PID aetiology was determined (CCR5 and RORC). Seven genes also had a weak positive correlation (R^2<0.90) between with CST (CD40, IL10, IL13, IL18, MYD88, NFKBIA, TLR5), with the strongest correlation being TLR5 with CST III (R^2=0.47). Pathway analysis was conducted and significantly expressed pathways (between cases and controls) included those with cell communication and the ability to detect bacteria and viruses. Overall, whilst the sample size was small, for the first time, a profile of an innate immune response was detected in women with PID. It would have been beneficial to have seen how much of these genes were also present at higher levels in the STI Test group and it is unfortunate that these samples were not able to be extracted to the quality needed. Hence, it may be feasible that a cervical host gene response profile that is diagnostic for PID could be detected, and also the findings support

that this study design and protocols being tested in this pilot study do work, with some potential for improvement.

4.4.1. Variation in gene expression is naturally different between cells and humans

Female genital tract secretions contain a wide variety of host defence molecules, along with adaptive immune system junctions for rapid response. To date, there has only been a few studies on this response in the context of pelvic inflammatory disease. In this study, it was found that lysozyme was significantly more highly expressed in cases than controls, although with a small sample size. In the actual samples 8/16 cases and 16/32 (note well, the extra cases and controls were not included in Chapter 2 or 3, due to ongoing recruitment as these haven't had their medical review at time of writing. Therefore only biospecimens received with no questionnaire or chart review, or 16S rRNA gene amplicon sequencing conducted) controls contributed to this signal, and of the 8 cases, 3/3 were from STI associated PID, 4/7 were from idiopathic associated PID and 1/2 from instrumentation (IUD) associated PID. However, differing populations and individuals show heterogeneity of responses to the same disease; this variability is classified as 'normal' [324, 325]. This variability has been reported for vaginal host response profiling (Figure 4.2), an example of this was shown by different studies in the 1990s whereby it was found that host response to the pathogen *Candida albicans* was variable amongst different people [326].

4.4.2. Lysozyme as an innate defence mechanism against pathogens

The *lyz* human gene codes for the enzyme lysozyme, which is an anti-bacterial protein, by targeting peptidoglycan a constituent of the cell wall. In an *in vitro* study of a pathogen community state type where a mix of 50% of *Gardnerella vaginalis* and 50% *L. crispatus, L. gasseri, L. jensenii, L. iners, Prevotella bivia, Bacteroides vulgatus, E. coli, Peptostreptococcus tetradius* and *C. albicans* it was found that recombinant human lysozyme along with metronidazole was able to significantly impact the biofilms of *G. vaginalis* and *L. iners* when compared to metronidazole alone or clindamycin alone [327]. Since most participants were found to have microbiome compositions consistent with CST III and IV (*L. iners* and *G. vaginalis* dominant respectively), this leads me to the point of considering whether the host response is releasing a sub-optimal concentration of lysozyme? Within the cervix, it is said that lysozyme is approximately 0.5ppm [322] while the study used 0.66ppm of lysozyme [327]. If hypothetically lysozyme was used in conjunction with antibiotics, it needs to be at a concentration that will be bactericidal, not that which is released by the human body. It would be valuable to investigate the secreted lysozyme in a wide range of PID cases and many studies state the importance of the vaginal first line of defence, which includes lysozyme [328-331].

In PID, the role of pattern recognition receptors (PRR) in recognising bacteria and viruses was an overexpressed pathway compared to controls, consistent with this, it was found that a single nuclear polymorphism in genes (MD2, CD14, TLR1 and CARD15) within this pathway were also associated with a high abundance of *G. vaginalis* and *A. vaginae* [332] (commonly found in women whose microbial CST is consistent with CST

IV). In another study using a 3-dimentional (3D) vaginal model, which was stimulated by colonisation of *L. iners,* the epithelia expressed multiple transcription factors (IRF1 and NFKBIA) and proinflammatory cytokines. Yet this activation only enhances cell-to-cell signalling and not cytokine secretion [333]. This is consistent with the findings in this present study, whereby it was determined that cell signalling was the cellular function that was found to be expressed at significantly higher levels in PID, and majority of cases had a cervicovaginal microbiota composition that was CST III (*L. iners* dominated). Further consistency with correlation of TLR5 with CST III, albeit it is a weak correlation that requires further investigation. However, due to study size and the interplay of epidemiological variance, specific genes were not found significant (i.e. when analysed by log-fold change and $\Delta\Delta C_t$) and further studies of the pattern recognition receptor pathway in PID, with a larger sample size needs to be conducted. This pilot study has informed that in future larger studies PRR pathways, cell death and signalling pathway and overall the innate immune system maybe a strong candidate for in-depth investigations.

4.4.3. Limitations

It is important to consider the limitations of this study, as designed as a pilot study to inform the parameters and protocols of a larger trial. Three other published studies identified different genes significantly upregulated in PID compared to their controls. Pentraxin 3 in one study on 64 PID compared to 70 controls was found to be significantly elevated in blood plasma levels [334]. In another study using enzyme-linked immunosorbent assay (ELISA) on blood plasma of 40 PID and 80 control participants, it

was found that the ratio of the matrix metalloproteinase-9 to matrix metalloproteinase-2 was predictive of PID [335]. These are proteinases responsible for the degradation of extracellular matrix in cells [336]. Neutrophil gelatinase-associated lipocalin (NGAL) is a stress secretory protein upregulated in epithelial cells under inflammation [337] which was found to significantly increase in plasma levels in 64 PID compared to 70 control participants; levels were found to reduce to control levels after PID treatment [338]. With all these significantly expressed genes mentioned above and in the introduction, this highlights the complexity and heterogeneity of the immune response in PID. However, these were all identified using blood as the testing medium, which I have mentioned in the introduction (section 4.1) is not routinely collected in most settings where PID is diagnosed. In fact, the genes alluded to in this paragraph are intended for the application of ELISA rather than a molecular test for RNA.

Whilst PID is an inflammatory condition, there would ideally be a broad investigation of many more genes, expanding upon the 84 innate and adaptive genes used in this study. Future studies should consider approaches such as RNA sequencing or a much larger set of genes for analysis such as by Nanostring nCounter™ [339, 340]. As seen in this Chapter and Chapter 3, DNA and RNA are difficult to extract from mucosal surfaces such as the vaginal and cervical swabs used here. Hence if RNA yield wasn't high or sufficiently pure for RNA sequencing, a panel for investigation of immune and metabolic pathways should be chosen with the pathways identified in Figures 4.7 and 4.8 considered. In addition, the significance of protein or RNA markers can then be validated to determine whether a biomarker panel is unique to PID compared to large cohorts of STI-positive women. A protein can be a biomarker, as this is currently being seen in

complicated cancer diagnosis, where mass spectrometry is used to identify proteins in participants [341]. However, a single gene is unlikely the answer, the aim in the future is to identify a set of markers at the cervical site that are diagnostically predictive of PID. As noted with this study and the aforementioned studies, it is challenging to recruit PID cases due to diagnostic methods currently in place (Chapter 1). Any future study design would ideally incorporate considerations around supporting what is needed for higher recruitment and in a larger number of sites to achieve the sample size.

However *lyz* in conjunction with an array of other significantly expressed genes could be predictive of PID, based on the results shown in Table 7, the following genes need further investigation and may play an essential role in diagnosis; CASP-1, IL18, and SLC11A1. As seen in Table 7 the p-values of ΔC_t for the case compared to control are 0.09, 0.09 and 0.07, and $\log_2$Fold change; -1.13, 1.70 and 2.98, respectively with a small sample size. In future studies, an increase in sample size would be needed to test if these are significantly upregulated in PID compared to controls, and also to compare to a STI infection group. In an animal model of the pathology of *C. trachomatis* (a known cause of PID as reviewed in Chapter 1) and the mouse analogue *C. muridarum* it was found that genetically caspase-1 (CASP-1) knockout mice had a significantly reduced upper genital tract pathologies compared to wild type mice after infection with bacterium [342]. If this gene is significantly expressed in future PID studies, could this lead to a furthering of the understanding of the inflammation process in PID, as seen in animal models? Furthermore, once CASP-1 is activated, it is able to process and secret mature IL18; another gene that should be studied in the context of PID [343, 344]. While the last gene mentioned SLC11A had not been studied well in the context of sexually transmitted

infections and consequent pathologies, it was shown that polymorphism in this gene is associated in increase infection of *C. trachomatis* and *Mycobacterium tuberculosis* [345, 346] (also a bacterium that can be detected within the female reproductive tract and cause PID [347]).

Furthermore, not every gene was detected in every participant. This could be due to many reasons, one possibility is the RNA sample quality, as this is from a cervix, time spent swabbing the site is minimal compared to the vagina (described in chapter 3.4). Unfortunately, technical replicates were unable to be conducted, these allows for the possibility of intra-RT-qPCR variability, this was combated by the positive PCR control (Kit supplied) and cDNA synthesis (Kit control) and threshold of detection. Whereby all of these controls had to result in the same Cq number (fluorescence number) on the RT-qPCR machine. Also, heterogeneity of responses should be expected as mentioned previously. Another issue that could be the reason is the amount of RNA extracted, firstly the reason for small amounts could be due to the high concentration of endogenous ribonuclease (RNAse) found in vaginal secretions during inflammation [348-351].

4.4.4. Future considerations

This is a novel study and being a pilot to evaluate the recruitment, study design and lab protocols in order to provide direction to future investigations in larger cohorts. Future improvements should consider the limitations experienced within this study. One critical example is the test group which had to be eliminated from further analysis for this component of the work. One contributing factor for low host signal detection is possibly

that these test group samples were held in storage for over three years; furthermore, shipment from Cairns to Sydney may play a role in the reduction of detectable signal. The location and time frames for extraction should be the same for future consideration. One other issue faced is that the test group was at a clinic for an STI check-up, and not all these women needed a cervical swab; hence this is a possibility as to why seven samples didn't have this swab.

Other considerations is the extraction protocol for RNA, in this Chapter, the kit protocol was followed. Due to the nature of mucosal samples, it leads me to the question of whether there are better collection or extraction methods that may prevent the high RNAse load from degrading sample RNA. Possibly the use of the RNA Direct-zol™ method as suggested by Alves *et al* [352], where they compared three extraction protocols (including the one used in this study) of oral swabs, and determined the RNA Direct-zol™ method yielded the highest and purest concentration of RNA. A future study with locally collected samples from the cervix to test and compare a few different extraction protocols should probably be conducted before a large PID cohort is recruited. Another point is that the participants didn't have an extra high vaginal swab for RNA extraction to compare to the cervical swab, could this swab be as valid or are PID markers highly tissue specific?

4.4.4. Conclusion

To date, this is the only study to my current knowledge that used a cervical swab to determine the host response by examining gene expression during PID. This method

for sample collection is important for analysis as it is already routinely collected in Australia already to diagnose any pathogens associated with PID. Although the sample size was small, this pilot study demonstrated lysozyme was a significantly more highly expressed gene in the cervical secretion of PID cases when compared to controls. However, more validation is needed in future studies with larger sample sizes and more genes included in the analysis. Further studies will allow for the vital opportunity to produce an accurate swab based diagnostic for PID.

Chapter 5 - Evaluation of genetic variants of Chlamydia, to examine if the loci are involved in virulence or pathogenesis that may interplay into pelvic inflammatory disease and other pathologies.

5.1. INTRODUCTION

5.1.1. *Chlamydia* and pelvic inflammatory disease

Pelvic inflammatory disease (PID) as discussed in Chapter 1, can be defined as, instrumentation (IUD), idiopathic, and pathogen associated. In terms of pathogen-associated PID, it is thought a pathogen ascends from the initial colonisation point of the endocervix or vagina, to the endometrium, and in some cases to the fallopian tubes [353]. Inflammation can occur at any point and the fundamental characteristics of PID include, but are not limited to, endometritis, salpingitis, and histological correlates of neutrophil influx (Chapter 1) [354].

The intracellular obligate Gram-negative organism *Chlamydia trachomatis* is responsible for 5-7% of hospitalised PID. In clinical settings, 39 -50% of cases of PID are due to *C. trachomatis* [202, 223] (Chapter 1). There are 15 serovars of *C. trachomatis* identified by the antigenic variation in the major outer membrane protein (MOMP), that are divided into three groups, A-C are ocular serovars, D-K are urogenital serovars, and L1-L3 are accountable for invasive lymphoma granuloma venereum [355]. Since the first publication of the full genome of urogenital *C. trachomatis* D/UW-3/Cx, there has been many sequenced strains of the different serovars with evidence that the species has a highly conserved genome [356]. It currently remains unclear what genetic loci(s) correspond to virulence functions or are associated with making variants more virulent. This is partially due to the lack of distinction (to date) of isolates correlating with specific pathologies or infection outcomes from the patients that they were derived. Further, while

mechanisms of cellular infection and *in vivo* models have been established, it is yet unclear as to the mechanisms of ascension in the human host that is a precursor to pelvic inflammation [357]. There have been few advancements in the characterisation of *C. trachomatis* due to the intracellular attributes of the organism. Only recently has there been some success in genetic modulation to understand virulence, keeping in mind the genetic modulation of *Escherichia coli,* has been established for over half a century [358]. Therefore, highlighting the significance of *in vivo* animal models and *ex vivo* sample collection of known isolates for the study of pathogenesis and virulence factors. Additionally, randomly generated mutants could be utilised in similar approaches, in order to investigate genetic loci associated with upper genital tract sequelae such as PID using *in vivo* models. However, there is a need for a suitable *in vivo* model for chlamydial pathology to be established.

5.1.2. *Chlamydia* and the murine model

Scientific investigation commonly relies on animal models to understand disease that could lead to the development of treatments or vaccines. Chlamydial research relies on the murine model, particularly in the vaginal infection of female mice, whereby pre-injection with progesterone increases susceptibility to organism [359]. For the model of sexual transmission, intravaginal inoculation of *Chlamydia* is performed, however, this inoculant load may differ and is typically much higher than that seen in humans [359]. In general most studies use *C. muridarum,* the mouse infecting species of chlamydia, that shows higher levels of infectivity and pathology that the human species. Mouse strains usually used are C57BL/6, BALB/C or C3H/HeJ, where C3H strains have been reported

to be more susceptible to human specific *C. trachomatis* [360-363]. The murine models are currently used either with human-specific *C. trachomatis* or with mouse-specific *C. muridarum*. However, strong immunopathological responses can only be monitored in the *C. muridarum* models due to the host-specific tropism of *C. muridarum* [364, 365]. Although, in some studies, the human specific *C. trachomatis* has been shown to induce salpingitis in mice (with less frequency) [366, 367], hence could variants of *C. trachomatis* with more pathogenic properties induce PID or salpingitis? Interestingly, *C. trachomatis* genetic variation has been used to study pathogenic factors [367-369], an example with a mutation in the plasmid open reading frame (CT135) impacted virulence in mice [367]. Specifically, the value of animal models should not be underestimated, in the case of *Chlamydia*, because *in vitro* experiments alone cannot shed light on the pathogenic properties of the organisms.

The C3H mouse model has some potential to show differences with *C. trachomatis* infections based on the previous data [360-363]. Although potentially very marked *in vivo* differences in infectivity and survival between *C. trachomatis* isolates may be apparent in C57BL/6 strains too. In both models there are real difficulties in seeing the level of pathology in mice infected with *C. trachomatis* and that in commonly used models like BALBC or C57BL/6 there is very little infection established and no pathology [368, 370]. Hence the preferred use of *C. muridarium* or development of more complex models described in Chapter 1. Hence, it is vital to note that since pathology detection is so low in any strain using *C. trachomatis,* in order to detect a difference, the variants must have a substantial biological impact on the mouse.

5.1.3. Chlamydial pathogenicity

While chlamydial serovars have been identified and characterised, especially urogenital serovars, there remains a need to untangle genetic factors that contribute to disease sequelae. It is currently unknown as to why only a portion of women with infection develop sequelae such as PID [371]. One consideration is that genetic variants of *Chlamydia* are not screened for in the current population and medical lab-based diagnostic protocols, so any contribution of strain features of chlamydia would not be known. A close relative of *C. trachomatis, C. muridarum,* a rodent pathogen displayed attenuated ability to infect the upper genital tract *in vivo* where mutations in specific genetic loci where characterised [372], supporting that particular loci may well be associated with more pathogenic outcomes in human infections. It was also found that chlamydial variants that lack a plasmid are less virulent than those with a plasmid [373-375].

5.1.4. Chlamydial variants

Variants of *Chlamydia trachomatis* were previously produced in our team via random chemical mutagenesis and a selective pressure experiment for resistance to a specific protease inhibitor (JO146) [376-379]. Whilst these variants are not expected to be related to PID, they do provide an opportunity to begin to investigate the animal model and genetic variation of *C. trachomatis*. Briefly, low-level ethyl methanesulfonate (EMS) was used as a mutagen since the incorporation of C-G into T-A transition mutations has

been previously demonstrated [380]. At 22 hours post-infection, infected host cells were exposed to EMS at 37ºC for one hour. The medium was removed and supplemented with fresh media containing 1 µg/ml cycloheximide following treatment with EMS, and the cells were incubated at 37ºC, 5% CO_2. Infected monolayers were collected and preserved at-80ºC in aliquots. Genetic variants were isolated by re-culturing in McCoy B cells and HEp2 cells to generate culturable pools of variants. Serial passage of chlamydia chemically mutated pools in the presence of JO146 selected for the survival of more resistant (or less susceptible) strains from the pools of mutants (26 passages of selection). Four of these cultures (1A, 1B, 2A, and 2B) were found to be less sensitive to the inhibitor when tested in the presence of JO146. Clonal isolates were cultured using plaque purification, and resistance of these isolates to JO146 was confirmed for up to 125 µM JO146. The isolates identified to be least susceptible to JO146 were termed 1B3 and 2A3, and these two isolates were further analysed here. The protease inhibitor used to produce these variants target the *Chlamydial* high temperature requirement A, which is a serine protease and chaperone. As the inhibitor illicit its effect this may causing an altered chaperone activity resulting in a difference in metabolism. Hence difference in proteins that scavenge for fatty acids.

Genomic variant characteristics enable the detection of single nucleotide variants (SNVs): both 1B3 and 2A3 had SNVs in the CT206 gene and were noted as acyltransferase. Both variants had SNVs in gene CT776, which encode for acylglycerophosphoethanolamine acyltransferase (aas), involved in fatty acid activation. In *C. trachomatis*, fatty acid chains can be synthesised using the type II FA cycle, and by acquiring the precursors (isoleucine, serine or glucose) from the host cell so *Chlamydia*

can make phospholipids [381]. There is no desaturase present in the genome (that oxidases the fatty acid chain and introduces a double bond), instead, *Chlamydia* has an acyl-acyl carrier protein (CT776 or Aas) that activates fatty acids acquired from the host cell to transfer them to chlamydial fatty acid and phospholipid synthesis [382]. This function has evolved in *Chlamydia* by separation of a single gene acyl-acyl synthase-transferase present in other bacteria into two functional genes [382]. It has substrate specificity for mono-unsaturated host cell fatty acids such as laurate. The fatty acids chains are extended to myristate and palmitate, or used for the synthesis of lipid A. Thus increasing the chlamydial repertoire of FA and likely also reducing energetic costs of fatty acid synthesis [382]. Although, type II FAS inhibitors may block the growth of *Chlamydia*, indicating autonomous synthesis is another major pathway in addition to the acquisition from the host [383]. Recently, host long-chain fatty acids synthases have been detected to be inside the chlamydial inclusion vacuole, and inhibition of these host proteins impeded chlamydial growth, indicating that chlamydial activation and use of host cell fatty acids are also critical [384]. Fatty acid composition of the lipid bilayer (both free fatty acids and as part of the phospholipids) determines the fluidity and structure of the membrane, can influence microdomains in the membrane critical for some proteins functions, and can influence cell signalling [385]. In this chapter, we will test the hypothesis that the fatty acid composition is altered by the CT776 (and CT206) mutations detected in these variants by profiling the fatty acid compositions, and explore the implications of these genetic variants for virulence using the mouse model. The impact of these variants on the cellular biology or pathogenesis of the *Chlamydia* has not been fully determined. The fatty acid composition of the *C. trachomatis* variants 1B3, 2A3 is

the focus of this analysis, in comparison to wild type *C. trachomatis* serovar D (UW-E/Cx) (CtD)

This study aimed to characterise chlamydial variants (1B3 and 2A3) using an *in vitro* and *in vivo* approach. This would be beneficial for future PID studies to have the capacity to show large differences in *in vivo* infectivity or pathology between *C. trachomatis* isolates, should PID relate to pathovariants rather than (or in addition to) variation in host responses. One isolate of C. trachomatis was cultured from this pilot study, although it was not further analysed, so further in vitro work was commenced with laboratory strains. In order to commence work to test and established a framework for PID *in vivo* work in future, this study took advantage of the existing chlamydial variants that may show large *in vivo* phenotypes. Given the functional impact of the amino acid change is not understood, such a big difference may have occurred *in vivo*, even though relatively little (no) impact was seen *in vitro* – but is that because *in vitro* the fatty acid supply is probably very high because it is a nutrient rich environment The overarching aim is to see whether variants of a pathogenic organisms have different pathogenic features to wild type that can be detected *in vivo* and *in vitro* that may help to establish a tool for animal model investigations of pathogenic variants associated with PID. More specifically this chapter aims to:

1. Analyse the fatty acid composition of the chlamydial variants relative to the wild-type strain (*C. trachomatis* serovar D (UW-E/Cx) (CtD)).

2. Determine if the variants had a different susceptibility to triclosan, a fatty acid inhibiting antibiotic, compared to the wild type strain.

3. Establish an *in vivo* mouse infection measured by shedding of chlamydial variants amongst two mouse strains

4. Conduct an *in vivo* mouse model to determine whether the variants were more likely to cause pathology in the mouse.

5.2. MATERIALS AND METHODS

5.2.1. Buffers and reagents for chlamydial culture

5.2.1.1. Supplemented Dulbecco's Modified Eagle's Medium - high glucose

Chlamydia was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5g/L) (D6546, Sigma-Aldrich, Australia), supplemented with 10% heat inactivated (54ºC for 45 minutes) foetal calf serum (FCS) (12003C-500ML, lot 14J211, Sigma-Aldrich, Australia), 10 mL of 200 mM of L-alanyl-l glutamine (Sigma-Aldrich, G8541-100ML, Germany), 50mg/mL of gentamicin (15750078, Life Technologies, New York) and 100mg/mL of streptomycin sulphate (11860038, Thermo Fisher Scientific, New York).

5.2.1.2. Dulbecco's Phosphate buffered saline (dPBS)

dPBS was used in wash buffer for staining and culture. 9.6 grams of Dulbecco's phosphate buffered saline (dPBS) modified without calcium chloride and magnesium chloride powder (D5652-50L, Sigma-Aldrich, Germany) was added to 1 L of milli Q H2O. This solution was autoclaved at 121ºC for 15 minutes (Systec D-200 Horizontal Benchtop Laboratory Autoclave, Systec GmbH, Linden, Germany).

5.2.1.3. Chlamydia storage buffer (SPG)

Chlamydia was stocked and stored in Sucrose phosphate glutamate (SPG) solution. SPG was prepared by combining; 13.68 mL of 0.5 M of di-sodium hydrogen phosphate dihydrate (71643, Sigma-Aldrich, Germany), 6.32 mL 0.5 M of sodium di-hydrogen phosphate monohydrate (S9638, Sigma-Aldrich, Germany), 0.736 g L-glutamic acid (A37840IN, Life Technologies, New York) and 85.575 g sucrose (15503022, Life Technologies, New York) in 900 ml Milli-Q water. The pH was adjusted to 7.2 and the total volume of the solution was adjusted to 1 L with Milli-Q water. The solution was filter sterilised using 0.2 µm pore-size filters (Corning, NY, U.S.A.), aliquoted in 50 mL tubes stored at 4ºC.

5.2.2. Chlamydial culture conditions

5.2.2.1. Mammalian cells used for chlamydial culture

McCoy B (ATCC: CRL-1696) cells were used for the culture of *Chlamydia,* particularly experiments where high yields for fatty acid analysis were required. The organism used were *C. trachomatis* serovar D (UW-E/Cx), *C. trachomatis* variant 1B3 and *C. trachomatis* 2A3, for the rest of the chapter they will be referred to as CtD, 1B3 and 2A3 respectively. The variants used in this study are described in Table 5.1. All *Chlamydia* used were cultured in DMEM and incubated at 37 ºC, 5% CO_2 (Heracell™ 150i CO2 Incubator, Thermo Fisher Scientific, USA). *Chlamydia* was used to infect T-75 culture flasks (156472, Thermo Fisher Scientific, USA) with 90% confluent McCoy B cells. The flasks were centrifuged at 500 × g for 30 minutes at 28 ºC. DMEM media containing 1 µg/mL of cycloheximide was replaced with fresh media at 4 hours (h) post-infection (PI), to inhibit protein synthesis in McCoy B cells. The cells were incubated up to the end of the chlamydial developmental cycle (i.e., 44 h PI) and were harvested by replacing the media with three mL SPG per flask. By using a cell scraper, the infected monolayer was gently removed from the flask. The cell lysate was transferred to a 50 mL Falcon tube containing sterile glass beads to an equivalent of 5 mL. The tubes were vortexed for two minutes. The cell lysate was then transferred to a new Falcon tube and was centrifuged at 800 × g for 10 minutes at 1 ºC to separate the host cell debris from Chlamydia. The supernatant was removed and aliquoted in 1.5 mL cryovials and stored at -80 ºC for further processing.

Cultures for high yields for fatty acid analysis were conducted by culturing four confluent (T-75) flasks for each variant and infecting with an M.O.I (Multiplicity of infection) of 3. These were then incubated at 37ºC with 5% CO_2 for 42 hours and harvested as described above. This process was completed for each strain individually and then repeated on different days for experimental replicates to achieve three harvests for each strain. These culture and isolation conditions were used for fatty acid analysis, triclosan sensitivity and purifying for mouse infection.

Table 5.1.Genomic characteristics of JO146 resistant isolates*

Gene locus[a]	**1B3**			**2A3**		
	Position[b]	SNV (nt[c])	Effect (aa[d])	Position[b]	SNV (nt[c])	Effect (aa[d])
CT206	232574	G→A	G→S	232340	C→T	Q→stop
CT390	-[e]	-	-	444970	C→T	Synonymous
CT404	-	-	-	463177	C→T	Synonymous
CT414	-	-	-	481998	C→T	A→V
CT474	-	-	-	548301	C→T	G→R
CT664	-	-	-	762369	G→A	A→T
CT776	911018	C→T	R→C	911217	G→A	S→N
CTDEC_p001	-	-	-	-	-	-
None (non-coding)	-	-	-	368480	C→T	non-coding

a: Gene protein products: CT206 = putative esterase; CT390 = LL-diaminopimelate aminotransferase; CT404 = SAM-dependent methyltransferase; CT414 = outer membrane protein pmpC; CT474 = uncharacterised protein; CT664 = adenylate cyclase-like protein; CT776 = acyl-acyl carrier protein synthetase; CTDEC_p001 = virulence plasmid integrase pGP7-D. Annotations retrieved from UniProtKB [386] with exception of CT776, obtained from Yao et al. 2015 [382], and CTDEC_p001, obtained from GenBank [387]

b: The wild type nucleotides noted with position in the genome

c:This is followed by letters in wild type, G = guanine, C = cytosine, followed by an arow indicating the variation in nucleotide. This was noted with letters; T = thymine or A = adenine

d: Variation in nucleotide resulted in change in codon (different amino acid). The arrow indicates the change from wildtype to variant. These acids are denoted by letters; R = arginine, C = cysteine, S = serine, N= asparagine, G = glycine, Q = glutamine, T = threonine, A = alanine, V = valine, stop indicates a stop codon, Synonymous indicates no change in amino acid and non-coding indicates codon does note recruit amino acid.

e: no change found indicated by the dash symbol "-"

*: reproduced from unpublished Huston lab work [378], all pathovariants were confirmed to still have the mutations by Natalie Strange prior to experimental work. Summary of results from the whole genome sequencing was also also conducted by Natalie Strange

5.2.2.2. Purification of Chlamydial culture

Purification of culture was vital as it is required to remove all host cell materials (McCoy B) for the further use in *in vivo* experiments and in order not to detect the fatty acids in the host cell. The stock solution of each replicate and strain was thawed and centrifuged at 18 000 ×g for 30 minutes at 4ºC. The supernatant was then discarded and for each individual replicate and strain, pellets were combined in a solution of 200 µL of SPG, resulting in 3 x 200µL experimental replicates for each strain.

5.2.3. Enumeration of inclusion forming units of chlamydia

A 96 well plate (Nunclon® MicroWell plates for automation 96 well (with lid), flat bottom, clear, 137103, Thermo Fisher Scientific, USA) was seeded with 20 000 cells per well (McCoy B). The monolayer was infected in a serial dilution with the chlamydial strains in triplicates. Infection was stopped at 40 hours (unless otherwise stated) hours post-infection (h PI) with methanol fixation. Chlamydial immunocytochemistry was conducted by permeabilising the cells with 0.5% Triton X-100 in dPBS (CAS Number 9002-93-1, Sigma Aldrich), followed by blocking with 1% Bovine Serum Albumin with dPBS (BSA 9048-46-8, Sigma Aldrich). Primary antibody (anti-HtrA rabbit sera at 1:500, with dPBS) along with DNA stain that shows the host cell nucleus clearly (DAPI CAS Number; 28718-90-3, Sigma Aldrich at 1:40000) was added to each well for 45 minutes (dark). Wells were washed four times with 0.2% PBST (dPBS with 0.2% Tween20) followed with the addition of secondary fluorophore-conjugated antibody (1:600 Anti-Rabbit IgG (H+L), CF™488A antibody produced in F(ab')2 fragment of goat Sigma Aldrich with PBS) for 45 minutes (dark). Wells were washed five times in PBST and stored in dPBS until analysis by microscopy.

The GE In Cell Analyser 2200 was used to image plates for enumeration of infectious chlamydia. Ten images were routinely collected from each well of the 96 well plate using the 10 × objective. The channels that detected in the fluorescence for FITC (475 – 650 nm) and DAPI (358 – 461 nm) were merged to ensure inclusions visible were in the host cell (quality control measure). *Chlamydial* inclusions were counted by

choosing a row were the row below and above the selected row decreases accuracy of counting range. For each strain there were 30 images with cell counts, the IFU/ml is calculated by the following formula: IFU/ml = Field of view (‘17’[based on 10 × objective and plate dimensions]) × cell count × dilution × infection volume. The data presented is the mean of n = 30 of this IFU/mL, and typically the standard error of the mean is presented. Each chlamydial variant was then standardised, so each variant was at 1×10^7 IFU/mL by dilution in SPG.

5.2.4. Western blot

A western blot was conducted to confirm the presence of chlamydial protein and the absence of host protein in the purified strains. 4 µl of each purified strain were prepared by adding 1 × sample buffer (NuPAGE™ LDS Sample Buffer 4 ×) and 1 × reducing agent (Invitrogen™ Novex™ 10X Bolt™ Sample Reducing Agent) to total reaction volume of 30 µl. Samples were heated at 100˚C for 10 minutes and centrifuged at for 10 min at 21 000 × g. These samples, with a molecular weight standard (SeeBlue Plus2 Prestained Standard (ladder), 500 µL) were run in a mini tank cassette gel at 200 V for 20 minutes. Western blot transfer was conducted using the life technologies iBlot2 transfer device in accordance with the manufacturer's instructions and protocol P0 (20-25V) for 7 minutes.

After transfer, the membrane was removed and blocked with TBS (tris-buffered saline) 0.1% skim milk powder for one hour on platform rocker at room temperature.

Anti-alpha-tubulin (1:5000) or anti-MOMP (1:500) was added in blocking buffer (TBS with 0.1% skim milk powder) for one hour at room temperature. The membrane was washed with TBST (TBS with 0.1% Tween20) four times, for five minutes each. Secondary HRP-conjugated antibody (1:10000) was added to 10 ml of TBS and incubated for one hour on a rocker at room temperature. Six TBST washes were conducted as described above.

Antibody-HRP binding was detected using room temperature ECL detection kit (ECL™ Prime Western Blotting System GE Healthcare, RPN2232) in accordance with the manufactures instructions. After incubation in the dark with the ECL reagent for five minutes, the chemiluminescence signal was detected using the GE Amersham imager 600.

5.2.5. Infectivity of variants

As different variant strains were used it was important to check that at the same MOI the same percent of host cells are infected for each strain. A 96 well plate (Nunclon® MicroWell plates for automation 96 well (with lid), flat bottom, clear) was seeded with 20 000 cells per well (McCoy B). The monolayer was infected at three different M.O.Is (0.2, 0.5 and1.0) in triplicates for each strain. Infection was stopped at 44 hours using a fixative, and immunocytochemistry to detect chlamydial inclusions and cell imaging was conducted as per section 2.3. Percent infectivity was calculated using the following formula: $\% = \frac{IFU}{host\ cells} \times 100$

5.2.6. Fatty acid extraction

Fatty acids were extracted from Chlamydia cultured to high yield (as per section 5.2.1.1) and isolated then purified as per section 5.2.1.2. The protocol for extraction of the fatty acids was in accordance with that protocol previously published [388]. Reagents for fatty acid extraction included: Reagent one (Saponification) contained a mixed solution of 45 g sodium hydroxide, 150 mL methanol and 150 mL of distilled water. Reagent two (Methylation) had a mix of 325 mL 6 N hydrochloric acid and 275 ml of methyl alcohol. Reagent 3 (Extraction) contained 200 mL hexane mixed with 200 mL of methyl-butyl ether. Finally, reagent four (Clean up) was a mix of 10.8 g sodium hydroxide dissolved in 900 mL distilled water.

40 µL of the purified standardised *Chlamydial* stock (1×10^7 IFU/mL, section 5.2.3) along with 500 ppm (500 mg/L) of internal standard (Methyl all-cis-7,10,13,16,19-docosapentaenoate, Sigma-Aldrich, Germany) was added to individual glass test tubes (with cap) followed by 1 mL of reagent one. This mixture was briefly vortexed before being placed in a heating block at 100 ºC for five minutes. The tubes were then vortexed for 15 seconds before being incubated at 100 ºC for 30 minutes. Tubes were then allowed to cool to room temperature before 2 mL of reagent two was added. This mixture was briefly vortexed before being heated for 10 ± 1 minutes at 80 ± 1 ºC. The tubes were allowed to cool before the extraction of methylated fatty acids. 1.25 mL of reagent three was added to each tube and placed on a clinical rotator at medium speed for 10 minutes. The tubes were then uncapped and the lower phase was pipetted out and discarded. Three mL of reagent four (wash) was added and tubes placed on a clinical rotator for five

minutes at medium speed. The upper organic phase was then pipetted into a gas chromatography (GC) vial for analysis by mass spectrometry.

5.2.6.1. Analysis of fatty acid composition using mass spectrometry

Fatty acid composition was analysed using a previously published protocol of gas chromatography-mass spectrometry (GC-MS) [388]. An external standard was used for quantifying fatty acids (Bacterial Acid Methyl Esters; BAMEs; 47080-U, Sigma Aldrich) when analysing the extracted samples from the isolates. In order to construct a standard curve the BAME solution was diluted in a series of 1, 10, 50, 75, 100 and 750 ppm before being placed in a GC vial for injection.

The fatty acids compared to BAMEs were analysed using the GC system coupled to 5973 Network MS with auto-injector (7683B series) and Auto sampler (7683 series) (Agilent 6890). The injection volume is set to one µL with the inlet temperature at 250 ºC. The injection sample was diluted in a split ratio of 5:1 (diluted five times) before separation of phases using the column (length 30 m, diameter 0.25 mm, film thickness 0.25 µm, Agilent HP-5MS). As the sample proceeded through the column the oven temperature was held at 135 ºC for four minutes; subsequently, the temperature was increased at 4 ºC per minute to reach 203 ºC. Following, the oven temperature is raised at 1 ºC per minute till 209 ºC is achieved, the temperature is raised at 4 ºC per minute till 250 ºC is achieved were it is held for 10 minutes. Finally, the oven temperature was increased at 20 ºC per minute till 290 ºC where this temperature is held for five minutes. This process was then repeated for every sample injection. The samples were then

processed through the MS instrument with a solvent delay of two minutes before a scan was conducted for particles between 29 to 450 Daltons. Analysis was conducted based on the intensity peaks of BAMES fatty acids (Figure 5.1) where retention time is the x-axis, (time it takes for fatty acid to come out of the column and register with machines detector) and intensity count the y-axis. In brief, the area of under the peak of the each diluted BAMES sample (mentioned above) was used to create a graph, x-axis = ppm (dilution series) and y-axis = area under the peak, this generated a formula for each BAMES acid (Appendix D). An example formula for undecanoate was: y=15733x-183008, using the area under the peak of the chlamydial samples (the y value), 'x' was calculated and this resulted with the concentration of acid.

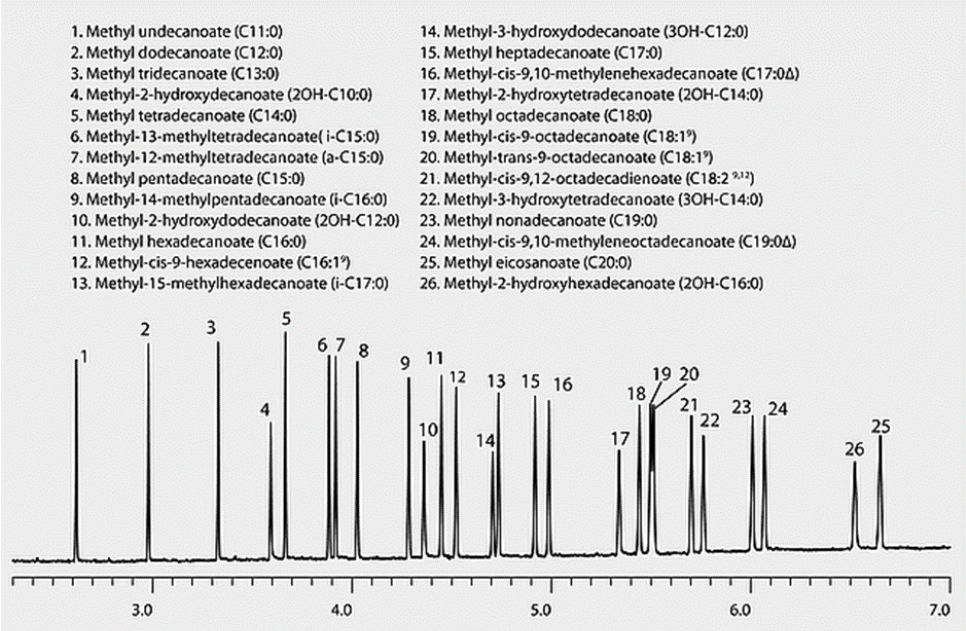


Figure 5.1. Constituent acids found in BAMES. This graph shows the 'retention time' (time taken for acid to get to detector in the machine) as the x-axis and intensity count as the y-axis. Larger acid have a longer retention time then smaller sized acids. (Reproduced from Sigma Aldrich, USA)

5.2.7. Analysis of susceptibility to triclosan

The effects of triclosan (a known inhibitor of fatty acids synthesis) on CtD, 1B3 and 2A3 were determined. Purified *Chlamydia trachomatis* CtD, 1B3 and 2A3 were cultured as per sections 5.2.1 – 5.2.2 and used for a susceptibility experiment in a 96 well plate (plate one) (Nunclon® MicroWell plates for automation 96 well (with lid), flat bottom, clear) that was seeded with 20 000 cells per well (McCoy B). The monolayer was infected with each chlamydial strain at a multiplicity of infection of 1.0. At 16 hours the infected monolayer was treated with five different concentrations of triclosan (0, 25, 100, 250 and 400 µg/mL) and two controls (100 nM of JO146 and 70% ethanol (solvent for triclosan)). The infection was stopped at 40 hours post-infection where 200 µL of SPG was added to each well and plate stored at -80 °C.

Chlamydial enumeration was then conducted as per section 5.2.3. Specifically, in cultures of 20 000 cells per well (McCoy B) in 3 × 96 well plates (Nunclon® MicroWell plates for automation 96 well (with lid), flat bottom, clear). The thawed infected monolayer from plate 1 was lysed using a pipette tip and used to infect the new monolayers in the same format (columns A-H, rows 1-12) using a dilution series (10^{-1} - 10^{-3}). Enumeration of chlamydial inclusions was conducted.

5.2.8. Mouse model of chlamydial reproductive tract infection

The *in vivo* growth and pathology inducing characteristics of the variant 1B3 in comparison to WT was addressed using infection models in female C57BL/6 and

C3H/HeJ mice. The C3H/HeJ mouse experiment also included the variant 2A3. The C3H/HeJ strain is known to have a prolonged and more intense infection of the human-specific *C. trachomatis* [360] strains (compared to the *C. muridarium* agent typically used in mouse models). Two groups of 10 mice were infected with CtD (wild type) and variant 1B3. Group 3 was the control which consisted of 5 mice infected with SPG only. The C3H/HeJ repeat experiment used one more group of 10 mice infected with variant 2A3.

Mice were subcutaneously injected with progesterone to synchronise the menstrual cycle seven days prior to the initiation of chlamydial infection. The mice were infected by vaginally administering of ultra-purified *Chlamydia* (1 $x10^7$ IFU/20 µL) or SPG with a volume of no more than 20 µL. Then 3, 7, 10 14 and 25 days post-infection the vagina was swabbed using a paediatric swab. The swab sample was stored in SPG and frozen at -80ºC for further analysis. At 26 days, post-infection all mice were culled and a series of pathology measures conducted. The first measure was the oviduct size. The right ovary and fallopian tube were placed in individual tissue cassettes and placed in formaldehyde for storage at room temperature for subsequent histological analysis. This procedure is seen in the flow chart in figure 5.2. It is important to note, mice infection, swabbing and culling was conducted at the University of Newcastle by Dr Jemma Mayall. All animal ethics and considerations were obtained by Dr Jemma Mayall for that site and full animal ethical approval was granted for this work

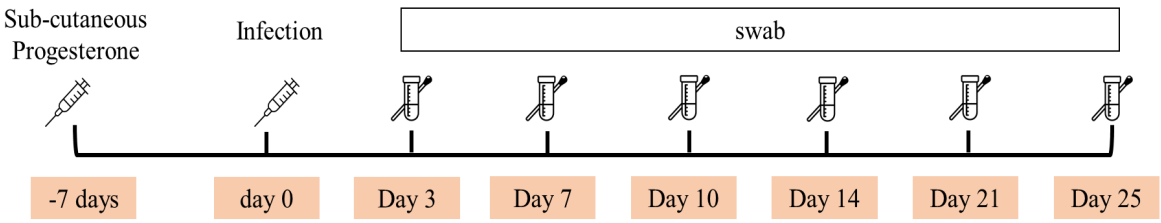


Figure 5.2. Mouse model flow chart. This shows the steps for mouse infection models. Injection with progesterone before Chlamydial infection followed by infection of WT(CtD), 1B3, 2A3 SPG. Swab collection days and final harvest on the 26th day.

5.2.9.1. Determination of chlamydial shedding from mouse

Using the same methods as section 5.2.3, the mouse swabs (6 in total per mouse) were used to determine the *chlamydial* shedding from the infection models. Comparison of shedding for the genetic variants and wildtype was conducted using analysis described in section 5.2.10.

5.2.9.2. Histological analysis of mouse tissue

Tissues were processed and stained with haematoxylin and eosin for histological analysis using published protocols [389]. In brief, cassettes were removed from the storage formaldehyde and placed in the Excelsior™ AS Tissue processor (Thermo Fisher Scientific, A82300001). Routine overnight processing was commenced on tissue samples that involved submerging the tissues in alcohol, xylene, and finally paraffin wax,

respectively. The processed tissues were then embedded with paraffin wax using HistoStar™ Embedding Workstation (A81000001, Thermo Fischer Scientific). The embedded tissue was then cut in 5 µm sections and place on a glass slide, and heat fixed. Using the Gemini AS Automated Slide Stainer (A81500001, Thermo Fischer Scientific) the haematoxylin and eosin stain program was selected and slides with tissue sections were stained.

5.2.9.3. Chlamydial chromosomal copy to enumerate yield

To confirm cell culture enumeration of chlamydial isolates a commercial TaqMan gene expression assay (Ba04646249_s1, 4448489 ThermoFisher Scientific) targeting the *tarp* gene was used for RT-qPCR as per manufacturer's instructions.

5.2.10. Statistical and graphical analysis

IFU/mL per day per mouse group, fatty acid analysis, and triclosan susceptibility data was graphed and analysed using GraphPad Prism version 8.00 [203]. This was used to calculate means, standard error of the mean (SEM) and p-values using independent non-parametric Mann-Whitney test and Kruskal–Wallis [204] where data was assumed not to be distributed normally. Turkey's non-parametric multi-comparison was also used for percent infectivity analysis. Where differences were observed between groups to a p-value of less than 0.05, this was considered significant.

5.3. RESULTS

5.3.1. Chlamydial purification showed no trace of host cell

Chlamydial variants were purified to completely remove traces of host cells as it was vital that there were no host cell contaminants for the experiments both *in vivo* and *in vitro*. Western blot of the proteins tubulin and chlamydial major outer membrane protein (MOMP) showed no detection of host cell tubulin within chlamydial samples (Figure 5.3). MOMP was detected in the purified samples indicating that *C. trachomatis* was present with the absence of host cell materials. Therefore *Chlamydia* samples were free from host cells and can be used in the experiments without host cell contamination that would be problematic.

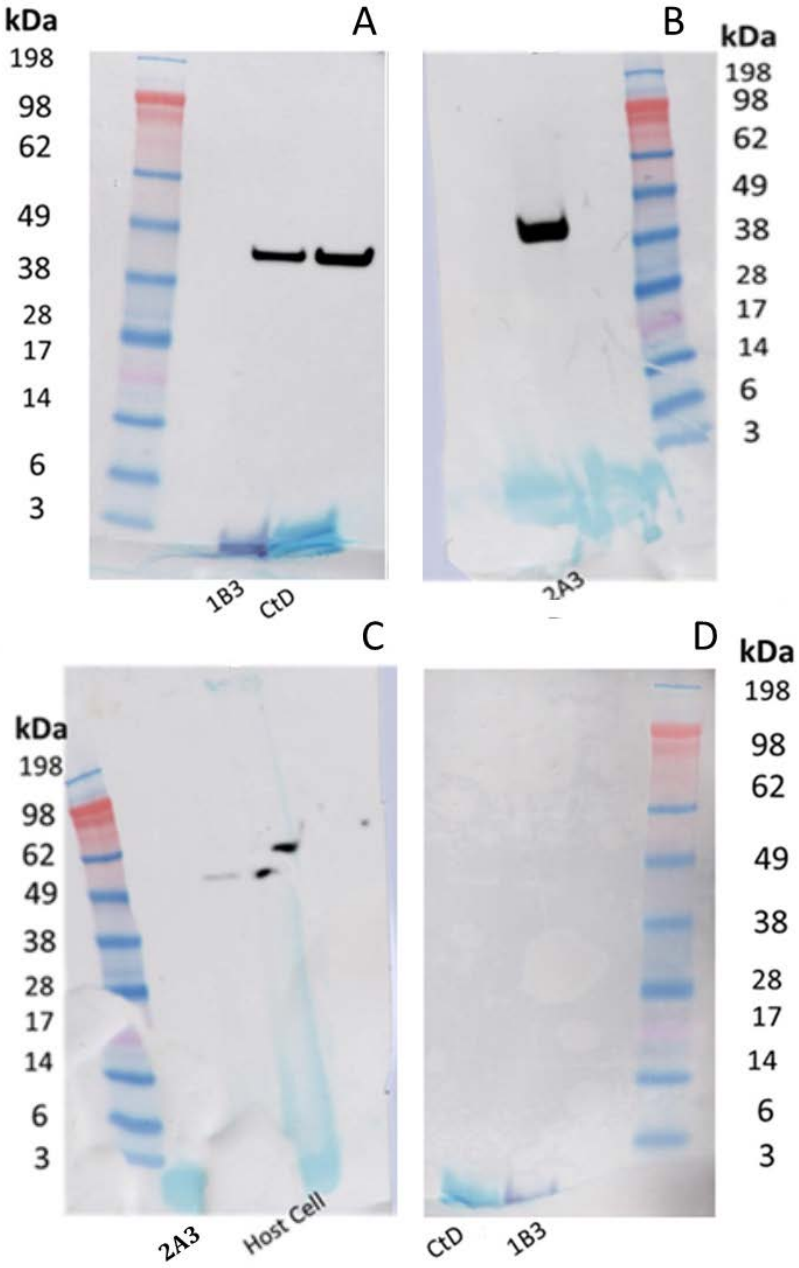


Figure 5.3. Western blot membrane for detection of Chlamydial MOMP. MOMP western blots are shown in A and B: Wild Type (noted as CtD), 1B3 and 2A3. Alpha-tubulin western blot is shown in panel C and D, where none of the Chlamydial samples had a positive host cell contamination. Panel A shows MOMP detected in samples 1B3,

wild type and Panel B shows the same for 2A3. Panel Cand D shows no detection of tubulin in samples with panel C containing the positive control (host cell).

5.3.2. *Chlamydia trachomatis* variants had a greater infectivity than the wild type

Differences in *Chlamydial* variant infectivity were assessed by determining the percent of inclusions formed in cell cultures after infecting host cells with different multiplicity of infection (MOI). Table 5.2 displays the percent infectivity in McCoy B cells calculated as per section 5.2.6. with standard deviation. The variants have significantly higher percent infectivity than wild type in MOI concentrations 0.5 to 1.0. Using the MOI of 0.2 the variant 2A3 had a significantly higher percentage infectivity than wild type.

Table 5.2. Percentage infectivity of the different variants

MOI[a]	**CtD%** ± SD[b]	**1B3%** ± SD[b]	**2A3%** ± SD[b]
0.2	7.13 ± 0.35	8.07 ± 0.32	11.16 ± 0.41
0.5	13.03 ± 0.65	18.48 ± 1.14	29.68 ± 1.06
1.0	23.29 ± 1.17	28.75 ± 1.25	42.85 ±1.62
	p-value[c]		
	CtD vs 1B3	**CtD vs 2A3**	**2A3 vs 1B3**
0.2	0.7762	0.0113*	0.0706
0.5	0.0003*	<0.0001*	<0.0001*
1.0	0.0003*	<0.0001*	<0.0001*

a: multiplicity of infection used for infecting monolayer with the bacteria

b: mean percent infectivity calculated by number of inclusions ÷ number of cells × 100, with standard deviation

c: p-value calculated using Turkey's non-parametric multi-comparison

5.3.3. Chlamydial variants contained a distinct fatty acid profile from the wild type

Fatty acids were detected in wild type and variants, with 16 acids detected in at least one sample (Table 5.3). Of the 16 acids, four were unsaturated fatty acids, three with one double bond and one with two double bonds. One unsaturated fatty acids of note was cis-9 octadecenoic acid, and this was detected in the variants but not wild type (1B3: 73.8±1.9 µg/mL and 2A3: 182.6±3.3 µg/mL, p-value <0.0001). Cis-9,12 octadecadienoic acid, was not detected in 1B3 but was found to be present at a higher concentration in 2A3 compared to wild type (CtD: 55.7±33.3, 2A3: 60.4±1.8 µg/mL, p-value =0.0125) Trans-9-octadecenoic acid was detected in significantly higher concentrations in wild type then variants (CtD: 165.7±24.6 µg/mL, 1B3: 24.9±3.4 µg/mL, 2A3: 48.7±1.0 µg/mL and p-value <0.0001). cis-9,10-methylene-octadecanoic acid was only detected in wild type (156.8±65.6 µg/mL, p-value <0.0001). These selected fatty acids are shown graphically in Figure 5.4.

Table 5.3. Fatty acid detected within Chlamydial variants

	Amount (µg/ml ± SEM)[b]			**p-value (two way ANOVA)[a]**		
Acid	**CtD**	**1B3**	**2A3**	**CtD vs 1B3**	**CtD vs 2A3**	**2A3 vs 1B3**
undecanoic acid (C11:0)	36.5 ± 0.9	31.5 ± 1.9	36.2 ± 2.5	0.9686	0.9999	0.9722
Dodecanoic acid (C12:0)	33.9 ± 0.5	27.6 ± 1.7	45.3 ± 2.7	0.9506	0.8473	0.6716

Tridecanoic acid (C13:0)	102.7 ± 2.2	20 ± 3.6	597.2 ± 19.1	0.0004*	<0.0001*	<0.00001*
tetradecanoic acid (C14:0)	34.1 ± 2.5	14.7 ± 1.6	24.4 ± 1	0.6202	0.8869	0.8869
13-methyltetradecanoic acid (i-C16:0)	76.1 ± 29	-	45.4 ± 2.5	0.0012*	0.3060	0.00788
12 methyltetradecanoic acid (a-C15:0)	45.4 ± 25.6	47.5 ± 2.1	115.7 ± 0.8	0.9944	0.0029*	0.00041*
cis-9-hexadecenoic acid (C16:1)	15.2 ± 1.5	13.8 ± 0.4	31.9 ± 0.6	0.9975	0.7015	0.6596
hexadecanoic acid (C16:0)	8.5 ± 0.2	-	17.7 ± 1.1	0.9119	0.8976	0.6716
15-methylhexadecanoic acid (i-C17:0)	112.8 ± 45.4	77.5 ± 1	153 ± 1.5	0.2107	0.1344	0.00013*
Heptadecanoic acid (C17:0)	24.2 ± 1.3	20.8 ± 0.6	20.1 ± 4.5	0.9853	0.9788	0.9994
cis-9,12 octadecadienoic acid (C17:2)	55.7 ± 33.3	-	60.4 ± 1.8	0.0232*	0.9722	0.0125*
cis-9 octadecenoic acid (C18:1)	-	73.8 ± 1.9	182.6 ± 3.3	0.0017*	<0.0001*	<0.00001*
trans-9-octadecenoic acid (C18:1)	165.7 ± 24.6	24.9 ± 3.4	48.7 ± 1.0	<0.0001*	<0.0001*	0.4885
octadecanoic acid (C18:0)	47.1 ± 16.8	49.9 ±2.4	105.5 ± 2.1	0.99	0.0163*	0.0235*
cis-9,10-methylene-octadecanoic acid (C19:0Δ)	156.8 ± 65.6	-	-	<0.0001*	<0.0001*	>0.9999

nonadecanoic acid (C19:0)	12.3 ± 1.6	-	-	0.8246	0.8246	>0.9999

a: non-parametric two way ANOVA

b: where there is a dash "-" there was no detection of the fatty acid in this sample

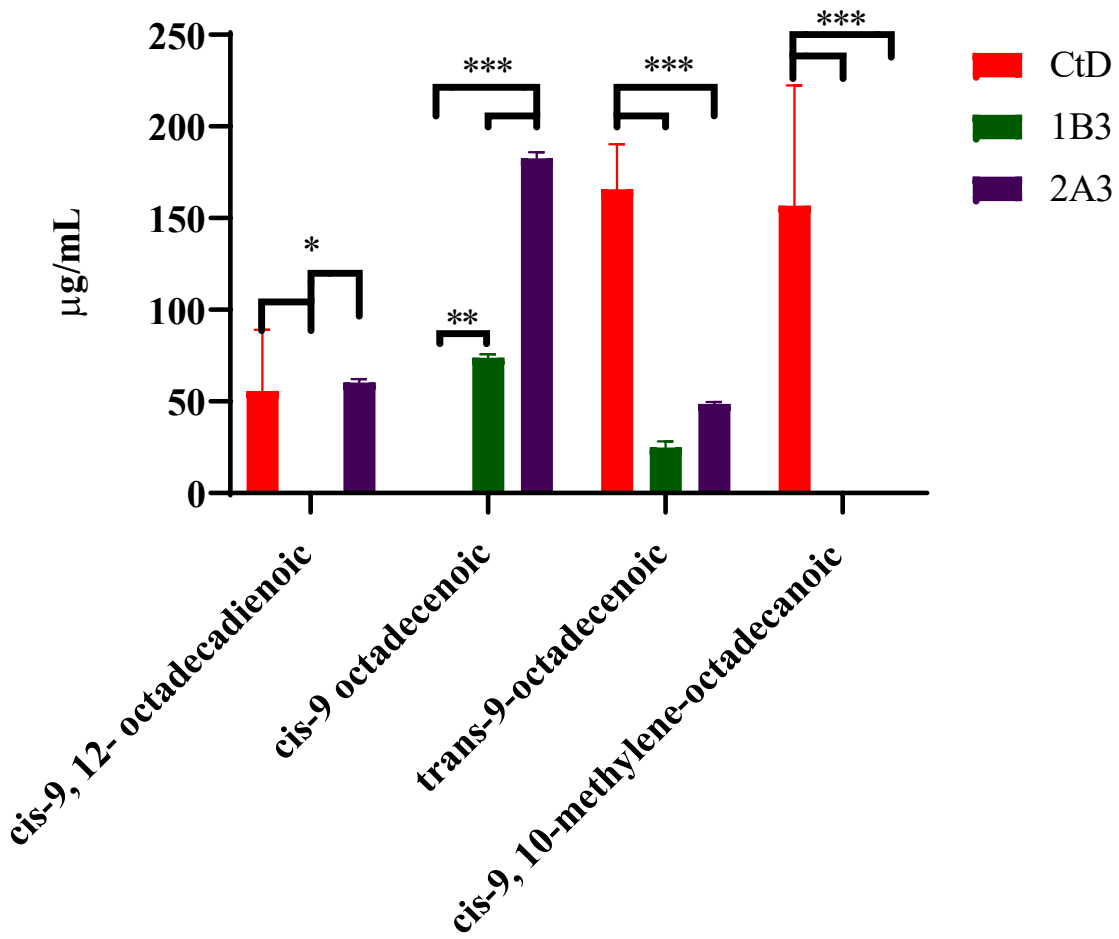
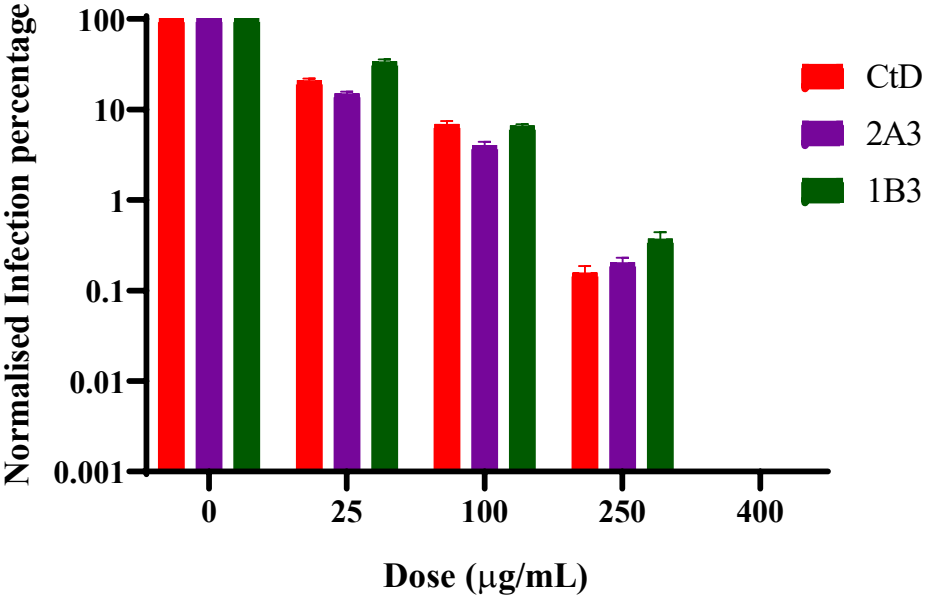


Figure 5.4. Fatty acids found at different concentrations in variants when compared to the wild-type. Column graph showing the concentration of detected fatty acids (µg/mL (y-axis)) within the different chlamydial variants. The graph shows mean and SEM of the fatty acids concentration for four acids that were detected to have significantly different

levels in variants compared to CtD. Asterix denote signficance; * ; p-value = 0.01-0.02, **; p-value = 0.001-0.009, ***; p-value <0.001

5.3.4. *Chlamydial* variants 1B3 and 2A3 were detected to be less susceptible to to triclosan than the wild type

Triclosan is a type II fatty acid synthesis inhibitor. The variants (as described in section 5.1.3.) have genetic alterations in the enzyme responsible for scavenging host fatty acids. *Chlamydial* variants were treated with triclosan in a dose range of 0 to 400µg/mL and analysed using percent infection during the infection to evaluate susceptibility. Infection was normalised to attain an infection percentage based on 0 µg/ml of triclosan dosage. The percentage of infectivity decreased as the dose concentration rose in all isolates as anticipated (Figure 5.5). The isolate 1B3 showed subtly reduced triclosan susceptibility compared to wild type with higher % infectivity at every dose tested compared to wild type and variant 2A3. However, this was only significantly higher at 25 µg/mL compared to wild type, and 25 µg/mL and 100 µg/mL compared to 2A3. Variant 2A3 appeared to be more susceptible, or similar in susceptibility as the wild type, with lower or similar % infectivity's at the two lower doses (p-value = 0.006 and p-value = 0.021 respectively at 25 µg/mL and 100 µg/mL compared to CtD), but conversely appeared subtly less impacted with a higher % infectivity that CtD at 250 ug/ml (albeit not significantly different).



Dose	Bonferroni corrected P-value		
	CtD vs 2A3	CtD vs 1B3	1B3 vs 2A3
0		n/a	
25	0.006	<0.001	<0.001
100	0.021	0.999	<0.001
250	0.378	0.094	0.487
400		n/a	

Figure 5.5. Triclosan dose-response of chlamydial variants. The dose of triclosan was normalised to 0 µg/mL at 100% infection percentage. At 400 µg/mL the infectious percentage was zero. At the bottom of the graph a table lists the p-values determined using the non-parametric Kruskal–Wallis test, corrected by the Bonferroni method.

5.3.5. Analysis of *in vivo* survival of chlamydial variants

The C57/BL6 mouse model of infection was firstly analysed by measuring and comparing the vaginal shedding of bacteria. The shedding CtD and variant 1B3 showed no difference over the infection course of 26 days when analysed by cultures from swabs

and determination of IFU/ml. At day 14, there was a mild a spike in bacterial shedding in CtD that wasn't as high in the variant 1B3, although this was not significantly different (Figure 5.6, panel A). Analysis of the chromosomal copy RT-qPCR on the *Chlamydia* Tarp gene (section 5.2.9.4) confirmed these results (Figure 5.6, panel C).

C. trachomatis CtD and variants were then tested to monitor infection in a more susceptible mouse model (C3H/HeJ). There was no difference in bacterial shedding over the course of 26 days when IFU/mL were enumerated from vaginal swabs collected every three days. (Figure 5.6, panel B). Representative images of *Chlamydial* IFUs for each mouse group across the days post-infection show the decrease in IFU's post-infection (Appendix D)

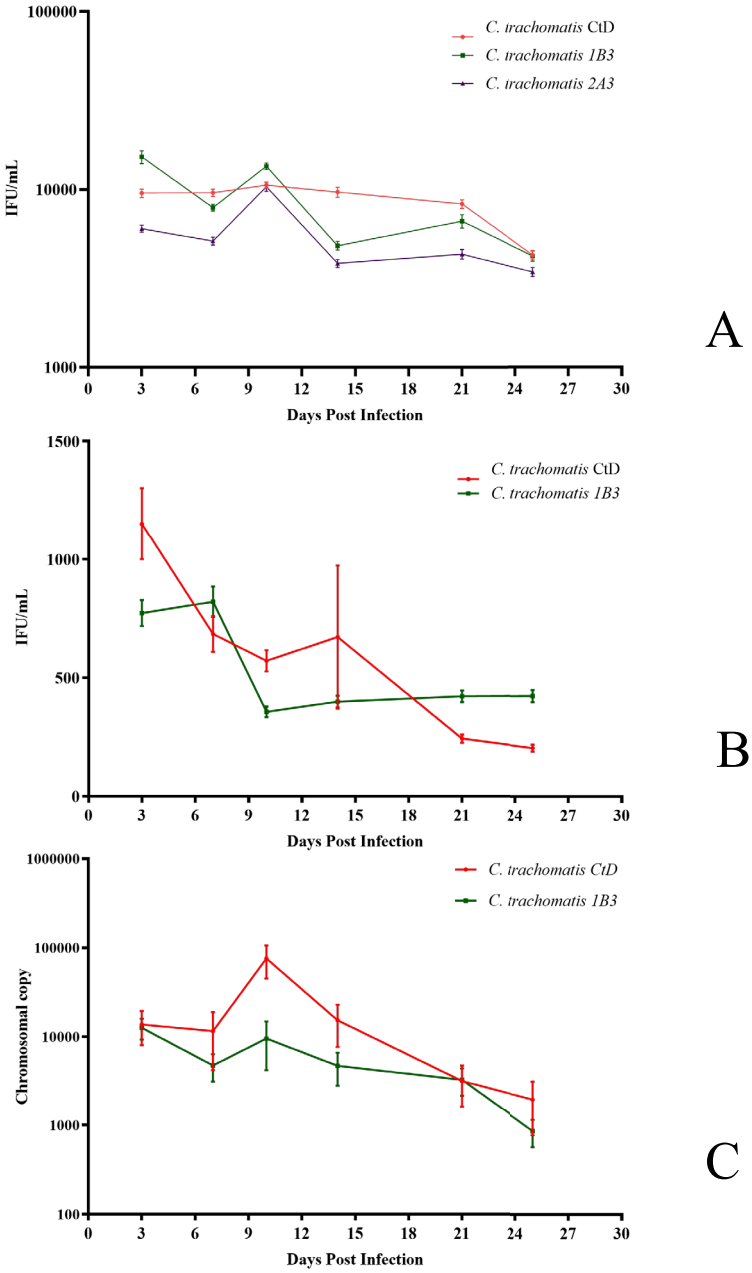


Figure 5.6. Chlamydial IFU/mL shedding after infection. Panel A is the graph is for the C57BL/6 mice and shows IFU/mL (y-axis) for CtD (red, circle) and 1B3 (green, square) and 2A3 (purple, triangle). Panel B is a line graph for C3H/HeJ mice that displays IFU/mL for CtD (red, circle), 1B3 (green, square). Panel C is the shedding of *Chlamydial* chromosome in C57/BL6 mice. The graph displays chromosomal copies (tarp PCR y-axis) shed from the vagina every three days for CtD (red, circle) and 1B3 (green, square)

(absolute quantities; y-axis). Shedding was monitored over 25 days with swab collection every 3 days with end point being the 26th day post-infection. The points indicate the mean with the error bars showing the standard error of mean.

5.3.6. Pathological analysis of *in vivo* chlamydial variants

Animal models are used in the study of human illness, and are mostly chosen for their resemblance to humans in terms of genetics, anatomy, and physiology, in order to indicate potentials of pathogen survival and potential of sequelae (e.g. PID). The cross-sectional area of the left and right oviduct was analysed and no difference was observed between wild type, 1B3, 2A3and SPG infected mice (both strains) (Figure 5.7). The cross-sectional area of the oviducts range from 3.5 – 5 mm^2 for all groups regardless of mice strain and if it was infected or the mock infected control (Figure 5.7). The mouse models were examined using histology to evaluate any signs of pathology in any of the upper reproductive tract organs. Histological sections of mice upper reproductive tract displayed no signs of inflammation or pathology (for both mice strains with all treatments including the negative control SPG). There was no sign of infiltration by leukocytes within the tissue in any case, which is used as a common sign of inflammation. Figure 5.8 displays representative images of the tissue sections from the analysis of the fallopian tubes, oviduct, and ovaries for each group of mice regardless of strain as both C57BL/6 and C3H/HeJ showing similar results.

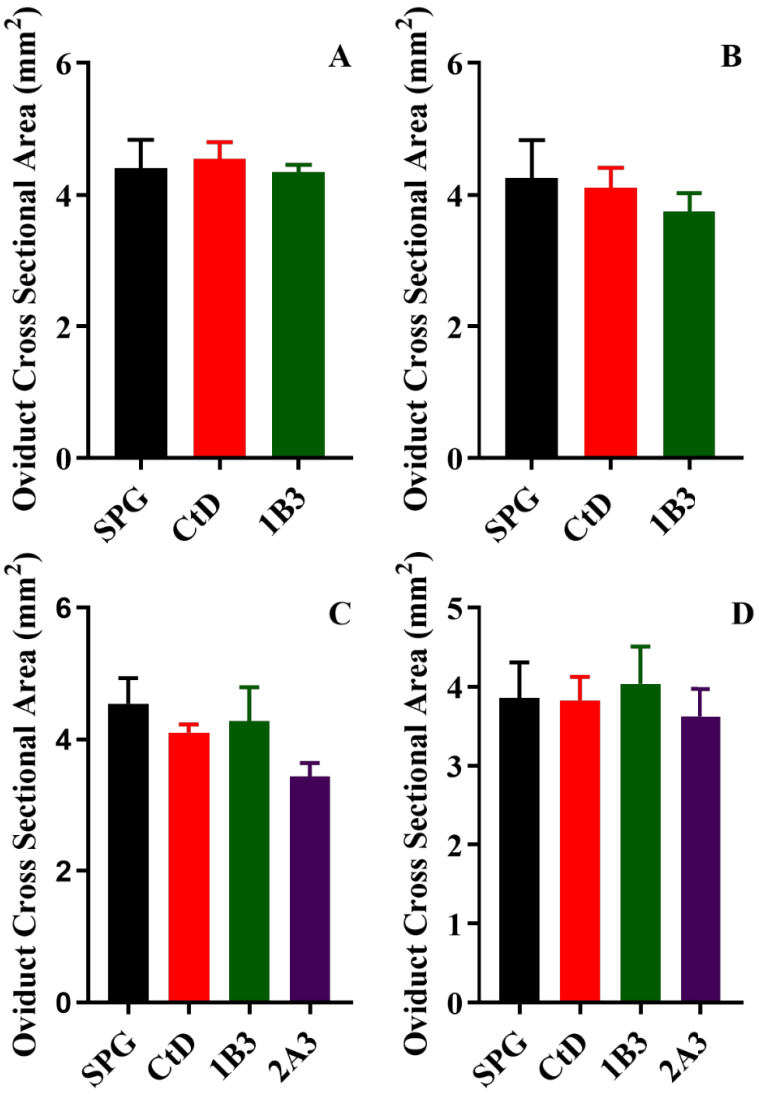
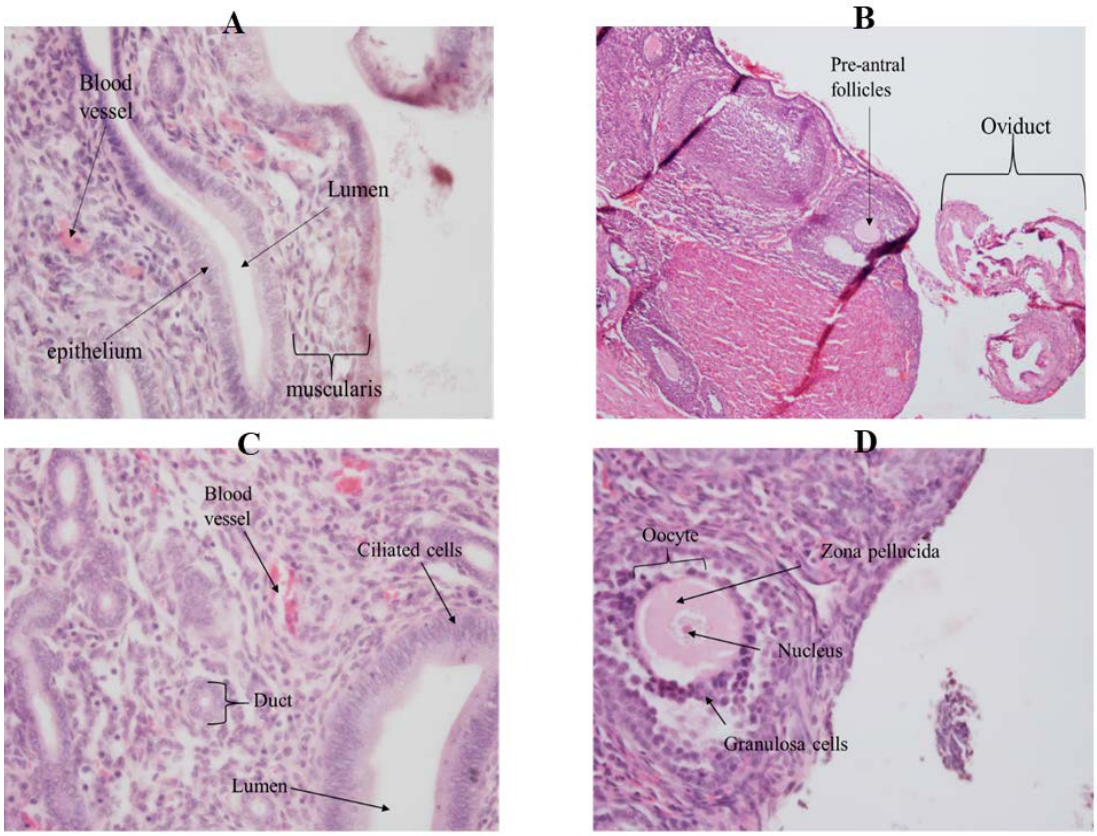
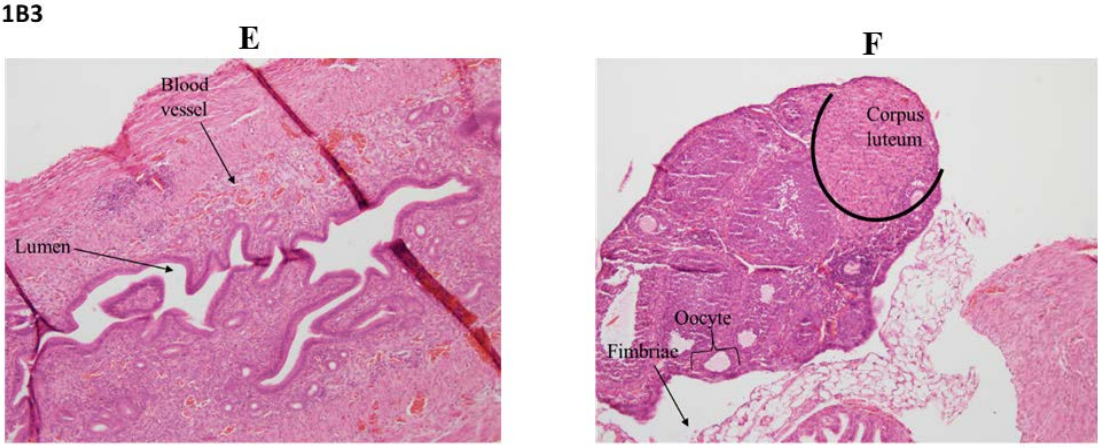


Figure 5.7.Mouse oviduct cross-sectional area. This bar graph displays the mean cross-sectional area of the mouse oviducts with a standard error of mean for the infections tested in C57/BL6 (Panel A and B) and C3H/HeJ (Panel C and D) mice. The x-axis indicate infection conditions; SPG: mock infected with storage buffer (black), CtD: wild type strain (red), 1B3; lab produced variant (green) and 2A3: lab produced variant (purple). Panel A and C are the left oviduct and B and D are the right oviduct measured in mm^2 (y-axis).

WT


A
Blood vessel
Lumen
epithelium
muscularis
B
Pre-antral follicles
Oviduct
C
Blood vessel
Ciliated cells
Duct
Lumen
D
Oocyte
Zona pellucida
Nucleus
Granulosa cells

1B3


E
Blood vessel
Lumen
F
Corpus luteum
Oocyte
Fimbriae

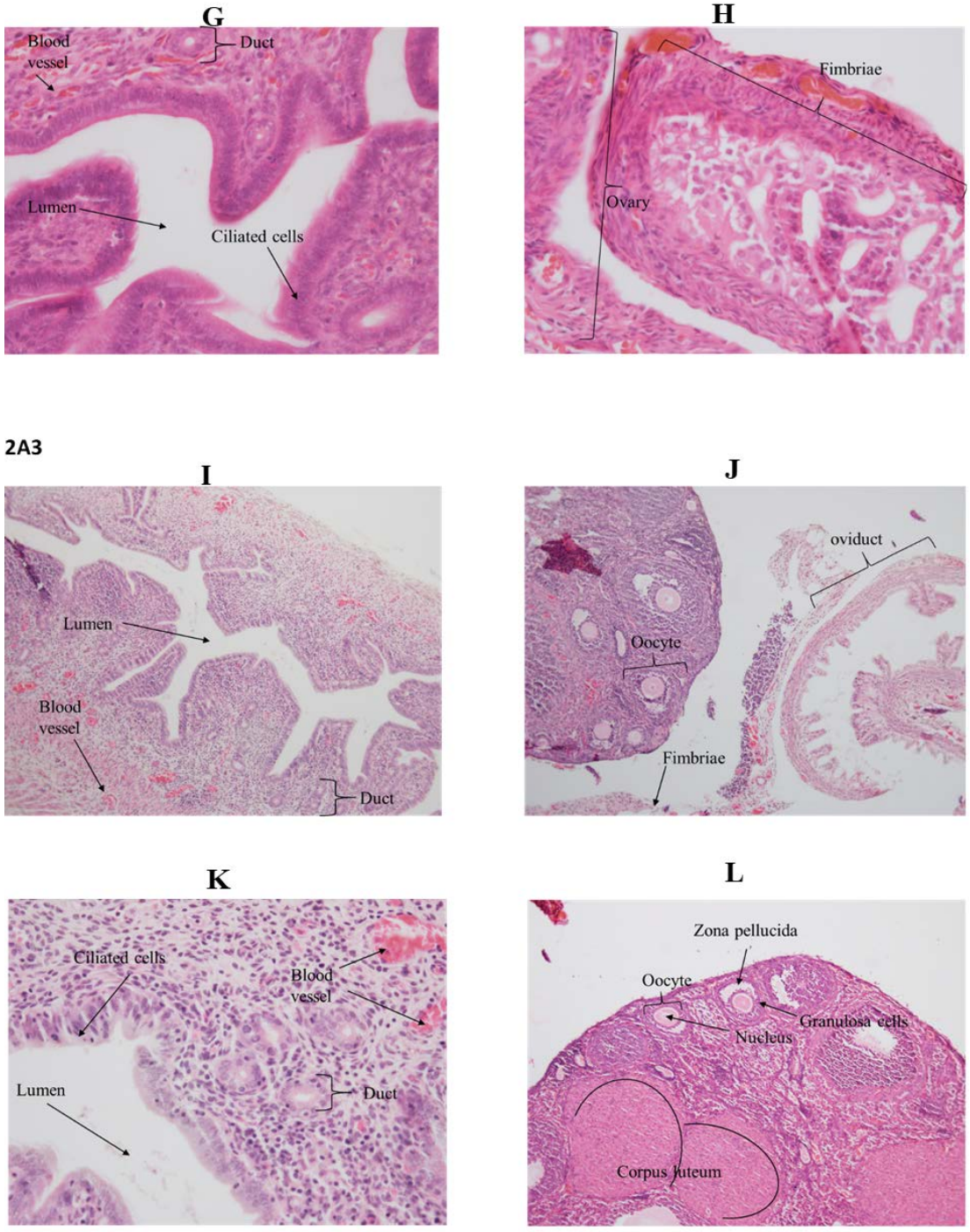


Figure 5.8. Haematoxylin and eosin-stained mouse upper reproductive tract. Panels A – D reveals fallopian epithelium (panels A, C), oviduct (panel B), and ovaries (panel D), of mice infected with CtD. Panels E-H come from 1B3 infected mice with fallopian epithelia (panels E, G), oviduct (panel H) and ovaries (panel F) . Panels I – L are from 2A3 infected mice with fallopian epithelia (panels I, K) , oviduct (panel J) and ovaries

(panel L). Note, whilst images are sourced from C3H/HeJ, both mice strains have similar observations.

5.4. DISCUSSION

Chemically generated variants previously developed in the lab were examined in an attempt to establish a framework approach to evaluate *C. trachomatis* pathology in our lab that could be used in future studies. Relevant to these particular strains, due to the genetic location of the variants was two phenotypic analysis. Firstly, fatty acids were successfully extracted from the bacterial strains and analysis found that there were different concentrations of four unsaturated acids compared to wild type. One of the variant, 1B3, had subtle indications of having less susceptibility to the fatty acid synthesis inhibitor type II (triclosan) compared to CtD. Although differences were subtle, and one variant did not show consistent susceptibility at different doses, so these results should be interpreted with caution. Analysis of the *in vivo* properties (survival and pathology) of the variants found no significant differences compared to the wildtype in either infection duration and shedding or pathology. However, as discussed below this likely reflects the challenges of mouse infection models for *C. trachomatis*.

5.4.1. Unsaturated fatty acids and their role in bacterial growth

These variants have a genetic change in the gene that encodes for the CT776, an enzyme that is reported to be a fatty acid host scavenger with a preference for palmitic acid [382]. This enzyme has been previously characterised and found to have an affinity

to monounsaturated fatty acid (double bonds) that *Chlamydia* isn't able to produce autotrophically [383]. It is important to note that *C. trachmatis* also has the ability to produce its own fatty acids and phospholipids by using scavenged host glucose, isoleucine and serine [381], but does not have a desaturase to introduce the double bonds. Hence, the detection of some unsaturated fatty acids in variants. Interestingly, variant 1B3 had no detected concentration of linoleic acid (cis-9,12 octadecadienoic acid) while oleic acid (cis-9 octadecenoic acid) was significantly higher in variant 1B3 and 2A3 compared to CtD. In a study on human bone marrow endothelial cells, it was found that linoleic acid upregulated and sustained the expression of ICAM-1 and VCAM-1 gene [390]. Oleic acid is found in 8% of the *Chlamydial* membrane as it cannot produce it autonomously, albeit 1B3 and 2A3 had it in abundance possibly due to another acyl-transferase as described in Recuero-Checa, e*t al* [384]. Another possibility is that the SNPs impact on, or only mildly vary the function of CT776 hence the results of the presence of oleic acid in the variants. This raises the question of altered affinity, whereby it is lost for oleic acid? In terms of biological growth, there was no differences in growth so the variants are still able to get the fatty acids needed either through another gene or CT776 function is not impacted by the polymorphisms.

A reminder that these variants were isolated due to less susceptibility of protease inhibitor JO146 (explained in [378]), hence I wanted to determine if the variations cause a change in their membrane to have a difference in fatty acid composition that in turn prevents penetration of triclosan and the JO146 molecule? While no specific variation in membrane specific genes were studied, an affect on the membrane can lead

to increase virulence and severity of disease, which has been seen in SNPs of the polymorphic membrane protein autotransporter (*pmpA, CT412*)[391].

The trans-isomer of oleic acid known as elaidic acid was found in higher concentrations in CtD than variants 1B3 and 2A3, whereby trans fatty acid are shown to be stimulators of the immune response by producing reactive oxygen species [392, 393]. Potentially, this could indicate that a shift in the isomer preferences of CT776 is related to the polymorphisms. Dihydrosterculic acid (cis-9,10-methylene-octadecanoic acid) has been detected in both Gram positive and negative bacteria, and it was found in CtD but not 1B3 or 2A3; however properties of cyclic fatty acids are yet to be understood [394]. This was not reported or tested or investigated to determine if it is a substrate of CT776. Through an extensive search of the literature, no known study has reported on dihydrosterculic acid in *Chlamydia.* This leads me to the question, could CT776 solely responsible for inclusion of this acid in the membrane? And could this acid (since being cyclic) be responsible for large membrane fluidity that in turn may impact on the penetration of the quite hydrophobic molecule JO146? In future experimentation, substrate specificity should be investigated on dihydrosterculic acid and other cyclic acids.

Antibiotic resistance in *Chlamydia* is extremely rare, whereby it has only been detected in laboratory settings and never rigorously confirmed *in vivo.* Only one of the variants seemed to be slightly less susceptible to triclosan [395]. The variants had a discrepancy in the fatty acid-related gene that lets them scavenge from the host cell. (i.e. CT776), so triclosan susceptibility the phenotype (one of many) has possibly changed.

Similarly, in a study using *C. suis* (host tropism for pigs) with a variation in plasmids, it was found that the tetracycline phenotype resulted in less susceptibility to the antibiotic [396]. This too was also found is heat sensitive mutations of *C. trachomatis,* where there was a variation in 12 genes that lead to phenotypic differences; however, authors don't show the data for this point they mention [397]. In this experiment % infectivity was used, and this may be not adequate enough a measure to see differences and the results should be interpreted with caution.

5.4.2. Chlamydial variants survive *in vivo*

While there was no detection of differences between the shedding and pathology due to the different strains in the mouse *in vivo* models, this was the first step towards determining if variants of chlamydia could show differences in the model *Chlamydial in vivo* mouse models. It is established in the literature that mouse models are not good for infection or pathology for *C. trachomatis* (as compared to *C. muridarum*) due to less survival and limited pathology as previously reported [398-401]. Yet, while *C. muridarum* illicit a long-lived defensive host responds to a synergistic inflammatory infection, *C. trachomatis*, when infected in a susceptible strain (C3H/HeJ), enables an infection of ample length to create an adaptive immune response relative to the C57/BL6 strain [38]. Interestingly, murine pathogen *C. muridarum* exhibits upper genital tract pathology and this is typically absent in human specific *C. trachomatis* (in mice). One relatively recent method to combat the lack of induction of pathology in upper genital tracts is to, infect the mice past the cervix (i.e. injecting the *Chlamydia* past the cervix known as transcervical infection) [402, 403]. While other animal models exist (pigs,

guinea pigs and non-human primates), no infection of *C. trachomatis* mimics the pathology, histology, and endocrinology of the human reproductive system or the pathogenesis and immune reactions that arise during human genital *C. trachomatis* infection. Non-human primate models more strongly mimic clinical illness, but their usage is hampered by financial, practical, and ethical considerations [38]. A limitation contributing to the lack of pathology could be the initial burden of the organism used for infection, could this be too high? It has been shown that a lower initial inoculum creates a persistent longer infection [404, 405]. In the future instead of trialling different inoculation doses, I would speculated a better result may be achieved if transcervical infection is undertaken. Interestingly, in a study using Dunkin Hartley strain guinea pigs, infection with *C. trachomatis* through vaginal infection withheld a sustained infection and produced pathology after 65 days [406]. For practicality and financial issues of sourcing transgenic mice, I speculate the guinea pig model might provide a better insight into pathology. Further form *in vivo* experimentation, *in vitro* investigations using a chemically defined media, such as, fatty acid deficient media should be investigated and a wider dosage range of triclosan, with the possible use of other antibiotics.

The limitations of the mouse models make it difficult to examine *ex vivo,* clinically sourced variants such as those from PID cases and if they are more or less virulent, or have features that are more include to produce pathology Furthermore, utilising strains that tend to have a disparity in infectivity and discrepancies in fatty acids, I aimed to examine whether any variations between such isolates can be detected in the mouse model, because there is a need a functioning animal model to examine *C. trachomatis* pathology for potential future studies. Also, because it was feasible that the

fatty acid phenotype may have been more marked *in vivo* compared the in vitro cell culture models that provide all the resources for maximal growth rather than challenges. If pathology was to occur in the C3H/HeJ mouse strain (more susceptible strain) it would probably only be if the variants somehow have higher pathology than CtD to the point that it could have been detected in the mouse models or if the variant that didn't seem to impact majorly on cell culture growth did impact *in vivo.* Or an alternative outcome could have been that the variants had a more marked phenotype *in vivo* and this may have limited their capacity to source fatty acids resulting in reduced establishment of an infection, which would have correlated to reduced shedding. The cell culture model is not likely to limit the source of fatty acids and so we aren't seeing any differences *in vitro*, not because the mutation isn't making a difference, but due to the culture conditions, in which case we may have seen them impacted in the mouse. In other words, in culture media and the host cells other variants were guaranteed a supply of fatty acids, from the host cells, however in the murine model they had find that for themselves. However, an alternative explanation is likely that SNPs are only making a subtle difference in the gene function or another pathway is used.

5.4.3. Conclusion

In summary, previously generated chemically induced variants, resistant to a protease inhibitor JO146, were investigated for the ability to scavenge unsaturated fatty acids. Due to the observation that the variants have SNV's in CT776 and in CT206, as CT776 is known to function in fatty acid acquisition from the host cell, and CT206 is a putative esterase that likely has a function in fatty acid metabolism. Four unsaturated fatty

acids were found to have different concentrations compared to the wild type (CtD). However, the data suggests that there is another method for intake of certain unsaturated fatty acids such as oleic acid, or that the mutations have only slightly altered the function of CT776. One of the variants was consistently less susceptible to the type II fatty acid synthesis inhibitor, triclosan, and two mouse models were examined using the vaginal infection model to compare the *in vivo* survival and any pathology induction from the strains. No difference was found in *Chlamydial* bacterial shedding and no pathology was detected in upper genital tract even with the wild-type. Hence, more work is needed on the analysis of these isolates and the significance of the fatty acid profile distinctions. Further, as part of establishing a pilot protocol for PID and strain pathology analysis, there has been little progress on this goal in this work unfortunately from this dataset. A mouse model for chlamydial PID is crucial for further evaluation of pathovariants but requires more comprehensive approaches, such as transgenic mice, guinea pigs and mini pigs as discussed in Chapter 1.

Chapter 6 . General discussion and future directions

6.1. The overall summary of findings

This prospective pilot study successfully completed the aim of providing future directions for more extensive studies. In chapter 2 demographic factors were deemed similar between groups, indicating no recruitment bias. This pilot study demonstrated that there is a need for further consideration of questionnaires design for larger study, with the possible use of a phone application or online survey. In Chapter 3, qPCR found that PID cases had a significantly higher bacterial load of *Prevotella spp* and *A. vaginae.* In this chapter, this pilot study informed future studies the need to consider other ecological assessment such as transcriptomics, virome analysis. In Chapter 4 *lyz* was found to be significantly higher in cases then controls. Regarding this chapter, this pilot study informed the importance of biospecimen transport and storage, where in larger studies recruitment should occur from a single state or region. In Chapter 5, it was attempted to investigate potential for an animal disease model using pathovarients of *C. trachomatis*. It was determined that a more humanised or guinea pigs to be used in future STI associated *in vivo* models. Overall, this pilot study enlightened the need to extensively consider experimental protocols and recruitment methods for a larger study.

6.2. The cervicovaginal microbiome and connection to the host response

The vaginal epithelial, mucosal and microbial environment are diverse, within an interrelated and mutually affecting relationship. The vaginal mucosa has a lower pH than any other mucosal surface, is under (cyclic) hormonal influence, and is often under immunological pressure. It is therefore important to consider how the microbiome

influences the host response. In Chapter 3 it was identified that women with PID frequently had a community state type that lacked *Lactobacillus crispatus* and was often dominated by *L. iners* or a mix of anaerobes. This observation was confirmed by quantitative species or genus specific PCRs, where I found significantly higher *Prevotella spp* and *Atopobium vaginae* levels in women with PID compared to controls. Also, cases with PID were also found to have a significantly high gene expression for pathways (between cases and controls) which included cell communication and the detection of bacteria and viruses.

It is known that the vaginal epithelium produces glycogen and this free glycogen is metabolised by *Lactobacillus spp* resulting in lactic acid [407] (Figure 6.1). This lactic acid creates a niche for acid tolerant organisms and in turn is associated with increased mucosal production by the epithelia [168]. Epithelial cells, as the frontline of immune defence in the vagina, express pattern recognition (PRRs), like Toll-like (TLRs) receptors that react with cytokines and chemokines (antimicrobials), to pathogen-associated molecular patterns (PAMPs) [408, 409]. It has been indicated that *Lactobacillus* does not activate the secretion of antimicrobial peptides, whilst anaerobic organism such as *A. vaginae* do as reviewed by Yarbrough *et al*[410] and also by Wira *et al* [411]. Hence, this supports that we need further investigation of antimicrobial peptide secretion due to CST III (*L. iners*) and secretion of lysozyme (Chapter 3 and 4 respectively) in PID and other reproductive tract conditions. In recent studies, it was also found that the vaginal mucosal barrier is dynamic and a denser, thicker layer is protective compared to less dense [412-414]. Furthermore, these studies suggest that the distinguishing characteristic of women with a high secretion of inflammatory cytokines is detection of proteins implicated in the

degradation of mucosal barrier structures [412-414]. Further investigation are required on the effects of *L. iners* and CST IV anaerobes and the inflammatory secretions produced that may lead to mucosal breakdown associated with PID.

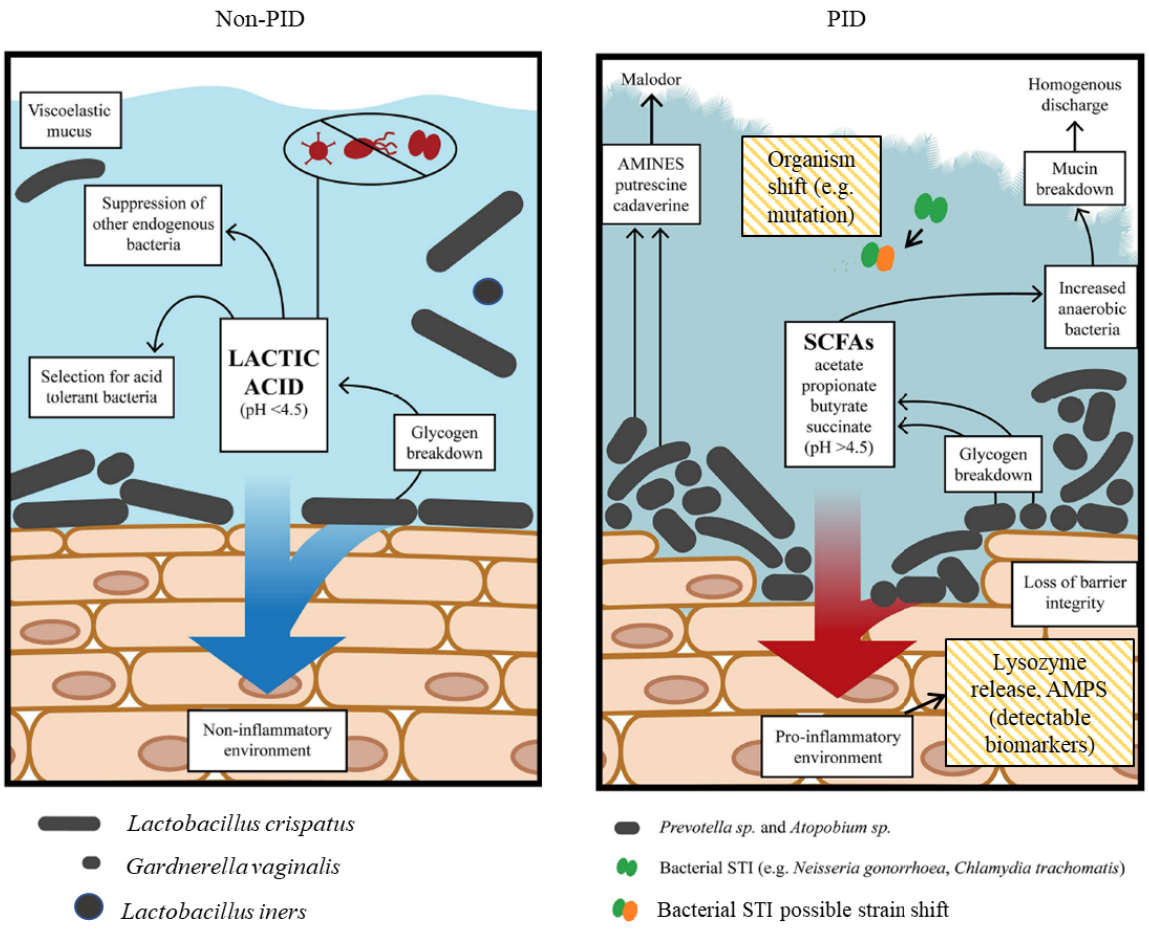


Figure 6.1. Vaginal epithelium in without and with PID. This directly reproduced image with alterations from findings in this project (from [168]). The image of non-PID epithelium displays the epithelium disseminating glycogen that is used by *Lactobacillus sp* to metabolise into lactic acid, creating an environment with a pH<4.5. This environment selects for acid tolerant bacteria and suppresses other bacteria. The epithetlium with PID displays, the intrusion of *L.iners, Prevotella sp and Atopobium sp*, causing a breakedown in the the epithelium and muscosal barrier. This can select for a

certain strain of bacteria to flourish with a proinflammatory environemnt with the release of lysozyme, AMPS and other possibl future biomarkers.

In a meta-RNA transcriptome sequencing study of four women between the ages of 18 to 40 it was suggested that *L. iners* in the presence of *L. crispatus* kept the vagina in a eubiotic asymptomatic state, whilst *L. iners* in the presence of anaerobes caused a breakdown in the mucosal layer, an increase of pH, release of short chain fatty acids resulting in a positive feedback loop [415] (Figure 6.2). Whilst this sample size is extremely small, the technology used is similar to 16S rRNA gene amplicon sequencing, but gives information on organism and the host concurrently. Similarly, with recent studies I too speculate that *L. iners* is a transitional state to CST IV [216, 416-418]. With this in mind, this cross-sectional study of women with PID taken a "snapshot" in time? A longitudinal study in a high-risk setting following those that develop PID using transcriptomics and RNA-sequencing for both host response and bacterial transcripts would help to unravel the interplay that results in progression to PID. However, a longitudinal study for a high-risk setting (e.g. sexual health clinic) isn't very feasible due to the fact that collection of swabs isn't required for all conditions in a sexual health clinic, secondly, diagnosis of PID is complicated (Chapter 1) this may cause a great variance in data collected, finally, only 10% of STI positive women may develop PID, so in order to recruit a significant number of cases the study would have to go for many years.

Overall, I speculate that the microbiome and dysbiosis is a factor leading to the development of PID. It is possible that vaginal dysbiosis facilitates particular pathogens or pathovariants or even some microbiome members that lead to PID. Hence one solution

that could aid this research is developing an animal model or something like an organ on a chip model to investigate the role of microbiome and host response, with differing pathovariants of pathogenic and non-pathogenic organisms.

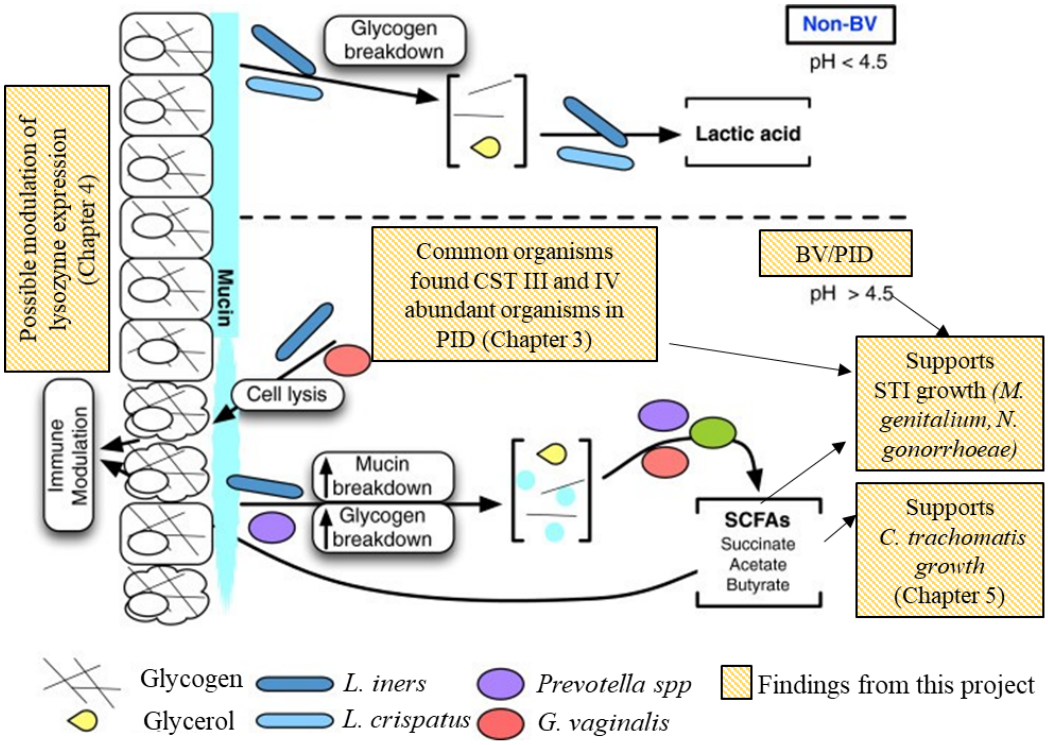


Figure 6.2. Predicted functions of microbiota in the vaginal epithelium. This image directly reproduced and ultered with resultant data from this study [415]. The figure displays two possibilities. In the non-BV, eubiotic state there is both *L. iners* and *L. crispatus* present, resulting in the breakdown of glycogen to produce an acidic eubiotic environment. In the BV state *L. iners* is present with anaerobes such as *G. vaginalis* and *Prevotella spp* which results in breakdown of mucus resulting in a increase of metabolisation by anaerobes producing short chain fatty acids causing further mucosal damage.

6.3. The cervicovaginal microbiome and antibiotics

As mentioned in Chapter 1, the treatment regimen for PID is a cocktail of antibiotic with a minimum of three antibiotics regardless of the region in world. Interestingly women who had PID were more likely to have taken antibiotics in previous three months to diagnosis (Chapter 2) when compared to controls. This gets me questioning whether the prior use of antibiotics increased the likelihood of PID onset. In an *in vitro* study of the effect of common antibiotics azithromycin and doxycycline (used in the Australian PID treatment guideline) on the main organisms of the five CST (*L. crispatus, L. jensenii, L. iners, L. gasseri* and CST IV representative *G. vaginalus*), it was found that *L. iners* and *G. vaginalis* were significantly resistant to azithromycin [216]. Interestingly, these antibiotics are the first line of defence from *C. trachomatis* and this STI has been associated with CST III or CST IV [419-421]. In other words, does a prescription of antibiotics lead to an altered microbiome that may increase the likelihood of PID and STI's being contracted after exposure? Subsequently, STI's are a known cause of PID. Furthermore, in a study of antibiotic depleted gut microbiome, antibiotic treatment started 3 days before the day of vaccination and continued until one day after for the antibiotics-treated group. This found significance that alterations in the gastrointestinal microbiota induced by antibiotics can influence immunogenicity in response to influenza vaccine [422]. Could this antibiotic induced community state type alter host response to certain microbes? Or alter the immune profile and susceptibility to infection susceptibility upon exposure as a consequence?

6.4. Time to rethink PID treatment and diagnosis?

To treat PID, medical practitioners must be able to diagnose PID objectively. To accomplish this a host response biomarker type of diagnostics could be a beneficial solution (Chapter 4) and the bimanual exam could consequently become a thing of the past for PID. In Australia, the antibiotics given upon a suspected PID diagnosis (before confirmation of STI). For example, as stated by the Australian sexual health guidelines, inpatient PID treatment includes 500 mg of azithromycin, whilst the same guideline suggests 1 g of azithromycin for treatment of *C. trachomatis* [22]. Furthermore, the mix of antibiotics has the risk of potentially contributing to antibiotic resistance, as discussed in Chapter 1. No one would be comfortable with not rapidly treating presumptive PID given the high risks of sequelae to individuals. On the other hand given the risks of PID, optimal treatment defined by what organisms are present administered rapidly with a comprehensive on-site molecular diagnostic of PID and pathogens present would have potential to be an improved treatment approach and maximise the use of most suitable antibiotics.

In a clinical trial of 68 women from Rwanda, tested the efficacy of Ecologic Femi+ vaginal capsule for recurrent bacterial vaginosis (EF+; Winclove Probiotics, Amsterdam, The Netherlands) and Gynophilus LP vaginal tablet (GynLP; Biose, Arpajon-sur-Cère, France) that contain lyophilized *Bifidobacterium bifidum* W28, *Lactobacillus acidophilus* W70, *L. helveticus* W74, *L. brevis* W63, *L. plantarum* W21, *L. salivarius* W24 and *Lcr Regenerans*, a culture of the *L. rhamnos*us 35 respectively [423]. While the authors found intermittent use of EF+ probiotic was more significant at

reducing shift of microbiome to a dysbiotic state, they were uncertain of significance due to small pilot size [423]. Firstly, a few studies have shown that prolonged use of low dose metronidazole, regardless of application method (2g per month oral or 0.75% gel intravaginally) reduced the chances of recurrence BV associated microbiome [424-426] compared to normal metronidazole 1 g daily for one week. Secondly, the above Rwanda trial didn't use clinically isolated *Lactobacillus* and no bacterium from protective community state types (i.e. *L. crispatus)*. Furthermore, it seems that a single bacterium probiotic cannot accomplish therapeutic benefits alone and either personalise or more complex probiotic formulations may be needed for vaginal conditions [427, 428].

There have been a few vaginal probiotic trials to date with limited success, some used food strain *Lactobacillus* and others used strains cultured from healthy women (vaginal and intestinal strains) [429-439]. The 11 studies mentioned use the following organism, *L. gasseri, L. rhamnosus, L. acidophilus, L. fermentum, L. casei var. rhamnosus, L. brevis* CD2*, L. salivarius subsp. salicinius, L. plantarum L.delbrueckiisubsp* lactis DM8909 and *L. paracasei.* Only one study conducted a trial on a probiotic containing *L. crispatus* (CST I) this study uses 78 women divided into placebo and treatment group. The treatment group was given an intravaginal capsule containing *L. crispatus* (10^9 colony forming units per gram), it was found that BV recurrence was significantly higher in the placebo group compared to treatment (p-value = 0.0497) [439]. Overall, the outcome of this aforementioned trial was looking at the prevention of recurrence of bacterial vaginosis (a condition associated with PID). However, an Australian clinical trial of combining *L. acidophilus* with oral antibiotic (metronidazole or clindamycin) found no effect in lowering the recurrence of BV [432]. It is possible that

L. crispatus could have been more successful because it makes better sense to include bacteria that are dominating a stable vaginal microbiota than other species.

While low dose antibiotic targeting anaerobic organism in combination with *L. crispatus* probiotics is showing promising therapeutics, there is the importance of prebiotics. In *Nature reviews,* Sanders *et al* reviewed the importance of prebiotics and probiotics in eliciting therapeutic effects, they highlight the success of prebiotics such as glucans and fructans that are well proven in the gut [440]. Furthermore, the importance of dietary fibre as a prebiotic for healthy gut microbes is starting to be understood as many studies suggest fibre as a prebiotic for gut microbial diversity [441-445]. Interestingly, in a small recent study of dietary fibre intakes effect on 108 women's vaginal flora, it was found that women who had higher dietary fibre intake had a lower odds ratio of BV associated microbiome (p-value = 0.049) [446]. While, fibre is becoming understood to be important, as shown in Figure 6.1, *L. crispatus* relies on glycogen, it has been suggested by many reviews that the use of oligosaccharides/polysaccharides such as glycogen may provide a higher chance in therapeutic effects of probiotics [447-450].

6.5. Future directions

This pilot study has uncovered many future directions in regards to protocols and recruitment procedures. One future trial could consider a cohort study over several years of a high risk population of getting PID, women that return with PID cases are then to be screened for host response as discussed in Chapter 4, further investigation in this cohort on the use of antibiotics and their effect on vaginal flora that may lead to PID.

Furthermore, collection of different women's representative CST flora with whole genomic sequencing, such as metagenomics analysis, and *in vitro* examinations of antibiotic effects. Using this information on the strains or pathovariants of vaginal flora need their correlation to be understood in terms of PID. Secondly vaginal organisms cultured from asymptomatic women, need their safety assessed as a probiotic, and safety of prebiotic containingfibre and glycogen or lactic acid. After safety, and *in vitro* and *iv vivo* analysis of the roles of each of these variables I hypothesise that a combined mix of *L. crispatus* strains along with low dose of metronidazole, and prebiotic is to be heavily investigate *in vivo* as potential treatment for idiopathic PID.

Further investigations of different anaerobes such as *G. vaginalis, Prevotella spp* and *A. vaginae* as it may be possible that specific strains or sub-species of these organisms may be associated with PID. This was the purpose of Chapter 5 and further investigation on differing strains or subspecies of *N. gonorrhoeae* or *M. genitalium* should be investigated *ex vivo.* In terms of IUD induced PID, prophylactic antibiotics have not been found to be advantageous; however, antibiotics used were broad-ranged and were less likely to treat for anaerobic vaginal flora only [451, 452]. Once, prebiotic and probiotics have been studied extensively *in vitro* a possible prophylactic clinical trial on women getting IUD insertion should be considered.

Throughout this dissertation, I stress the importance of an *in vivo* animal model that can help understand the role of differing strains or pathovariants of organisms. It is unfeasible for a longitudinal trial of women at high risk of developing PID, firstly, as discussed in Chapter one around 10% develop PID. Secondly, constant cervical swab are

not only painful but can elicit a host response. Overcoming this obstacle would be a humanized transgenic animal model, that is ethical, able to handle and accommodate relatively easily. This can be trialled either through the use of McGreen (Cfs1r-EGFP) inbreed with C57BL/6 mice (Chapter 1) or possibly the guinea pig model discussed in Chapter 5.

6.6. Conclusion

In this project, a prospective pilot study recruited a case, control and test group, recruitment was difficult and this is a consideration for future PID studies. Nevertheless, there did not appear to be any large differences in demographic factors for the group recruited so the recruitment protocol was likely appropriate. Through 16S rRNA gene amplicon sequencing, it was found that PID cases predominantly had a cervicovaginal community state type of III (*L.* iners) and IV (anaerobic community). Utilising quantitative PCR it was determined that PID cases had significantly higher copy load of *A. vaginae* and *Prevotella spp.* Whilst only 84 innate and adaptive host response genes were investigated in this study and the test group weren't included, it was found that the antimicrobial lysozyme was expressed at significantly higher levels in cases than controls and cell-cell communication pathways were present at significantly higher levels in PID cases than controls. In the hope of investigating variants of chlamydia to help establish a mouse model where large infectivity or pathology differences could be detected, *in vivo* and *in vitro* analysis of *C. trachomatis variants* was conducted In conclusion, an infection of pathovariants of *C. trachomatis* was established in both C3H/HeJ and C57BL/6 mice even though no pathological outcomes were observed.

Finally in the hope of future PID studies, I have constructed Figure 6.3 for consideration. The whole aim to diagnose any disease is to accomplish it objectively with high sensitivity and specificity informing adequate treatment protocols. Hence the potential to reduce the need for the bimanual exam of the cervix, to be replaced with simple swabs detecting host specific genes, representative organisms that may be associated with PID at specific quantities or thresholds, or certain pathovariants, and of course presence of an STI. Based on this, the possibility to reduce the amounts of antibiotics used in PID arises where a combination of antibiotics targeting the exact composition of organisms observed could be the result of such a diagnostic test. Ultimately, with a treatment protocol that includes pre and probiotics are used for treatment and prophylactic prevention of further PID incidences would be a future innovation that could develop.

Overall, this pilot study has established that the recruitment, specimen processing and analysis protocols are effective. The results indicate that there are potential microbiota that may influence the disease highlighting the challenges of understanding PID from microbiome perspective. Importantly it found that the bacterial load of *Atopobium vaginae* and *Prevotella species* were higher in women with PID. Secondly, human immune gene expression profiles were found to correlate with PID, and these biomarkers could be used for future molecular diagnostic of PID with further supporting data. The lysozyme gene was found to be expressed higher in PID than in controls and pathway analysis identified important cell signalling pathways associated with PID that help to understand the pathogenesis of this disease. The final study of *Chlamydial* variants

highlight that there is a need for a *in vivo* model of PID, especially in terms of investigating different organisms and potential pathogenic factors.

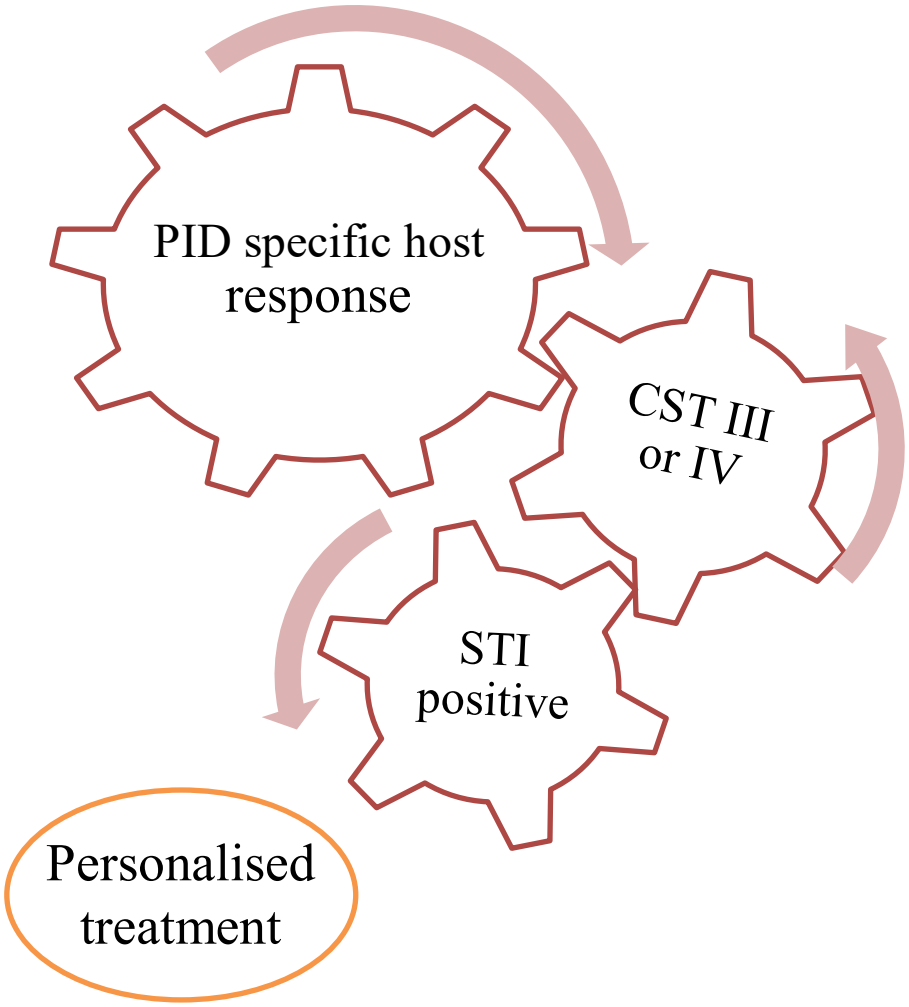


Figure 6.3. Diagnostic protocol for PID for future consideration. This protocol is intended for the removal or reduced need of the bimanual examination of the cervix. Cervical swabs are taken to test for host response biomarker array, STI's and representative vaginal flora concentrations. The diagnostic test could also be used to guide personalised and specific treatment regimens, especially if developed into on site point of care test.

Appendix A

PARTICIPANT INFORMATION SHEET AND INFORMED CONSENT

CASE PARTICIPANT - recruited prior to diagnosis during consultation

A pilot study to identify microbial and immune biomarkers for improved diagnosis of pelvic inflammatory disease

Dr Huston, Clinical Associate Professor Bateson, Ms Estoesta, Dr Sweeney, Ms Fleming, Ms Duggan, Dr Stewart, and Dr Burke

HREC FPNSW R2016-07,

INTRODUCTION

You are invited to participate in a study investigating a medical condition called pelvic inflammatory disease (PID). PID is an infection of the uterus (womb) and sometimes the fallopian tubes usually caused by a sexually transmissible infection. If PID is not diagnosed and treated promptly it can lead to serious problems including infertility. In this study we are recruiting a group of women who are diagnosed with PID known as the 'case group' and another group who do not have PID known as the 'control group'. You have been invited to participate in the 'case group'. This invitation is because your doctor thinks it is possible that you have PID and will be examining you to check for this.

PURPOSE OF THE STUDY

We are doing this research to find out the causes of PID and whether there are any differences in swabs taken from the cervix and vagina of women with PID and women in the control group who do not have PID. The results from the study will help improve the tests we use to detect PID in the future.

STUDY PROCEDURES

If you agree to be in the study you will be asked to consent to participate by signing the consent forms. Before you sign the consent form you can ask the doctor any questions that you might have.
After giving consent for the study, and when being examined to check if you may have PID, the doctor or nurse will collect three additional swabs from your cervix and one from your vagina. These swabs will be sent to a research laboratory at the University of Technology in Sydney (UTS) where they will be tested and compared with the swabs from women without PID.
We will then ask you complete a brief questionnaire about your gynaecological and sexual health.
We also request your consent to access your Family Planning NSW medical record for the purposes of the study so we can check the results of the tests that have been carried out today by your doctor.

RISKS

In this study the doctor or nurse will take three extra swabs from the cervix and one from the vagina. There are no known risks from providing these extra swabs except for possible minor discomfort. Taking these extra swabs will not affect the outcome of the other swabs taken as part of your consultation today.

PARTICIPATION IN THE STUDY

It is your decision about whether you participate in the study or not. If you decide not to participate in

you receive at
relevant infor

your doctor or nurse and the lead clinical researchers at FPNSW will know that you have participated in the study. Each participant is assigned a unique study code (included in this form) which is used by the researchers in place of your name and other identifying information. If you agree to participate in the study you will be asked to sign the consent form and complete the questionnaire. The doctor or nurse will collect the study swabs after taking swabs as part of your consultation. After this visit there is nothing further you need to do. While we will be unable to provide you with your individual swab results, the overall results from the study will be published and made available through the Family Planning NSW website (www.fpnsw.org.au). This report will not identify any individual study participants.

You may choose to leave the study at any time by contacting the chief researcher Dr Wilhelmina Huston on 02 95143449 or (Wilhelmina.Huston@uts.edu.au) and telling her your unique study code on the form below. The clinic will ask the researchers to take your swabs out of the study. Your swabs, Questionnaire and other data will then be destroyed and your medical record will not be examined for the study. You will also be given a Study Withdrawal form which can be returned to the Family Planning NSW research centre should you choose to leave the study.

If you have any concerns or wish to make a complaint about the conduct of the study you can call the UTS Research Ethics Committee on 02 9514 2478, or the Family Planning NSW Ethics Committee on 02 8752 4338 or ethics@fpnsw.org.au.

POSSIBLE BENEFITS OF PARTICIPATING

This research is designed to support the development of new tests, new medicines or other diagnostic tools for PID. There will be no direct benefits to you from participating in this study and you will not receive payment for your participation.

STUDY FUNDING

The study is being funded by a research grant from University of Technology Sydney to investigator Dr Huston.

PRIVACY

The information and samples that you provide us will be used in research publications and presentations but will not identify you in any way. All the information collected will be de-identified before the data analysis.

Pre-set de-identified code:

SIGNATURES

By signing and dating this Consent Form, you agree to have additional cervical and vaginal swabs taken and to complete the Questionnaire. By signing the Consent Form you also agree to Family Planning NSW doctors and nurses accessing your medical record and sharing relevant information with the researchers.

CONSENT FORM

Signature Page

To participate in this study, this page must be signed by you.

By signing this page, you are confirming the following:

- You have read all of the information in this Information Sheet, and you have had time to think about the study requirements.
- All of your questions have been answered to your satisfaction.
- You voluntarily agree to have additional cervical samples taken and to complete the study Questionnaire.
- You authorize the study doctor and researchers to access the test results, the Questionnaire information and relevant information for the study from your medical record as described in the Information Sheet.

One copy of the Consent Form is kept by you and the other by the researchers.

_____

_____

Signature of participant

Date (ddMMyy)
(Participant must date)

_____

_____

Name of participant (Print)

_____

Name of Clinician

_____

_____

Signature of Clinician

Date (ddMMyy) Clinician must date

Clinician – confirm urine Hcg test today indicates participant is not pregnant Y ☐ N☐

STUDY WITHDRAWAL FORM

A pilot study to identify microbial and immune biomarkers for improved diagnosis of pelvic inflammatory disease

Dr Huston, Clinical Associate Professor Bateson, Ms Estoesta, Dr Sweeney, Ms Fleming, Ms Duggan, Dr Stewart and Dr Burke

HREC approval numbers: FPSNW R2016-07, UTS xxxx

If you ever chose to leave this study you can call/email Dr Wilhelmina Huston 02 95143449 (Wilhelmina.Huston@uts.edu.au) and tell her this study code:

De-identified code:

If you have any concerns or want to make a complaint about the study, please contact:

UTS Human Research Ethics Committee 02 9514 2478 or
Family Planning NSW Ethics Committee ph: 07 8752 4338 or
Study investigator: Dr W Huston ph 02 95143449, email: Wilhelmina.Huston@uts.edu.au

You will not need to give your name to leave the study if you can give the above code.

Should you choose to leave the study all of your swabs and study-related information will be destroyed.

PARTICIPANT QUESTIONAIRRE

A pilot study to identify microbial and immune biomarkers for improved diagnosis of pelvic inflammatory disease

Dr Huston, Clinical Associate Professor Bateson, Ms Estoesta, Dr Sweeney, Ms Fleming, Ms Duggan, Dr Stewart, and Dr Burke

HREC Approval Number: FPNSW R2016-07and UTS XXXX

De-identified code:

Please complete the following information and give the completed questionnaire to the doctor or nurse when you have finished:

DATE: _____

Age: _____ (month /year of birth) : _____

Main current contraceptive method: (tick appropriate box)

☐combined hormonal contraceptive pill ☐progestogen only pill (mini-pill)

☐vaginal ring; ☐DMPA (depot provera injection)

☐ implant (implanon) ☐ copper IUD ☐hormonal IUD (Mirena)

☐condoms ☐diaphragm

☐male or female sterilization ☐ natural family planning/ fertility awareness

☐ withdrawal ☐none

Have you started or changed to this method of contraception in the past 12 months?

☐ Y ☐ N

If yes, which month/year did you start: _____

In the past 12 months have you had either a vaginal thrush infection (candida) and/or bacterial vaginosis? ☐ Y ☐ N ☐ Don't know

If Yes: ☐ once ☐2-3 times ☐ 4 or more times

Have you ever had previous treatment for pelvic inflammatory disease (PID) ☐Y ☐N

If yes, how many times: _____
When was this (years): _____
☐ Don't know

Have you any antibiotics for any other reason in the past 3 months (in addition to any you may have had for PID) ☐Y ☐N

Have you ever been diagnosed with chlamydia? ☐Y ☐N If yes which years(s): _____
Have you ever been diagnosed with gonorrhoea? ☐Y ☐ N If yes which year(s): _____

Are you currently pregnant or suspect that you might be pregnant? ☐Y ☐N
Have you ever been pregnant ☐Y ☐N
Number of pregnancies: ____
Number of live births: _____
Did any pregnancy involve assisted reproduction technologies such as IVF? ☐ Y ☐ N

Has it ever taken more than one year of trying to become pregnant? ☐ Y ☐ N

Past diagnosis of gynaecological conditions (e.g. ectopic pregnancy, endometriosis, polycystic ovarian syndrome, fibroids, ovarian cysts)

Please list with approximate years
__

Number of sexual partners in past 3 months: ☐0 ☐1 ☐ 2-4 ☐ >5
Number of sexual partners in the past 12 months : ☐0 ☐1 ☐ 2-4 ☐ >5-7 ☐ >7

Thank you for your time in completing this form. Please return it to reception in the provided envelope before you leave.

Cairns Sexual health recruitment kit

PATIENT INFORMATION SHEET AND INFORMED CONSENT

Investigation of the host and pathogen factors which are associated with different disease presentation of genital *Chlamydia trachomatis* and *Neisseria gonohorreae* in women.

Dr Wilhelmina Huston, Dr Jane Hocking, and Professor Peter Timms

Protocol Number _____

INTRODUCTION

You are invited to participate in a study to look at the biological aspects important in sexually transmitted infections that lead to pelvic inflammatory disease. You have been invited to participate because you currently have a sexually transmitted infection with *Chlamydia* or gonococcus.

Your participation is completely voluntary, you can decide not to participate and this will have no impact on your clinical care.

If you decide not to participate, it will have no effect on your future treatment.

PURPOSE OF THE STUDY

The purpose of this study is to identifybacterial and human factors that lead to the development of pelvic inflammatory disease (PID) in some women with sexually transmitted infections. Samples from your vagina, cervix, blood and saliva will be collected by your health care professional. These samples will be analysed in a research laboratory. Additionally, if you so choose, your immune DNA sequence can also be analysed.

STUDY PROCEDURES

You will be asked to consent to participate in this study (if you choose to do so) by signing the consent form after you have read this information sheet in full. You will complete a patient history form which will help the research study. This will be followed by collection of these samples by your health care professional, : Samples will include 2 vaginal and 3 endocervical swabs, blood, and saliva for the purposes of this study.

The study will use a part of your blood samples or the saliva specimen to do some genetic tests to see if immune gene sequences are associated with PID. The study will not look for known disease risk factors like cancer genes will NOT be tested, and for this reason you will not be provided with any genetic results. Please note if you are not comfortable with genetic testing but wish to complete the remainder of the study you can indicate on the below consent form and your samples will not be genetically tested and will only be used for the other components of the study.

RISKS

This study will involve a collection of extra samples. There are no known additional risks from providing these extra samples as these samples would routinely be collected during a sexual health check up. Some of the samples we collect will not be immediately analyzed so there is a risk that some samples will be collected, but not analysed.

Your samples will be given a code (de-identified) and stored prior to provision to the research team at QUT laboratories where the analysis will be done. Therefore your identity will remain confidential and will not get disclosed to anyone other than your health care professional and research lead investigators.

If you choose to withdraw from the study you may do so at any time without penalty. You can withdraw from the study by contacting the Cairns sexual health clinic Phone 07 42264769, and providing the study name and your name. The clinic will look up the code for your sample and advise the research group to withdraw your samples and any data from the study. The samples and data will be destroyed in a secure manner.

PARTICIPATION IN THE STUDY

Your agreement to participate is entirely voluntary. You may refuse without any penalty or loss of benefits to which you are otherwise entitled. If you do agree to participate, you will be required to sign the consent, complete the history form, and samples for the research study will be collected. You will be reimbursed for the cost of your time spent on the study to a total of $50.

If you have any concerns or wish to make a complaint regarding the conduct of this study you can notify the Cairns Human Research Ethics Committee; 07 4226 5312, Cairns.Ethics@health.qld.gov.au.

POSSIBLE BENEFITS

This research may contribute to the creation of new laboratory tests, new medicines or other items that may be commercially valuable to the sponsor. The sponsor has no plans to provide you, either now or in the future, any compensation, royalty or any other financial benefit that might result from any product, procedure, or other item that may be developed or any information or data that is derived from this research.

PRIVACY

In addition to the uses and disclosures of your personal health information described in the Consent Form for the main study, information gathered from future research on these samples will be used in research publications and presentations. Participants will never be identified and data will typically be presented as grouped data.

SIGNATURES

By signing and dating this Consent Form, you authorize the collection and provision of blood, vagina, cervix and salvia samples and your patient history form to the research study.

CONSENT FORM
Attachment 1
Signature Page

To participate in this study, you or your legal representative must sign this page.

By signing this page, you are confirming the following:

- You have read all of the information in this Consent Form, and you have had time to think about it.
- All of your questions have been answered to your satisfaction.
- You voluntarily agree to have samples taken from you and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- You authorize the study doctor and sponsor to use and disclose your data as described in this document.
- You have received a copy of this Consent Form to keep for yourself.

_____
_____
Signature of Subject

Date (ddMMyy)
{add time of day if appropriate]
(Subject must personally date)

_____
_____
Name (Print or Type)

Subject Initials and Number

_____
Name of Investigator/Clinician

_____
_____
Signature of Investigator/Clinician

Date (ddMMyy)
(Investigator must personally date)

I hereby indicate that I DO NOT want to provide a sample for genetic testing but agree to the remainder of the study Yes ☐(ticking here means no genetic testing)

PATIENT INFORMATION SHEET AND INFORMED CONSENT

Investigation of the host and pathogen factors which are associated with different disease presentation of genital *Chlamydia trachomatis* and *Neisseria gonohorreae* in women.

Dr Wilhelmina Huston, Dr Jane Hocking, Professor Peter Timms, and Dr Darren Russell

Protocol Number _____

Age: (month and year of birth)

Current STI:

Current contraceptive method:

Previous contraceptive method (s):
If changed over past few years please list all and estimates of changes:

Number of pregnancies in the past: -

Number of live births: -

(where any of these involving assisted reproduction technologies?) Y ☐ N ☐

Have you ever tried to fall pregnant for greater than 1 year Y ☐ N ☐

Past history of gynecological or genital infections or conditions (including surgery) – eg ectopic pregnancy, PID endometriosis, previous STI:

Any abnormal pap smear results:	
Any history of pelvic inflammatory disease:	
Sexual History – Lifetime sexual partners	1 ☐2-5 ☐>5 ☐
Sexual History – Sexual partners in past year	1 ☐2-5 ☐>5 ☐
Current smoker	Y ☐ N ☐
Past smoker	Y ☐ N ☐(number of years:)
Ethinicity	

Appendix B

Possible combinations of adaptors used to annotate samples.

Forward primers	1	2	3	4	5	6	7	8
Row1	i7_1	i7_1	i7_1	i7_1	i7_1	i7_1	i7_1	i7_1
Row2	i7_1	i7_1	i7_2	i7_2	i7_2	i7_2	i7_3	i7_3
Row3	i7_3	i7_3	i7_4	i7_4	i7_4	i7_4	i7_4	i7_5
Row4	i7_5	i7_5	i7_5	i7_5	i7_5	i7_5	i7_5	i7_5
Row5	i7_6	i7_6	i7_6	i7_6	i7_6	i7_7	i7_7	i7_7
Row6	i7_7	i7_7	i7_7	i7_8	i7_8	i7_8	i7_8	i7_9
Row7	i7_9	i7_9	i7_9	i7_9	i7_9	i7_10	i7_10	i7_10
Row8	i7_10	i7_10	i7_10	i7_10	i7_10	i7_11	i7_11	i7_11
Row9	i7_12	i7_12	i7_12	i7_12	i7_12	i7_12	i7_14	i7_14
Row10	i7_15	i7_15	i7_15	i7_15	i7_15	i7_16	i7_16	i7_16
Row11	i7_16	i7_16	i7_16	i7_16	i7_16	EMPTY	EMPTY	EMPTY
Reverse primers	1	2	3	4	5	6	7	8
Row1	i5_1	i5_2	i5_3	i5_6	i5_7	i5_11	i5_12	i5_16
Row2	i5_17	i5_18	i5_1	i5_2	i5_3	i5_15	i5_7	i5_18
Row3	i5_21	i5_22	i5_3	i5_5	i5_17	i5_18	i5_20	i5_1
Row4	i5_2	i5_5	i5_6	i5_7	i5_11	i5_16	i5_17	i5_18
Row5	i5_3	i5_5	i5_7	i5_12	i5_20	i5_2	i5_3	i5_5
Row6	i5_8	i5_12	i5_18	i5_6	i5_8	i5_11	i5_20	i5_1
Row7	i5_2	i5_3	i5_11	i5_12	i5_18	i5_1	i5_2	i5_6
Row8	i5_7	i5_11	i5_12	i5_15	i5_18	i5_7	i5_8	i5_22
Row9	i5_2	i5_3	i5_5	i5_6	i5_16	i5_20	i5_8	i5_20
Row10	i5_2	i5_6	i5_16	i5_17	i5_20	i5_1	i5_7	i5_11
Row11	i5_15	i5_17	i5_20	i5_21	i5_22	EMPTY	EMPTY	EMPTY

Code used in R studio with thresholds shown

Each operation is separated into section with the description of each section beginning with a # symbol.

```
#import feature table
   feature<- as.matrix(read.table("data/feature-
table3.tsv.txt", sep="\t", header=TRUE,
```

```
row.names=1))
feature<- otu_table(feature, taxa_are_rows = TRUE)
taxa_are_rows(feature)
#import taxonomic table
   tax<- read.table("data/taxa3..txt", header=TRUE,
row.names=1)
tax <- as.matrix(tax)
#import metadata table
   ap <- sample_data(read.csv("data/meta4.csv", header
= T, stringsAsFactors = F, sep = ",", row.names = 1))
   #import Tree
   tree<- read_tree("data/tree.nwk")
   #import and read DNA sequences
   refseq<- readDNAStringSet("data/sequences.fasta"
   #Merge into phyloseq object
   pid<- merge_phyloseq(feature, tax, map, tree,
refseq)
   #to make data easier for plotting
   pidplot <- psmelt(pid)
   #To collapse OTU table
   pidglom <- tax_glom(pid, taxrank = "Species")
# Check range of coverage on samples
sample_sums(pid)
#rid samples less than 2000 sequences
   pid_fil <- prune_samples(sample_sums(pidglom)>2000,
pidglom)
pid_fil
sort(taxa_sums(pid_fil))
fivenum(taxa_sums(pid_fil))
#rarefy
   pid_fil_rare <- rarefy_even_depth(pid_fil, 2000,
rngseed = 711, replace = FALSE, trimOTUs = TRUE)
```

```
#make a data frame for annotations on heat map
   an <- data.frame(sample_data(pid_fil_rare)) %>%
select("cst", "disease_type", "Cause")
#distance matrix jenson shannon divergence
   js_d <- phyloseq::distance(pid_fil_rare, method =
"jsd")
#hierarchy cluster
hc <- hclust(js_d, method = "ward.D")
plot(hc)
#select for top20 sequences
   top20 <- names(sort(taxa_sums(pid_fil_rare),
TRUE)[1:20])
top20otu<- data.frame(otu_table(pid_fil_rare)[top20,])
#create heatmap annotations rules and colour codes
   ha <- HeatmapAnnotation(df= an, col = list(cst =
c("I"= "magenta", "II"= "navy", "III"= "cyan", "IV" =
"green"),  disease_type = c("cst_control" = "orange",
"case " = "red", "control " = "blue")),
annotation_legend_param = list(title_gp =
gpar(fontsize=8,fontface = "plain"),  grid_height =
unit(4, "mm"), grid_width = unit(4, "mm"),labels_gp =
gpar(fontsize = 8)))

   #create heat map with annotations
   Heatmap(top20otu,
col=colorRamp2(c(0,2000),c("white", "red")),
cluster_columns = hc,  show_row_dend = FALSE,
row_names_side = "left",  heatmap_legend_param =
list(color_bar="continuous", title=NULL,
legend_direction = "vertical",labels_gp =
gpar(fontsize = 8)), top_annotation = ha,
column_names_gp = gpar(fontsize = 8), row_names_gp =
```

```
gpar(fontsize = 8), row_names_max_width = unit(8,
"cm"), column_dend_reorder=FALSE)
```

Qiime 2 output

Taxonomic output

	Genus	Species
92b27a4dc9c24dca6950fe2782187bd2	Lactobacillus	crustorum_mindensis_farciminis
f6887b0842412eab02716b1f6e742c29	Campylobacter	fetus_mucosalis_hyointestinalis
7d41c30f8e9f9feed729cc1d01d6a5cb	Aurantimonas	altamirensis
823b15e5143d4d8c60840d4e3758415c	Agromyces	cluster54
00d17926e7931e484a1777c9c4f6ef42	Porphyromonas	circumdentaria
7d44a933e28eece971639d26792ed705	Parvimonas	micra
d5a57fcbe2ecfda6f73993333262c6e8	Parvimonas	micra
fef850ec606a4cfa4eac96ae8013b512	Anaerococcus	hydrogenalis
240d660a2cb2d9cf7dfc0b72637566a2	Moryella	indoligenes
55a9780e2b2bcd9880268ca9c6de08d8	Peptoniphilus	ivorii
b96b14b21e3df89d55694257a852fb5a	Anaerococcus	lactolyticus_cluster
694963973fd9cbede1b2315452bf403d	Anaerococcus	lactolyticus_cluster
df0c0c043ee0c06a64074df03ab437df	Catonella	morbi
a1a6d097168ade201a731e5a1aad2e8c	Anaerococcus	tetradius
da4e1c7a46f03e021fe12a99311c2d9d	Anaerococcus	tetradius
d7107134e1ca8f5ee4d12fc8b429cb7f	Peptoniphilus	lacrimalis
1c2d3042fee6080118ddd7dec75dcaa1	Peptoniphilus	lacrimalis
6ddb27e7040497489912b0b92f820717	Eggerthella	sinensis
0a88125c91933744b0a9c8a62b7f179d	Peptoniphilus	lacrimalis

222c5cb114a655cd19f1a0e7b0308f00	Eggerthella	sinensis
c1a623dddbe7c2d2c5bcee8da642a2cd	Peptoniphilus	indolicus
e5e597fb0e108a998edb1bf32624246d	Campylobacter	ureolyticus
3a096adebdd0dc685b1e59f17208fa9f	Anaerococcus	octavius
b641ba20b982fc8803f4d5a72951f738	Finegoldia	magna
173109738d2ce05d5803f9ae02723bef	Sneathia	amnii
d5d9cf6ec2c66edd80232606ef4a7870	Pseudobutyrivibrio	ruminis
8731e32cd658484dd9b85eb1cb5bbbd7	Peptostreptococcus	anaerobius
1dc809cc8fec227dbefb54a67a7c90a9	Clostridium	perfringens
33ca91a9b90c3e7558bf8c3cc77311d6	Moryella	indoligenes
a764e6002d763c8adca217dd47646ac4	Sneathia	sanguinegens
13c2d3993b648b4fdfdd8f9b0c5d5f75	Bacteroides	coagulans
c4499478bb06ee69aa2e4d6f0c2f1d9d	Sneathia	amnii
2ae8b832d36eac522d7896d924afa29f	Peptostreptococcus	anaerobius
37d979c91e8f98d9bc7eb2cab1ebd0e7	Anaerococcus	hydrogenalis
94fcbb065f2f1a7e32295e1912210b33	Anaerococcus	hydrogenalis
55f18552dd06640be5dba66882d6296d	Sneathia	amnii
655148a7be947a738a8f05bdc7d09a00	Sneathia	amnii
14086bf69adef0c07cbfa505027690a7	Sneathia	amnii
21ebf2098d292b612a46fa3ecc38d37b	Fusobacterium	cluster48
aca9c18de0b81c5c913a912bed399247	Clostridium	colicanis
3a7d1cff0da953a6da1b97346963162e	Porphyromonas	gulae
4813fd718df2a7097c75e29c85ef8545	Porphyromonas	gulae
5a9cdb608601ebdd727047b1f40dc2a0	Clostridiales	BVAB3
f8523341ef05293f779b05d0a32559f4	Clostridium	cluster32

2588fc6a47baf5003993950743023b85	Porphyromonas	gingivalis
88d8c8dbcf5ebfa5dcef5c7b94e47234	Clostridiales	BVAB2
1885d76a2cae0671ee312ae505262751	Fusobacterium	cluster48
2eb1a1635314206c8375ccf37f1e7866	Fusobacterium	cluster48
932cb3d4bd0b114df294ece9b57f613e	Arcanobacterium	hippocoleae
b46290a7e7e8a013d15ae115df7be381	Propionibacterium	acnes
2fafa564c2719a61111e60d871474202	Gardnerella	vaginalis
157a4f681867948ceef1373cbf553704	Clostridiales	BVAB2
3727719b1ce601249c61ef2b07611ff2	Corynebacterium	cluster45
eee5b0a244e9980e56af1adfbdf9bd1a	Gardnerella	vaginalis
0db04616a39f91c5877ee5012ceb9d88	Gardnerella	vaginalis
642b390682ddfc81603a803db82336a0	Gardnerella	vaginalis
45055322c4c1a9130482af3089bc498e	Gardnerella	vaginalis
4cd688484604fe730150ede46df2f3b9	Bifidobacterium	catenulatum_pseudocatenulatum
2796278e9815f5eeb4a85b6b525251b2	Bifidobacterium	scardovii
c7fd1f31d42ce443e6745f95c1455a86	Gardnerella	vaginalis
005a5ab88e653923731daa74f31189d1	Atopobium	parvulum
db24588954fe9c9d6a0c47a9a5afee0a	Atopobium	parvulum
a4bcffec013b27ad38d78bb4dce40e54	Atopobium	parvulum
afb3fa9f41340f8f692f92b7b8a6dbfa	Gardnerella	vaginalis
019de7ddca84848474cc40d52279b9cd	Gardnerella	vaginalis
d367aa1bb14c10c0e23d349c239746c5	Gardnerella	vaginalis
1b9364170b89535b50fc14582efe9544	Gardnerella	vaginalis
41c260a5a2d4329b373a22ba47a66c8d	Mobiluncus	curtisii
8a2d913c8c7abcf4141b831197c5ce2f	Bacteroides	thetaiotaomicron_faecis

49bebb8b8d685d926fdff31c79fca3ff	Porphyromonas	bennonis
be64ef27c34b19e96a9baac061af790f	Staphylococcus	cluster47
ce4246da0399994ea27727ae4a6d4951	Veillonella	montpellierensis
ab7d5669efc815284bd4463d0956ffff	Veillonella	montpellierensis
61ee20f3d2b163ad712609877bdb50b3	Bacteroides	helcogenes
1a85b275df97268a8448d92686d6caa1	Veillonella	montpellierensis
5e0c66e4b6a33d1370eece00174632ad	Granulicatella	adiacens
01705f98f28a0e75c9e1c2cbd5740278	Prevotella	salivae
4dff66a2b9f805b9a149de401875ee51	Actinomyces	hongkongensis
794b5b55c497e330e1c3a5540792318b	Prevotella	salivae
a720a8539334b471e95e54cdda7d32bc	Prevotella	amnii
52f97ea00523e41810c1e80f36c54d7d	Prevotella	cluster2
129dbab4d0762e7aeb18605e6dfde720	Prevotella	salivae
3dd11cd8d837ee7b65077526e9e985a8	Prevotella	amnii
8cf3b46ec0ee0a1433bf61de838f5210	Prevotella	salivae
4a8ba5ad218c2097800e6d703d65545f	Prevotella	amnii
09af411f10a838d6d9a377204651f45f	Bacteroides	helcogenes
b1851a75b5226fbfe5dcce69b59f2753	Prevotella	amnii
fe8f03e340dd79dd754998bb63108f5b	Prevotella	amnii
1081c2db60227699383c0999eda3720b	Dialister	invisus
369909c9dc4734a31191339f0a63826d	Prevotella	veroralis
4398458d8ee7cc2c9a8530b449aa2263	Prevotella	veroralis
3ba5dd1f991e1f87f45c984c8ab1985d	Prevotella	corporis
cf7a5a9b8a884f39a688ff14d344fbd8	Porphyromonas	uenonis_asaccharolytica
7b1b09acfd835fd5c857040a7877942c	Prevotella	salivae

cbdb7a22f68ec728abdf9479ce72591c	Prevotella	salivae
80535cec1c7ca7e2b4f9a6e36b3a80b9	Porphyromonas	uenonis_asaccharolytica
d76bdc0be49c6e958cb31ffc6b6671c9	Prevotella	veroralis
8b8947f65fbc5da1a2c3c1dd219de2ca	Prevotella	salivae
a806a77110119a1b5460fbe950039b5d	Prevotella	salivae
fc57a523741afbe0dda790c49f9b3963	Prevotella	salivae
3c0d59bebb889645417ab78065ab2a74	Prevotella	salivae
1f659c2e2dc17b0dc653f29c3e561158	Dialister	micraerophilus
130c4246b2289321f45e25bc004c09df	Dialister	micraerophilus
06e0211af12bdce3588a04c24723f52d	Prevotella	melaninogenica_cluster
bb92a082e9e9498697372651fb664907	Actinomyces	meyeri_odontolyticus
094bc2552a8485f6adf042f7fc29ae21	Prevotella	melaninogenica_cluster
c7eb68d4c6f37c31d29be141855d8f00	Mobiluncus	mulieris
abfb3d134423d2dcc91426af4fd4107f	Prevotella	disiens
ca429895c1bc96ec32eb213c140a240c	Veillonella	montpellierensis
48306b07215ad1214e0a91fb49457b1f	Delftia	acidovorans_lacustris_tsuruhatensis
6c844319fb4c325ad4d562615de89654	Neisseria	gonorrhoeae
73840 12c56edee30a176794788d870ac	Prevotella	bivia
9bd75ea8d9c84b12c8c56e63dc6cc6d5	Prevotella	bivia
f97e72e8d90d26270005222fcc33579d	Prevotella	bivia
c48c24dd8608c55b5f44d26da11da097	Lactobacillus	iners
995ce5a9a8f54565206f5d5cacc34935	Bacillus	cereus
2bfbc924b20e2e0eff318e3fccb77d1b	Pseudomonas	aeruginosa
77f9a9d797da4d81274ae09635146bec	Lactobacillus	reuteri
ee45b62c049bd5a45fc3977522eb1653	Sutterella	sanguinis

eaeef7529b8dd89cc823d7bc16b3eed3	Veillonella	atypica_dispar_parvula
524212e12c0b6be52c708270bee8da44	Gemella	palaticanis
346fa622953646572f1ea6c293ef9759	Lactobacillus	vaginalis
af9bae5316b65a05a9fd21f04fcf49d7	Lactobacillus	oris_antri
41a216772cd0b215bba598ddf8ff92cc	Veillonella	montpellierensis
9331967e7c34533dd22068cfa9478b44	Acinetobacter	baumanii
b530e65a6ceb31e0b0e71bcfacbe39c3	Veillonella	atypica_dispar_parvula
74ee5548a0f6384ff93445304c3d5a31	Granulicatella	elegans
314f742ebfa7a810cd5b2500fe68ad65	Lactobacillus	vaginalis
504bca99568460db5508b09c8c516cf9	Aerococcus	christensenii
7d0649a3f08e3b27d2a0a2938cd025e6	Veillonella	atypica_dispar_parvula
75a21e57f25652583c9548e6e55bf645	Aerococcus	christensenii
1254ff16cf5ed1f4d678ce4944f31f86	Lactobacillus	pontis_frumenti
bc0f635657ee2b0a492ddebe205c1638	Mycoplasma	hominis
6d6e6d09a0f3bc61070e7aec4cc59510	Gemella	morbillorum_sanguinis_haemolysans
02dbca99907241c148e78cb667ab5bc3	Veillonella	montpellierensis
97ea33a76dc6685a39d57ffe849813d4	Dialister	micraerophilus
4dd76aadefbd4fc4d78c844ce7cf52c4	Lactobacillus	crispatus_cluster
0568ef5802d7c9ea6c9d439a0ece5891	Veillonella	montpellierensis
dead0a7bc2c11459fac230388398ce85	Lactobacillus	crispatus_cluster
f7b439a234bf31bfe5a8f70ad2aeb4c6	Lactobacillus	crispatus_cluster
0b5cc2c9311cc7f7c409fc43787c6ae2	Streptococcus	alactolyticus
32c2bdf7e511bd272a75f0fdbd352fec	Streptococcus	alactolyticus
fdffe19ce185c8a8b4176d6cff2cfb5f	Staphylococcus	cluster47
d0e8561a38677fc0d57baab5cf03acf9	Ureaplasma	cluster23

9aaa45d9f78dae30ac21c2f9fb9f0096	Lactobacillus	crispatus_cluster
c3973f7553ad338426cf46263aa3915c	Lactobacillus	crispatus_cluster
0d8cf11f8ef46a677d101a376641649c	Lactobacillus	crispatus_cluster
5da77409e851d8334658354c42130754	Streptococcus	mutans
992afe80517981011395d2e70db96e16	Lactobacillus	gastricus
5e06d07bc48380171a7e1d7888a7803a	Staphylococcus	cluster47
b126a90fbb24ddb0984b2db7ad939244	Staphylococcus	cluster47
ebab2ee03653037be3aa4919ef6635aa	Streptococcus	cluster29
fb5cc48dfa01cf86992468807a2ecfde	Lactobacillus	crispatus_cluster
33e50442924d964c430745520cacae29	Streptococcus	cluster29
a59ca3c0c056e39a009ec72c3a8ea2e8	Lactobacillus	crispatus_cluster
cc7007a50b31ecce4cd21fdc7eacc705	Chlamydia	trachomatis
015a52068e8c46f263abaacc53f5f845	Enterobacteriaceae	cluster31
3c66488df17299f6f46b606e201cbace	Streptococcus	dentapri
575f70a7f05645be88a6d8467c930499	Lactobacillus	iners
20615da4664d17be70d8ec74ee9f5c07	Staphylococcus	cluster47
3c23eb18a0d619c5779f9ecca353e851	Ureaplasma	cluster23
9e2ec2739d160cdb248036bf9da98fe9	Enterobacteriaceae	cluster31
baa922ef3873b1111aca6df0de664ab9	Streptococcus	parasanguinis
fb849f83f595f259e47b5edb8cc0c57e	Enterobacteriaceae	cluster31
4bae9869668a5b717af27cc1cd136574	Streptococcus	salivarius_thermophilus_vestibularis
e38e56b9ae640770031d1dd0121f7262	Enterobacteriaceae	cluster31
95e8fdcafcf87a453613396a38fbed59	Dialister	micraerophilus
6175184d8afcd96c312ad7e5acd966f7	Dialister	propionicifaciens
0a1de5891637cc656ec97ba023409a29	Dialister	micraerophilus

7cf2b2a27ef108a5e21ceebc448cb682	Enterococcus	faecalis
c458737885b52b35a2898f81662ab0de	Lactobacillus	gasseri_cluster
3de42fe1fbaa8969942cc97077421cbf	Dialister	micraerophilus

Feature table

https://drive.google.com/file/d/1_z2k751nXk5lKzdVL7J2uq3A1pg070_D/view?usp=sharing

Feature Tree

https://drive.google.com/file/d/19YbMh0MNeUu9wdtl9Q4ceMmTcbosH1Sy/view?usp=sharing

Sequences File

https://drive.google.com/file/d/19YbMh0MNeUu9wdtl9Q4ceMmTcbosH1Sy/view?usp=sharing

Appendix C

Code used to create cluster linkage dendrogram

Each operation is separated into the section with the description of each section beginning with a # symbol.

```
#Import ΔC_T table
Feature ← as.matrix(read.table("data/fold4.txt", sep="", header=TRUE, row.names=1)) colnames(feature) <- gsub(pattern = 'X', replacement = '', colnames(feature))
#Import participent metadata
map<- read.table("data/metadata.csv", header = TRUE, sep = ",", row.names = 1)
#make a data frame for annotations on heat map
an <- data.frame(map) %>% select("cst", "disease_type", "current_STI", "Cause")
#Merge into phyloseq object
PID <- merge.data.frame(feature, map)
#distance matrix
dis<- ape::dist.gene(PID, method = "pairwise", pairwise.deletion = F, variance = F)
#hierarchy cluster
hc <- hclust(dis, method = "median")
plot((hc))
#create heatmap annotations rules and colour codes
```

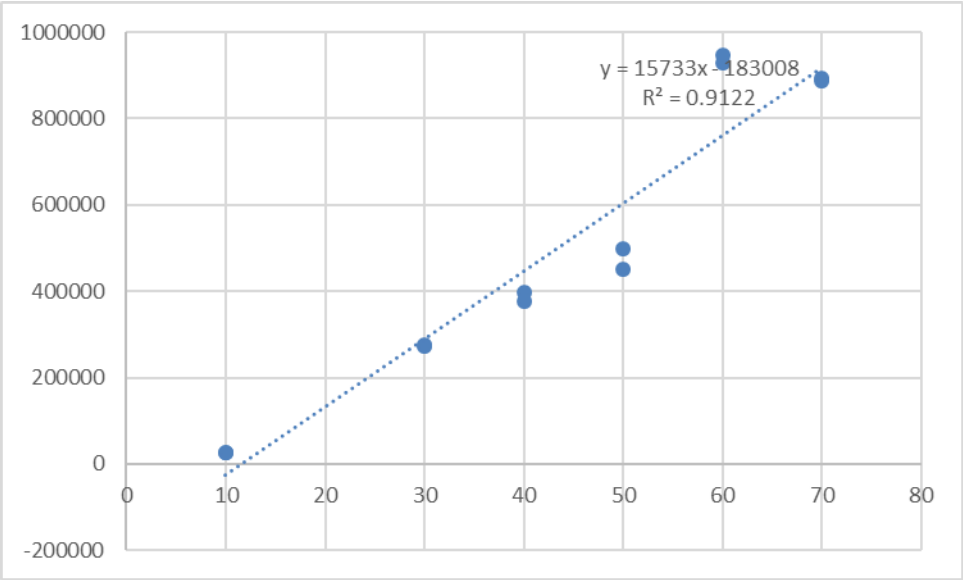
```
ha <- HeatmapAnnotation(df= an, col = list(cst = c("I"= "magenta", "Empty"= "white", "III"= "cyan", "IV" = "green"), disease_type = c("case" = "red", "control"= "blue"), current_STI = c("Chlamydia" = "light green", "Gonorrhoea"= "dark red", "Mycoplasma" = "grey", "Empty"= "white", "None" = "White" )), annotation_legend_param = list(title_gp = gpar(fontsize=8, fontface = "plain"), grid_height = unit(4, "mm"), grid_width = unit(4, "mm"),labels_gp = gpar(fontsize = 8)), show_legend = TRUE)
```

#create heat map with annotations

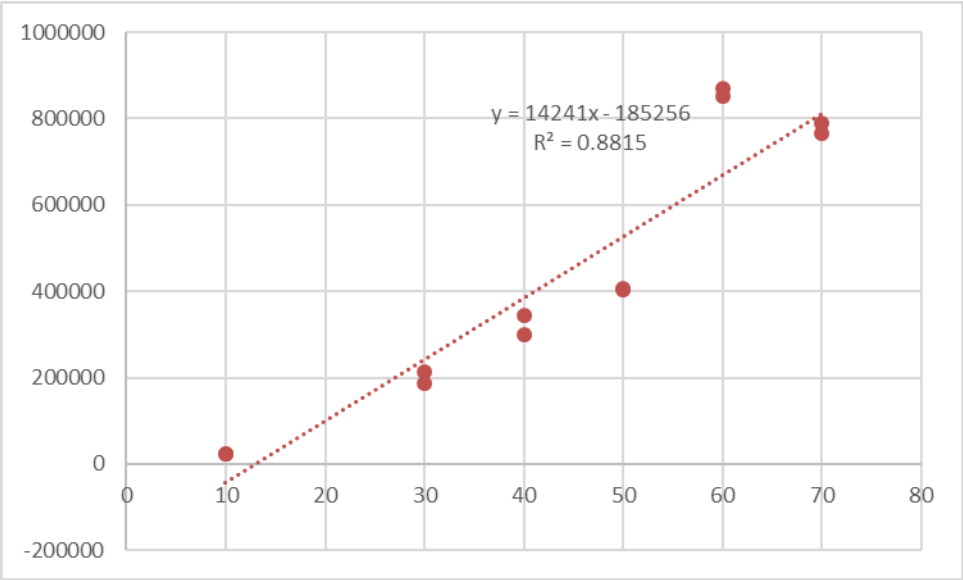
```
Heatmap(feature, col=colorRamp2(c(-3,0,3),c("blue", "white", "red")), clustering_distance_columns = "euclidean",clustering_distance_rows = "euclidean", show_row_dend = FALSE, row_names_side = "left", heatmap_legend_param = list(color_bar="continuous", title=NULL, legend_direction = "vertical",labels_gp = gpar(fontsize = 4)), top_annotation = ha, column_names_gp = gpar(fontsize = 8), row_names_gp = gpar(fontsize = 8), row_names_max_width = unit(8, "cm"), column_dend_reorder=FALSE)
```

Appendix D

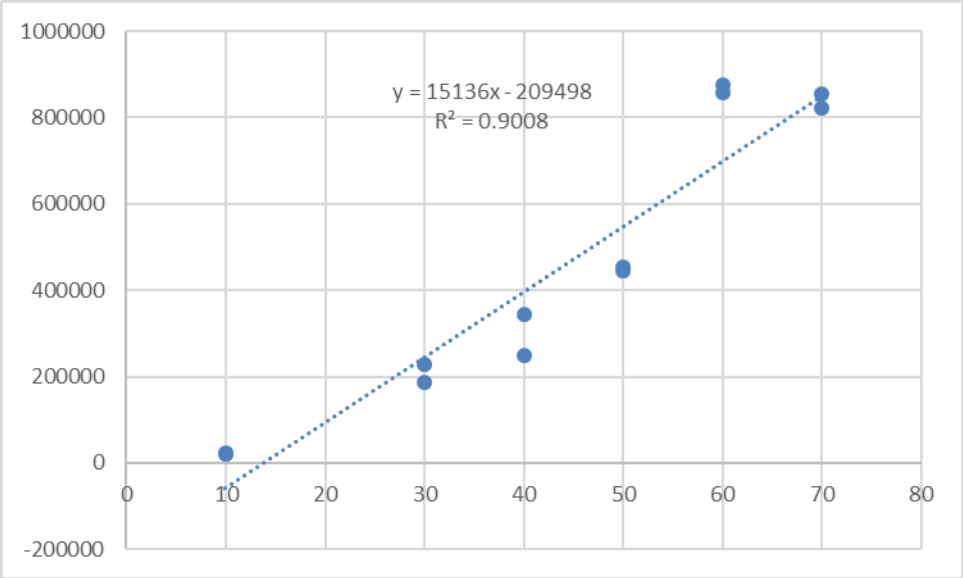
undecanoic acid



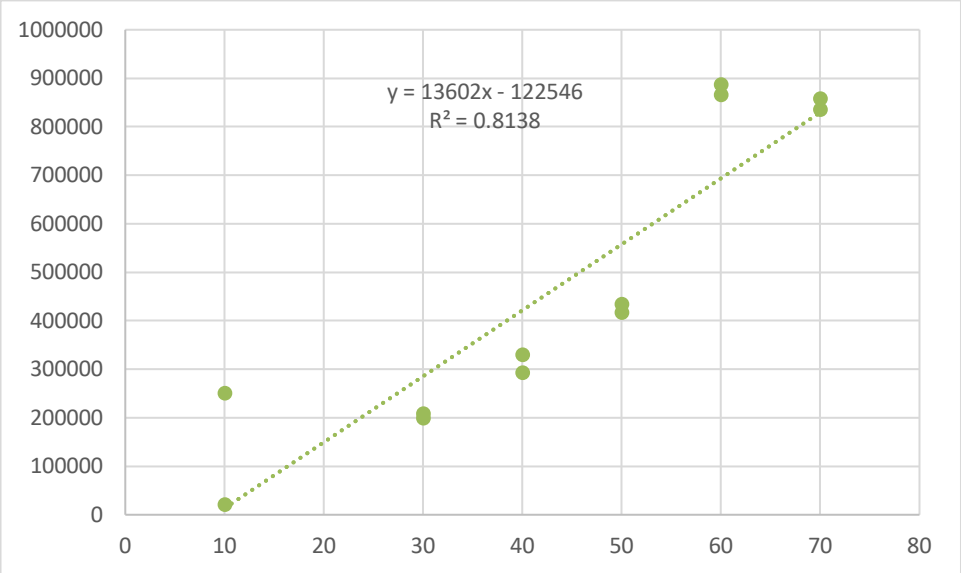
Dodecanoic acid



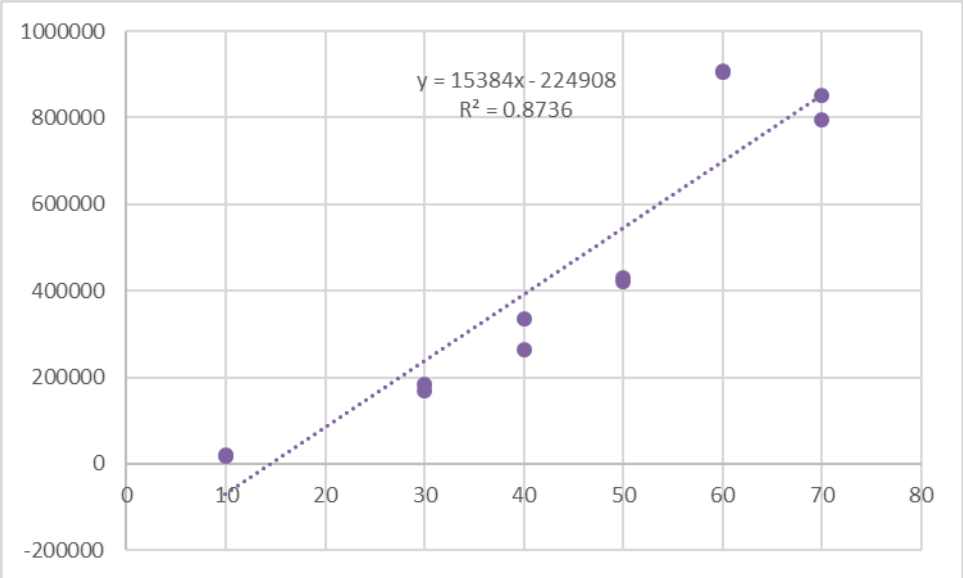
Tridecanoic acid



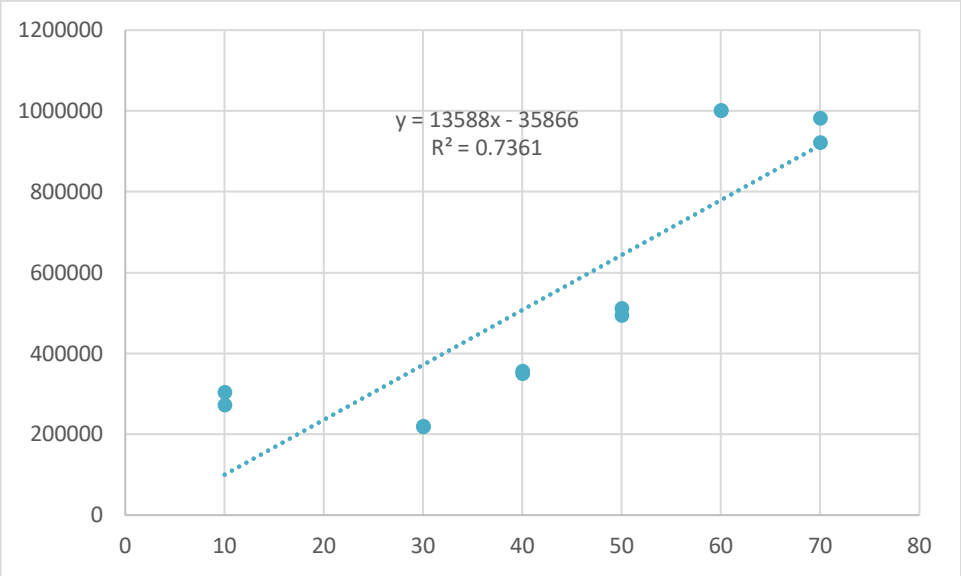
Tetradecanoic acid



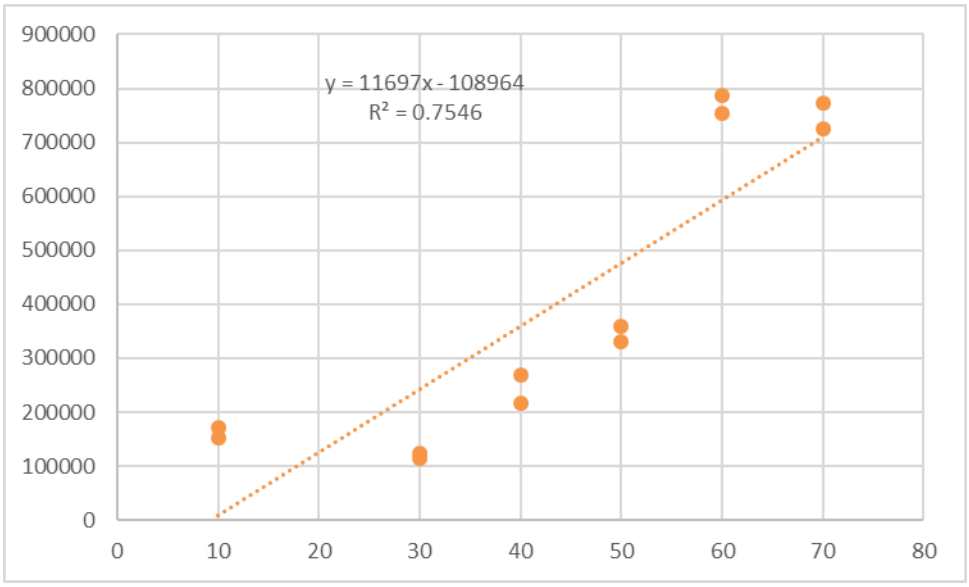
13-methyltetradecanoic acid



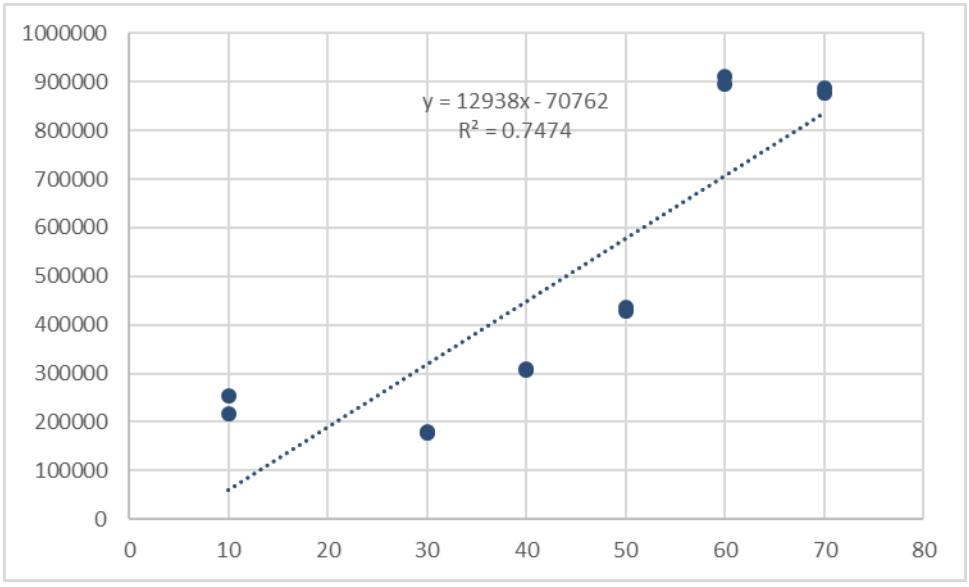
12-methytetradecanoic acid



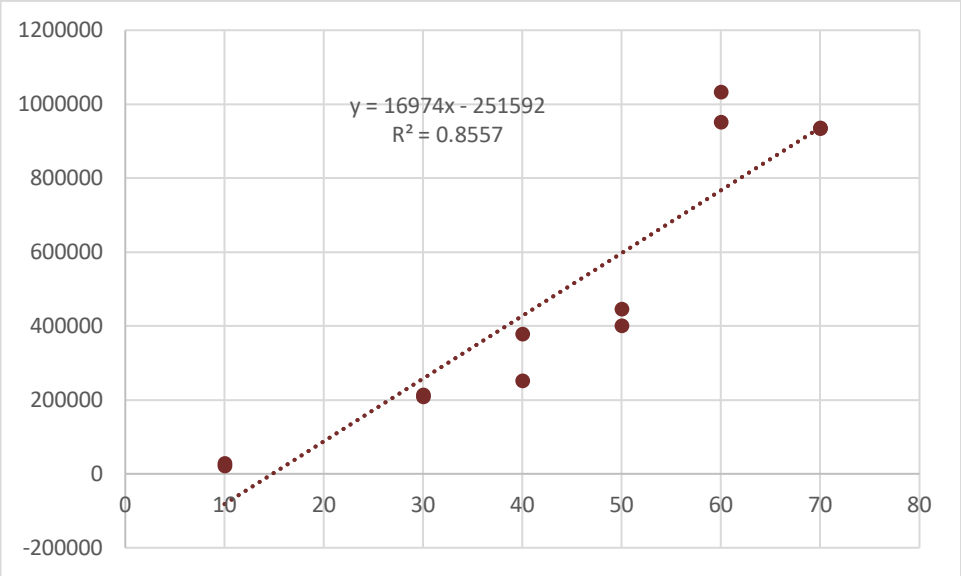
Cis-9-hexadecanoic acid



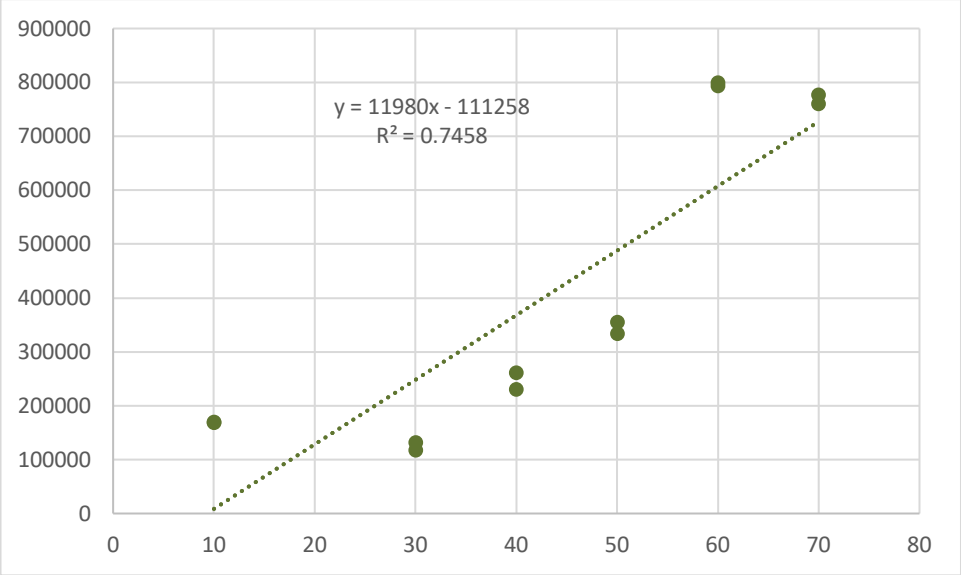
Hexadecanoic acid



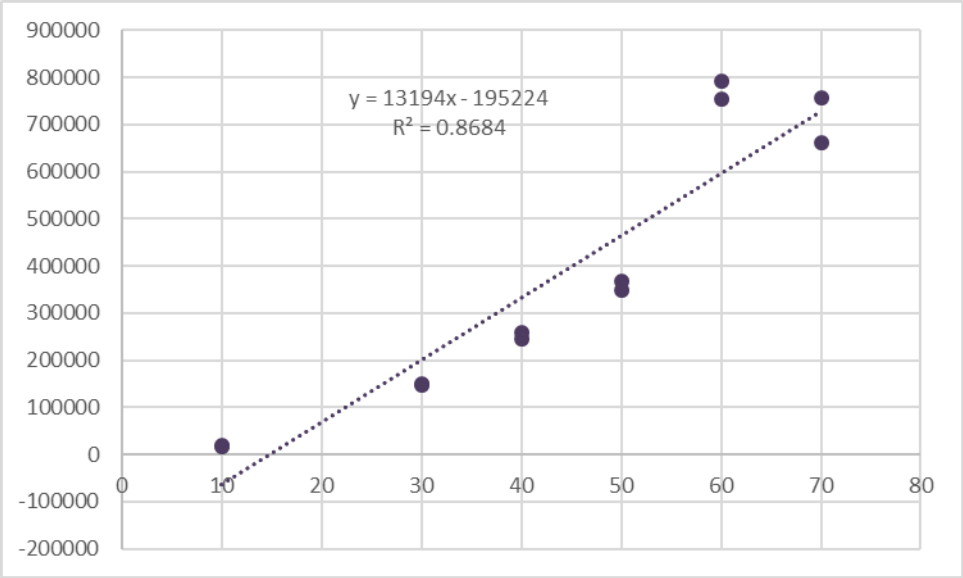
15-methylhexadecanoic acid



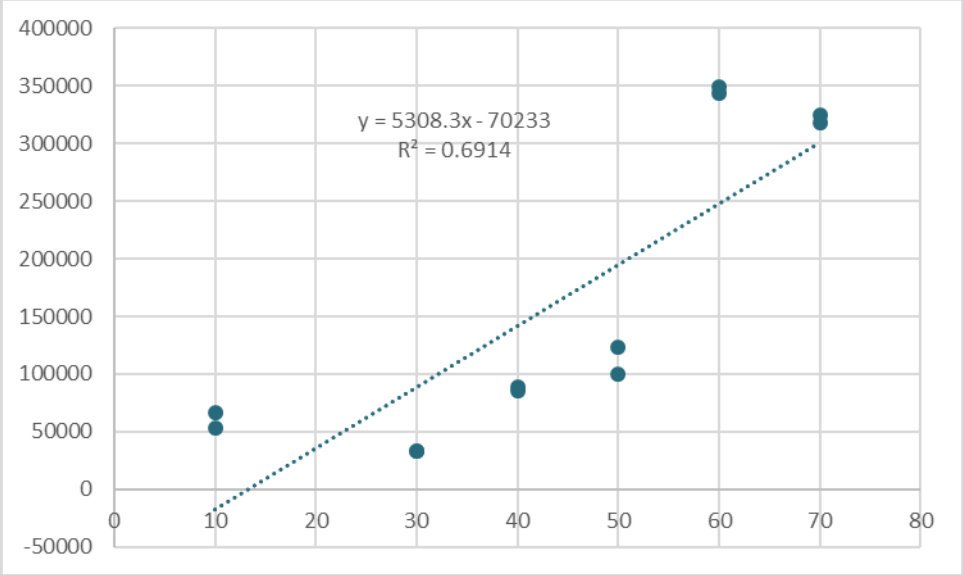
Cis-9, 10-methylene-hexadecanoic acid



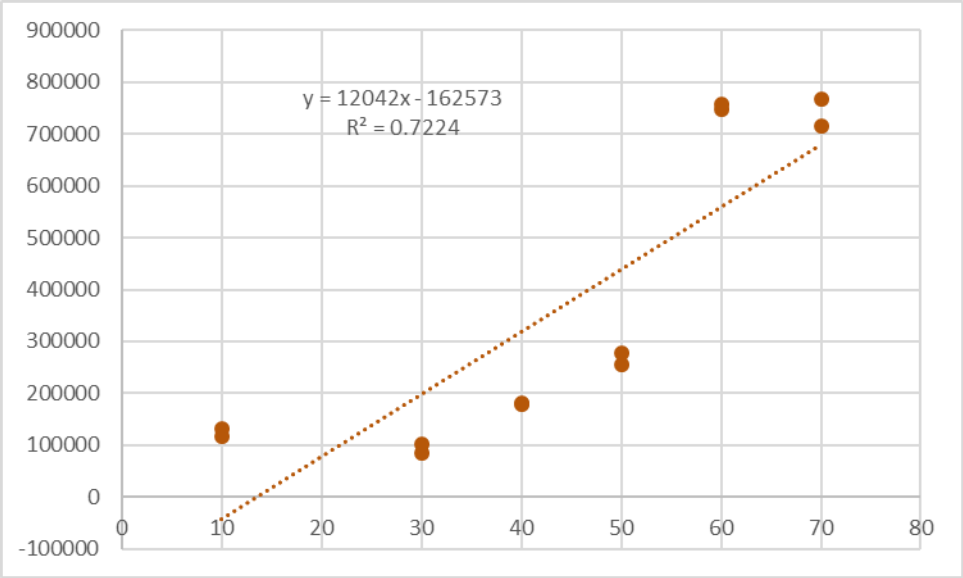
Heptadecanoic acid



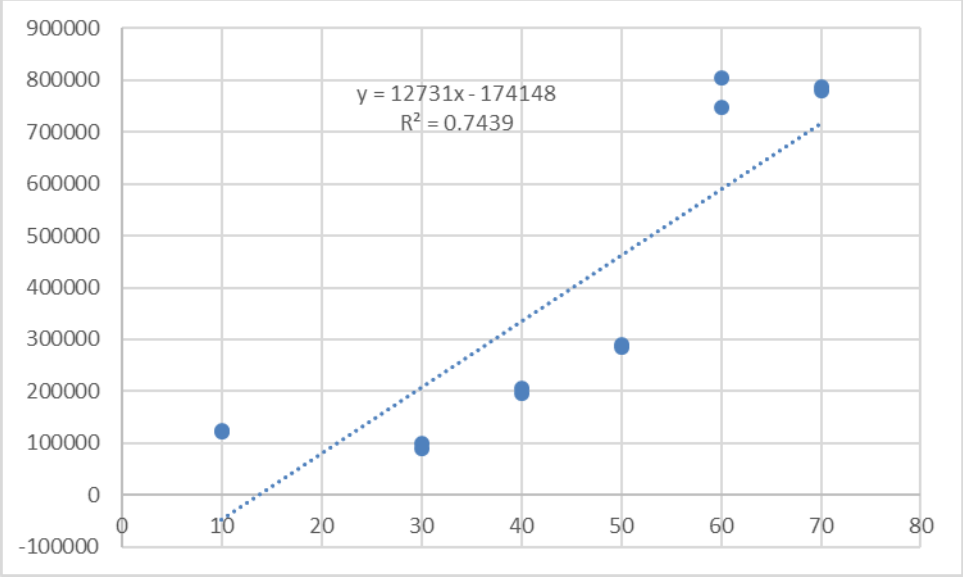
Cis-9, 12-octadecanoic acid



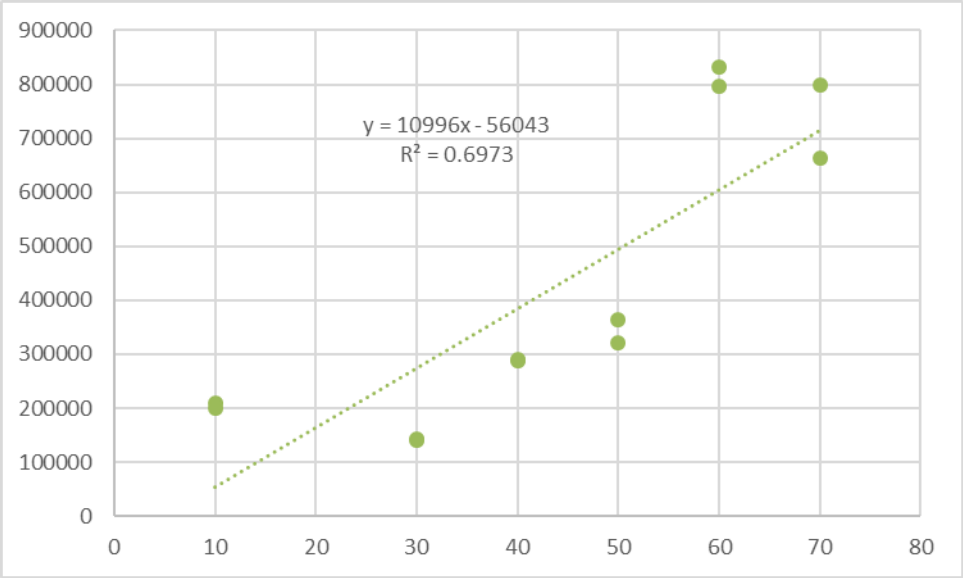
Cis-9 octadecanoic acid



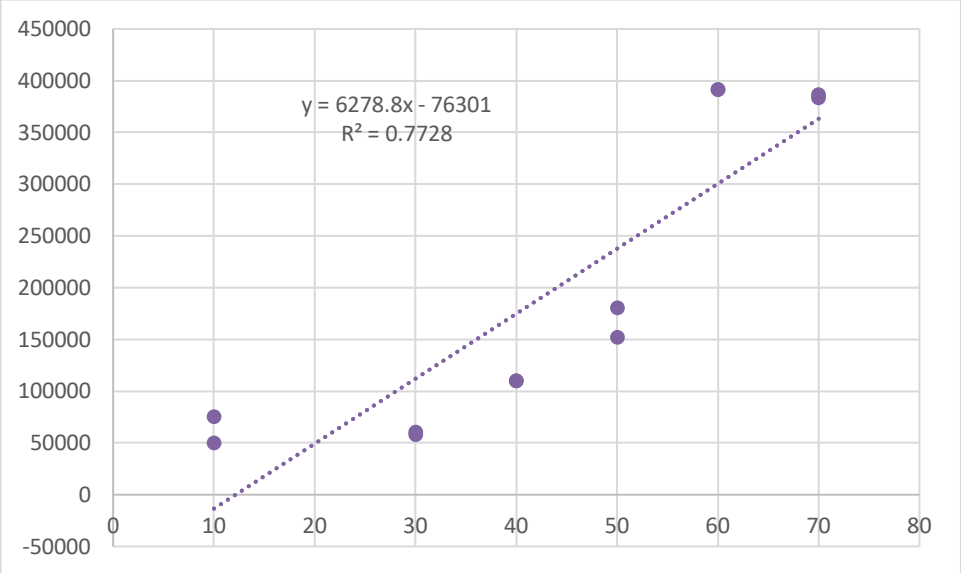
Trans-9-octadecanoic acid



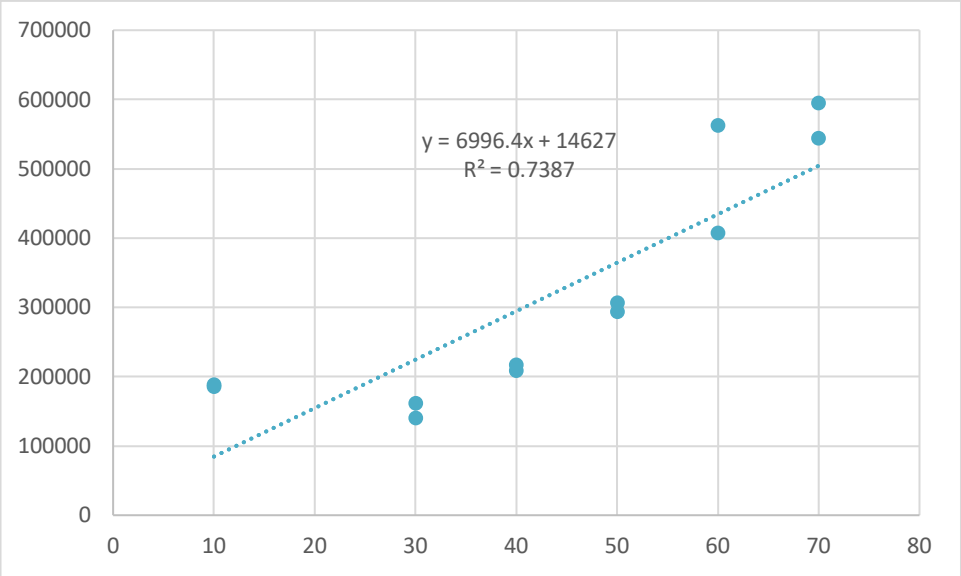
Octadecanoic acid

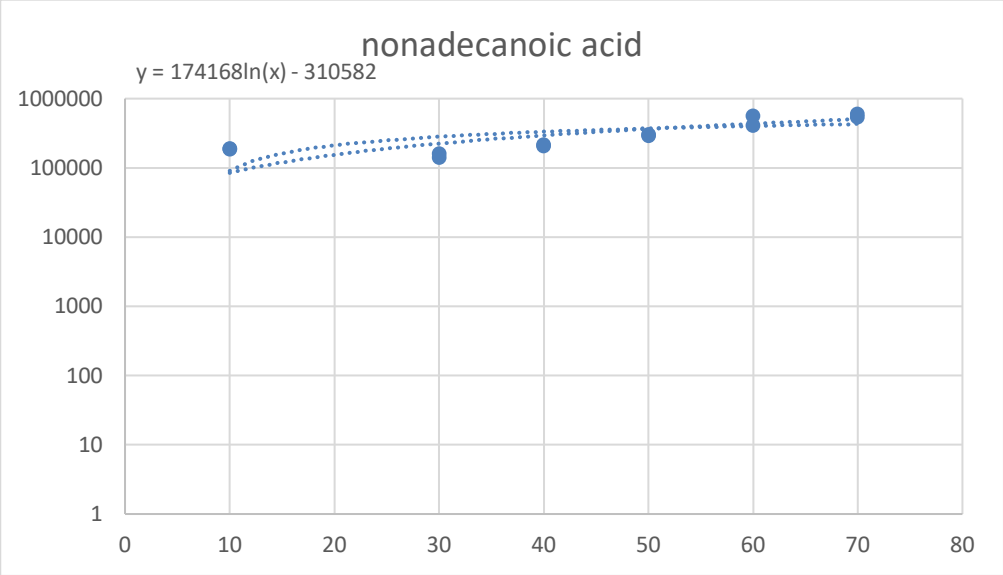


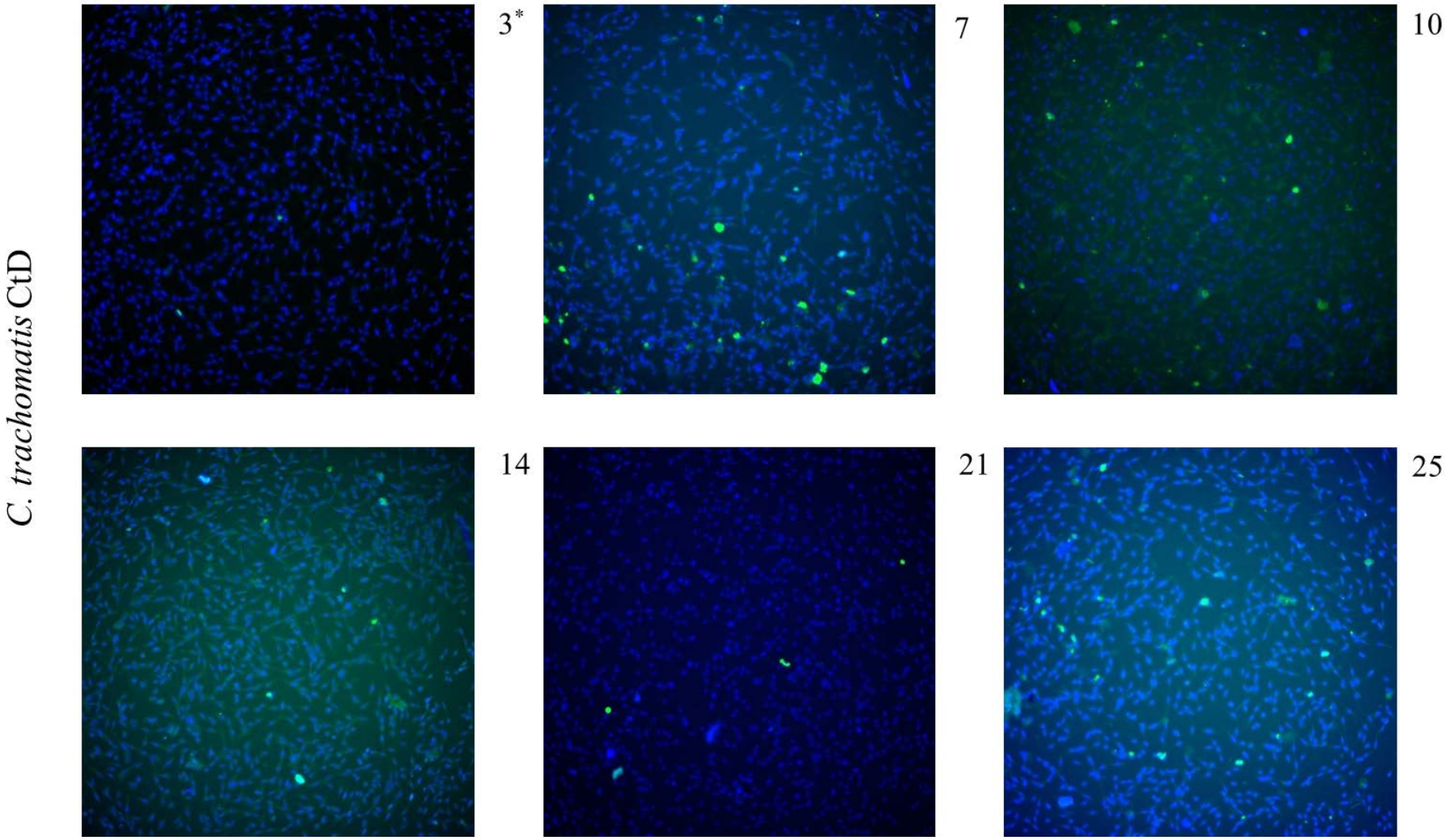
cis-9,10-methylene-octadecanoic acid

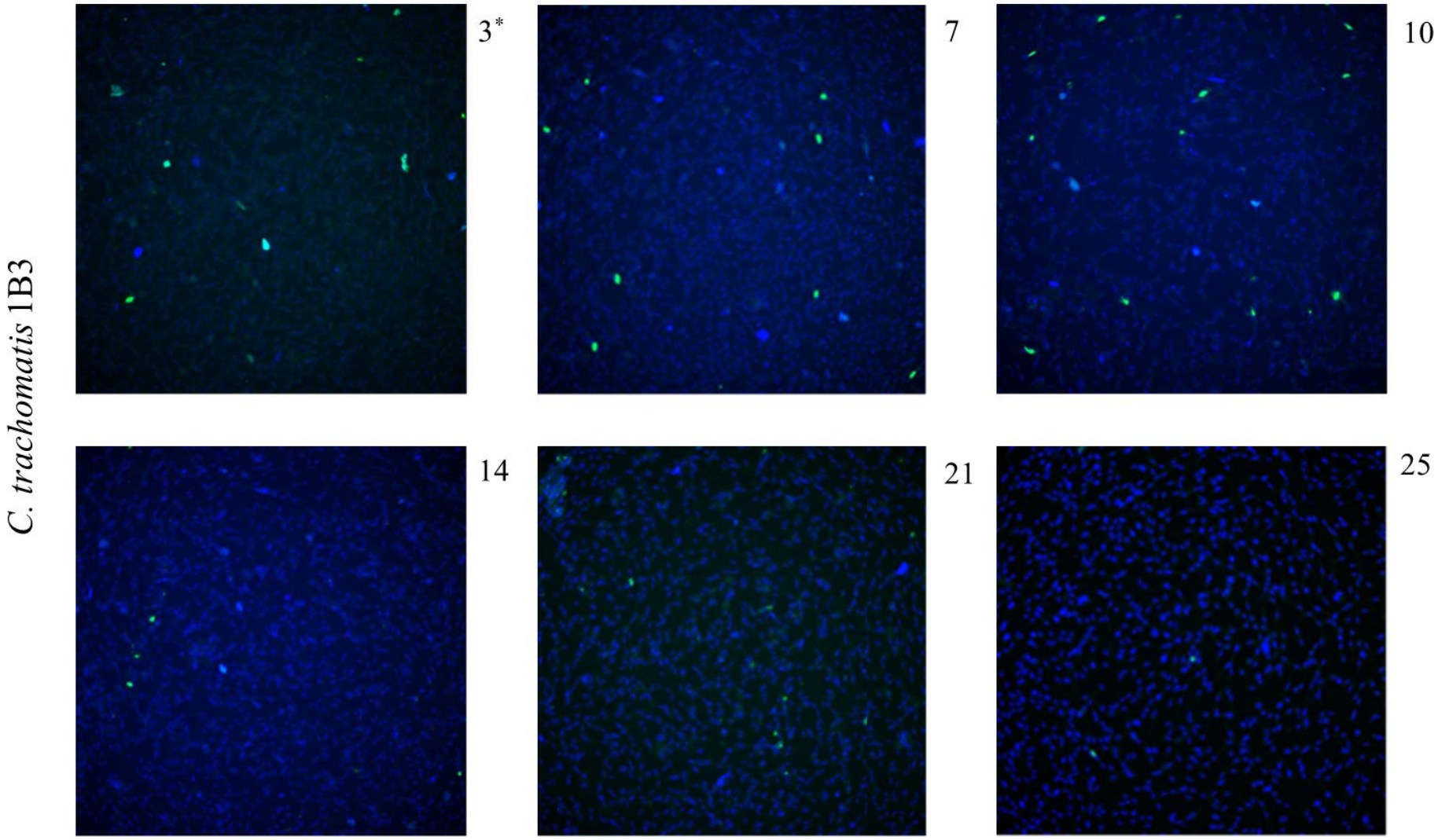


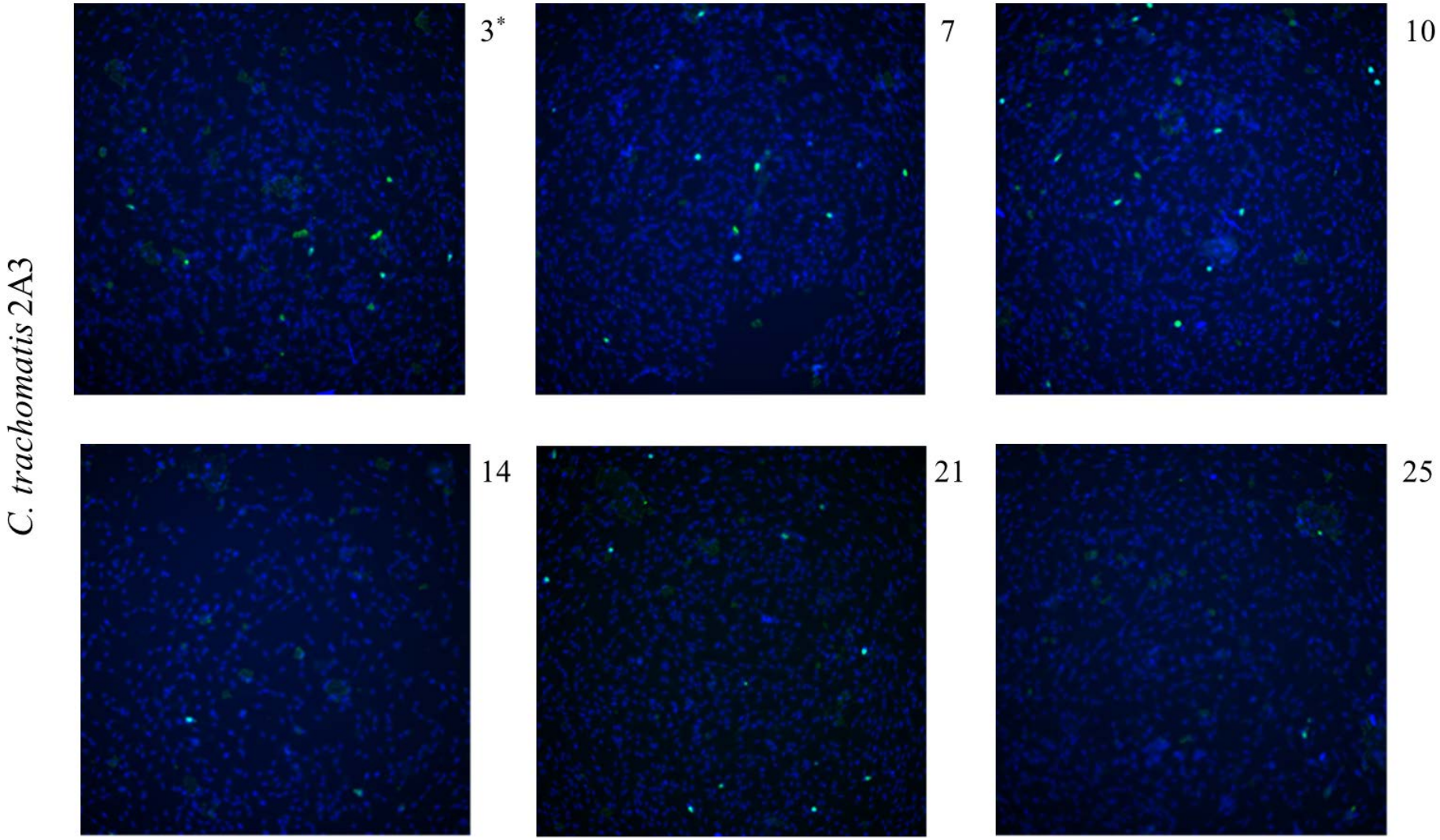
Nonadecanoic acid


700000
600000
500000
400000
300000
200000
100000
0
0
10
20
30
40
50
60
70
80
y = 6996.4x + 14627
R² = 0.7387


nonadecanoic acid
y = 174168ln(x) - 310582
1000000
100000
10000
1000
100
10
1
0
10
20
30
40
50
60
70
80


C. trachomatis CtD
3*
7
10
14
21
25


C. trachomatis 1B3
3*
7
10
14
21
25



Representative image for each day post-infection from all mouse models. Images captured on GE health Incell analyser 2200 at 10 x magnification using a FITC and DAPI channel. * Note well images for all dpi was captured at a 1:2. The FITC channel stains for the Chlamydial inclusions (green) and the DAPI channel stains for the McCoy B host cell (blue). Days post infection shown on the top right, chlamydial variant shown on the left

Bibliography

1. Mitchell, C. and M.J.I.D.C. Prabhu, *Pelvic inflammatory disease: current concepts in pathogenesis, diagnosis and treatment.* 2013. **27**(4): p. 793-809.
2. Ross, J.D.J.C.o.i.i.d., *Pelvic inflammatory disease: how should it be managed?* 2003. **16**(1): p. 37-41.
3. Darville, T. and T.P.I.D.W.J.S.t.d. Proceedings, *Pelvic Inflammatory Disease: Identifying Research Gaps-Proceedings of a Workshop Sponsored by DHHS/NIH/NIAID November 3-4, 2011.* 2013. **40**(10): p. 761.
4. Brunham, R.C., S.L. Gottlieb, and J.J.N.E.J.o.M. Paavonen, *Pelvic inflammatory disease.* 2015. **372**(21): p. 2039-2048.
5. Kreisel, K., et al., *Prevalence of Pelvic Inflammatory Disease in Sexually Experienced Women of Reproductive Age - United States, 2013-2014.* MMWR. Morbidity and mortality weekly report, 2017. **66**(3): p. 80-83.
6. Reekie, J., et al., *Hospitalisations for pelvic inflammatory disease temporally related to a diagnosis of Chlamydia or gonorrhoea: a retrospective cohort study.* 2014. **9**(4): p. e94361.
7. Bender, N., et al., *Chlamydia infection, pelvic inflammatory disease, ectopic pregnancy and infertility: cross-national study.* Sex Transm Infect, 2011. **87**(7): p. 601-8.
8. Goller, J.L., et al., *Trends in diagnosis of pelvic inflammatory disease in an Australian sexual health clinic, 2002–16: before and after clinical audit feedback.* 2019. **16**(3): p. 247-253.
9. Jacobson, L., L.J.A.J.o.O. Weström, and Gynecology, *Objectivized diagnosis of acute pelvic inflammatory disease: diagnostic and prognostic value of routine laparoscopy.* 1969. **105**(7): p. 1088-1098.
10. Bevan, C., et al., *Clinical, laparoscopic and microbiological findings in acute salpingitis: report on a United Kingdom cohort.* 1995. **102**(5): p. 407-414.
11. Munday, P.J.J.o.I., *Pelvic inflammatory disease—an evidence-based approach to diagnosis.* 2000. **40**(1): p. 31-41.
12. WestrÖM, L., et al., *Pelvic Inflammatory Disease and Fertility: A Cohort Study of 1,844 Women with Laparoscopically Verified Disease and 657 Control Women with Normal Laparoscopic Results.* Sexually Transmitted Diseases, 1992. **19**(4).
13. Sellors, J., et al., *The accuracy of clinical findings and laparoscopy in pelvic inflammatory disease.* 1991. **164**(1): p. 113-120.
14. Cacciatore, B., et al., *Transvaginal sonographic findings in ambulatory patients with suspected pelvic inflammatory disease.* 1992. **80**(6): p. 912-916.
15. Patten, R.M., et al., *Pelvic inflammatory disease. Endovaginal sonography with laparoscopic correlation.* 1990. **9**(12): p. 681-689.
16. Tukeva, T.A., et al., *MR imaging in pelvic inflammatory disease: comparison with laparoscopy and US.* 1999. **210**(1): p. 209-216.
17. Fiddes, P., et al., *Attitudes towards pelvic examination and chaperones: a questionnaire survey of patients and providers.* 2003. **67**(4): p. 313-317.

18. Stewart, R.A., M. Jill Thistlethwaite, and G.J.A.J.o.G.P. Rebecca Evans BSpExSc Hons, *Pelvic examination of asymptomatic women: attitudes and clinical practice.* 2008. **37**(6): p. 493.
19. Yanikkerem, E., et al., *Women's attitudes and expectations regarding gynaecological examination.* Midwifery, 2009. **25**(5): p. 500-508.
20. Hilden, M., et al., *Women's experiences of the gynecologic examination: factors associated with discomfort.* 2003. **82**(11): p. 1030-1036.
21. Morris, G.C., et al., *A cross-sectional study showing differences in the clinical diagnosis of pelvic inflammatory disease according to the experience of clinicians: implications for training and audit.* 2014. **90**(6): p. 445-451.
22. Alliance, A.S.H., *Australian STI management guidelines for use in primary care.* 2016.
23. Perry, T.R. and J.J. Schentag, *Clinical Use of Ceftriaxone.* Clinical Pharmacokinetics, 2001. **40**(9): p. 685-694.
24. Brogden, R.N. and A. Ward, *Ceftriaxone.* Drugs, 1988. **35**(6): p. 604-645.
25. Alam, M.J., et al., *Ceftriaxone resistance patterns of uropathogens isolated from urinary tract infection patients in selected areas of Dhaka city, Bangladesh.* 2017. **6**(5): p. 28-34.
26. Freeman, C.D., N.E. Klutman, and K.C. Lamp, *Metronidazole.* Drugs, 1997. **54**(5): p. 679-708.
27. Dhand, A. and D.R. Snydman, *Mechanism of Resistance in Metronidazole*, in *Antimicrobial Drug Resistance: Mechanisms of Drug Resistance*, D.L. Mayers, Editor. 2009, Humana Press: Totowa, NJ. p. 223-227.
28. Smith, A., *Metronidazole resistance: a hidden epidemic?* British Dental Journal, 2018. **224**(6): p. 403-404.
29. Cunha, B.A., C.M. Sibley, and A.M. Ristuccia, *Doxycycline.* Therapeutic drug monitoring, 1982. **4**(2): p. 115-135.
30. Joshi, N. and D.Q.J.A.o.i.m. Miller, *Doxycycline revisited.* 1997. **157**(13): p. 1421-1428.
31. Poncin, T., et al., *Multidrug-resistant Neisseria gonorrhoeae failing treatment with ceftriaxone and doxycycline in France, November 2017.* 2018. **23**(21): p. 1800264.
32. Carmine, A.A., et al., *Cefotaxime.* Drugs, 1983. **25**(3): p. 223-289.
33. Robertson, M.B., et al., *Ceftriaxone and cefotaxime use in Victorian hospitals.* 2002. **176**(11): p. 524-529.
34. Bonomo, R.A., et al., *Cefotaxime-resistant bacteria colonizing older people admitted to an acute care hospital.* Journal of the American Geriatrics Society, 2003. **51**(4): p. 519-522.
35. Dunn, C.J. and L.B. Barradell, *Azithromycin.* Drugs, 1996. **51**(3): p. 483-505.
36. Peters, D.H., H.A. Friedel, and D. McTavish, *Azithromycin.* Drugs, 1992. **44**(5): p. 750-799.
37. McMullan, B.J. and M. Mostaghim, *Prescribing azithromycin.* Australian prescriber, 2015. **38**(3): p. 87-89.
38. Balfour, J.A.B. and L.R. Wiseman, *Moxifloxacin.* Drugs, 1999. **57**(3): p. 363-373.
39. Keating, G.M. and L.J. Scott, *Moxifloxacin.* Drugs, 2004. **64**(20): p. 2347-2377.
40. Couldwell, D.L., et al., *Failure of moxifloxacin treatment in Mycoplasma genitalium infections due to macrolide and fluoroquinolone resistance.* 2013. **24**(10): p. 822-828.
41. Oldenburg, C.E., et al., *Emerging moxifloxacin resistance in Pseudomonas aeruginosa keratitis isolates in South India.* 2013. **20**(3): p. 155-158.
42. Quentin, R., et al., *Pelvic inflammatory disease: medical treatment.* 2000. **92**(2): p. 189-192.

43. Ness, R.B., et al., *Effectiveness of inpatient and outpatient treatment strategies for women with pelvic inflammatory disease: results from the Pelvic Inflammatory Disease Evaluation and Clinical Health (PEACH) Randomized Trial.* 2002. **186**(5): p. 929-937.
44. Washington, A.E., et al., *Assessing risk for pelvic inflammatory disease and its sequelae.* Jama, 1991. **266**(18): p. 2581-6.
45. Brunham, R., et al., *Etiology and outcome of acute pelvic inflammatory disease.* 1988. **158**(3): p. 510-517.
46. Haggerty, C.L., et al., *Lower quality of life among women with chronic pelvic pain after pelvic inflammatory disease.* 2003. **102**(5): p. 934-939.
47. Ness, R.B., et al., *Chlamydia Antibodies, Chlamydia Heat Shock Protein, and Adverse Sequelae After Pelvic Inflammatory Disease: The PID Evaluation and Clinical Health (PEACH) Study.* 2008. **35**(2): p. 129-135.
48. Safrin, S., et al., *Long-term sequelae of acute pelvic inflammatory disease: a retrospective cohort study.* 1992. **166**(4): p. 1300-1305.
49. Rein, D.B., et al., *Direct medical cost of pelvic inflammatory disease and its sequelae: decreasing, but still substantial.* 2000. **95**(3): p. 397-402.
50. Patrick, D.M.J.C.J.o.H.S., *CHLAMYDIA CONTROL: COMPONENTS OF AN EFFECTIVE CONTROL STRATEGY TO REDUCE THE INCIDENCE OF CHLAMYDIA TRACHOMATIS.* 1997. **6**(2).
51. Haggerty, C.L. and R.B. Ness, *Epidemiology, pathogenesis and treatment of pelvic inflammatory disease.* Expert Review of Anti-infective Therapy, 2006. **4**(2): p. 235-247.
52. Kildea, S., F.J.J.A. Bowden, and N.Z.J.o.P. Health, *Reproductive health, infertility and sexually transmitted infections in Indigenous women in a remote community in the Northern Territory.* 2000. **24**(4): p. 382-386.
53. Washington, A.E., W. Cates, Jr, and A.A. Zaidi, *Hospitalizations for Pelvic Inflammatory Disease: Epidemiology and Trends in the United States, 1975 to 1981.* JAMA, 1984. **251**(19): p. 2529-2533.
54. Simms, I., et al., *Risk factors associated with pelvic inflammatory disease.* 2006. **82**(6): p. 452-457.
55. Goller, J.L., et al., *Characteristics of pelvic inflammatory disease where no sexually transmitted infection is identified: a cross-sectional analysis of routinely collected sexual health clinic data.* 2017. **93**(1): p. 68-70.
56. Haggerty, C.L., B.D.J.I.d.i.o. Taylor, and gynecology, *Mycoplasma genitalium: an emerging cause of pelvic inflammatory disease.* 2011. **2011**.
57. Mears, A., J.S.J.T.O. Bingham, and Gynaecologist, *Pelvic inflammatory disease.* 2004. **6**(3): p. 138-144.
58. Bradshaw, C.S., et al., *Prevalent and incident bacterial vaginosis are associated with sexual and contraceptive behaviours in young Australian women.* 2013. **8**(3): p. e57688.
59. Marrazzo, J.M., et al., *Risk factors for cervicitis among women with bacterial vaginosis.* 2006. **193**(5): p. 617-624.
60. Haggerty, C.L., et al., *Bacterial vaginosis and anaerobic bacteria are associated with endometritis.* 2004. **39**(7): p. 990-995.
61. Wiesenfeld, H.C., et al., *Lower genital tract infection and endometritis: insight into subclinical pelvic inflammatory disease.* 2002. **100**(3): p. 456-463.
62. Peipert, J.P., et al., *Bacterial Vaginosis as a Risk Factor for Upper Genital Tract Infection.* 1998. **53**(3): p. 150-151.

63. Ness, R.B., et al., *A Cluster Analysis of Bacterial Vaginosis–associated Microflora and Pelvic Inflammatory Disease.* American Journal of Epidemiology, 2005. **162**(6): p. 585-590.
64. Taylor, B.D., T. Darville, and C.L. Haggerty, *Does Bacterial Vaginosis Cause Pelvic Inflammatory Disease?* Sexually Transmitted Diseases, 2013. **40**(2).
65. Haggerty, C.L., et al., *Identification of novel microbes associated with pelvic inflammatory disease and infertility.* Sexually Transmitted Infections, 2016. **92**(6): p. 441.
66. Petrina, M.A., et al., *Susceptibility of endometrial isolates recovered from women with clinical pelvic inflammatory disease or histological endometritis to antimicrobial agents.* 2019. **56**: p. 61-65.
67. Jaiyeoba, O., G. Lazenby, and D.E. Soper, *Recommendations and rationale for the treatment of pelvic inflammatory disease.* Expert Review of Anti-infective Therapy, 2011. **9**(1): p. 61-70.
68. Stepanović, S., et al., *Staphylococcus sciuri: an unusual cause of pelvic inflammatory disease.* 2005. **16**(6): p. 452-453.
69. Jaiyeoba, O., G. Lazenby, and D.E.J.E.r.o.a.-i.t. Soper, *Recommendations and rationale for the treatment of pelvic inflammatory disease.* 2011. **9**(1): p. 61-70.
70. Brunham, R.C. and J.J.N.r.i. Rey-Ladino, *Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine.* 2005. **5**(2): p. 149-161.
71. O'Connell, C.M. and M.E. Ferone, *Chlamydia trachomatis Genital Infections.* Microb Cell, 2016. **3**(9): p. 390-403.
72. Hybiske, K. and R.S. Stephens, *Exit strategies of intracellular pathogens.* Nature Reviews Microbiology, 2008. **6**(2): p. 99-110.
73. Abdul-Sater, A.A., et al., *Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen Chlamydia trachomatis.* 2009. **284**(39): p. 26789-26796.
74. Skipp, P.J., et al., *Quantitative proteomics of the infectious and replicative forms of Chlamydia trachomatis.* 2016. **11**(2): p. e0149011.
75. Muvunyi, C.M., et al., *Chlamydia trachomatis infection in fertile and subfertile women in Rwanda: prevalence and diagnostic significance of IgG and IgA antibodies testing.* 2011. **26**(12): p. 3319-3326.
76. Newman, L., et al., *Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting.* 2015. **10**(12): p. e0143304.
77. Igietseme, J.U., F.O. Eko, and C.M.J.E.r.o.v. Black, *Chlamydia vaccines: recent developments and the role of adjuvants in future formulations.* 2011. **10**(11): p. 1585-1596.
78. Lewis, D., et al., *The prevalence of Chlamydia trachomatis infection in Australia: a systematic review and meta-analysis.* 2012. **12**(1): p. 113.
79. Steiner, K.C., et al., *Field-delivered therapy increases treatment for chlamydia and gonorrhea.* 2003. **93**(6): p. 882-884.
80. Kong, F.Y.S. and J.S.J.B.i.d. Hocking, *Treatment challenges for urogenital and anorectal Chlamydia trachomatis.* 2015. **15**(1): p. 1-7.
81. Cappello, F., et al., *Chlamydia trachomatis infection and anti-Hsp60 immunity: the two sides of the coin.* 2009. **5**(8): p. e1000552.
82. Hjelholt, A., et al., *Tubal factor infertility is associated with antibodies against Chlamydia trachomatis heat shock protein 60 (HSP60) but not human HSP60.* 2011. **26**(8): p. 2069-2076.

83. Linhares, I.M., S.S.J.C.S. Witkin, and Chaperones, *Immunopathogenic consequences of Chlamydia trachomatis 60 kDa heat shock protein expression in the female reproductive tract.* 2010. **15**(5): p. 467-473.
84. Di Felice, V., et al., *Is chlamydial heat shock protein 60 a risk factor for oncogenesis?* 2005. **62**(1): p. 4-9.
85. Stephens, R.S.J.T.i.m., *The cellular paradigm of chlamydial pathogenesis.* 2003. **11**(1): p. 44-51.
86. Stephens, R.S., *The cellular paradigm of chlamydial pathogenesis.* Trends Microbiol, 2003. **11**(1): p. 44-51.
87. Cunningham, K., et al., *The IL-6 response to Chlamydia from primary reproductive epithelial cells is highly variable and may be involved in differential susceptibility to the immunopathological consequences of chlamydial infection.* 2013. **14**(1): p. 1-13.
88. Menon, S., et al., *Human and pathogen factors associated with Chlamydia trachomatis-related infertility in women.* 2015. **28**(4): p. 969-985.
89. Lan, J., et al., *Chlamydia trachomatis and ectopic pregnancy: retrospective analysis of salpingectomy specimens, endometrial biopsies, and cervical smears.* 1995. **48**(9): p. 815-819.
90. BARLOW, R.E., et al., *The prevalence of Chlamydia trachomatis in fresh tissue specimens from patients with ectopic pregnancy or tubal factor infertility as determined by PCR and in-situ hybridisation.* 2001. **50**(10): p. 902-908.
91. Wyrick, P.B.J.T.J.o.i.d., *Chlamydia trachomatis persistence in vitro: an overview.* 2010. **201**(Supplement_2): p. S88-S95.
92. McSheffrey, G.G. and S.D. Gray-Owen, *Neisseria gonorrhoeae*, in *Molecular Medical Microbiology*. 2015, Elsevier. p. 1471-1485.
93. Kenneth, J. and C.J.S.M.M.M.-H.C.I. George, *An introduction to infectious diseases.* 2004: p. 14-17.
94. Bowen, V.B., et al., *Gonorrhea.* Current Epidemiology Reports, 2017. **4**(1): p. 1-10.
95. Risser, W.L., et al., *Current perspectives in the USA on the diagnosis and treatment of pelvic inflammatory disease in adolescents.* 2017. **8**: p. 87.
96. Organization, W.H., *Prevalence and incidence of selected sexually transmitted infections, Chlamydia trachomatis, Neisseria gonorrhoeae, syphilis and Trichomonas vaginalis: methods and results used by WHO to generate 2005 estimates*. 2011, World Health Organization.
97. Kularatne, R.S., et al., *Adult gonorrhea, chlamydia and syphilis prevalence, incidence, treatment and syndromic case reporting in South Africa: Estimates using the Spectrum-STI model, 1990-2017.* 2018. **13**(10): p. e0205863.
98. Papp, J.R., et al., *Recommendations for the laboratory-based detection of Chlamydia trachomatis and Neisseria gonorrhoeae—2014.* 2014. **63**: p. 1.
99. Manis, R., B. Harris, and P.J.J.o.c.m. Geiseler, *Evaluation of Gonozyme, an enzyme immunoassay for the rapid diagnosis of gonorrhea.* 1984. **20**(4): p. 742-746.
100. pdf, W.H.O.J.U.h.a.w.i.i.b.-e., *WHO guidelines for the treatment of Neisseria gonorrhoeae. 2016.* 2016.
101. Brown, L.B., et al., *Neisseria gonorrhoeae antimicrobial susceptibility in Lilongwe, Malawi, 2007.* 2010. **37**(3): p. 169-172.
102. Prevention, C.f.D.C.a. *Antibiotic-Resistant Gonorrhea Basic Information*. 2019 [cited 2020; Available from: https://www.cdc.gov/std/gonorrhea/arg/basic.htm.
103. Unemo, M. and W.M.J.C.m.r. Shafer, *Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future.* 2014. **27**(3): p. 587-613.

104. Hill, S.A., T.L. Masters, and J.J.M.c. Wachter, *Gonorrhea-an evolving disease of the new millennium.* 2016. **3**(9): p. 371.
105. Wolfgang, M., et al., *PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae.* 1998. **29**(1): p. 321-330.
106. Bhat, K., et al., *The opacity proteins of Neisseria gonorrhoeae strain MS11 are encoded by a family of 11 complete genes.* 1991. **5**(8): p. 1889-1901.
107. Britigan, B.E., M.S. Cohen, and P.F.J.N.E.J.o.M. Sparling, *Gonococcal infection: a model of molecular pathogenesis.* 1985. **312**(26): p. 1683-1694.
108. Elkins, C., et al., *Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated.* 1992. **6**(18): p. 2617-2628.
109. Gregg, C.R., et al., *Toxic activity of purified lipopolysaccharide of Neisseria gonorrhoeae for human fallopian tube mucosa.* 1981. **143**(3): p. 432-439.
110. Ayala, P., et al., *Neisseria gonorrhoeae porin P1. B induces endosome exocytosis and a redistribution of Lamp1 to the plasma membrane.* 2002. **70**(11): p. 5965-5971.
111. Halter, R., J. Pohlner, and T.F.J.T.E.j. Meyer, *IgA protease of Neisseria gonorrhoeae: isolation and characterization of the gene and its extracellular product.* 1984. **3**(7): p. 1595-1601.
112. Razin, S., et al., *Molecular biology and pathogenicity of mycoplasmas.* 1998. **62**(4): p. 1094-1156.
113. Tully, J., et al., *A newly discovered mycoplasma in the human urogenital tract.* 1981. **317**(8233): p. 1288-1291.
114. Taylor-Robinson, D., *Mycoplasma genitalium-an up-date*. 2002, SAGE Publications Sage UK: London, England.
115. Tully, J.G., et al., *Urogenital challenge of primate species with Mycoplasma genitalium and characteristics of infection induced in chimpanzees.* 1986. **153**(6): p. 1046-1054.
116. Totten, P.A., L.E. Manhart, and A. Centurion-Lara, *PCR detection of Haemophilus ducreyi, Treponema pallidum, and Mycoplasma genitalium*, in *Molecular Microbiology*. 2011, American Society of Microbiology. p. 397-413.
117. Cohen, C., et al., *Detection of Mycoplasma genitalium in women with laparoscopically diagnosed acute salpingitis.* 2005. **81**(6): p. 463-466.
118. Clausen, H.F., et al., *Serological investigation of Mycoplasma genitalium in infertile women.* 2001. **16**(9): p. 1866-1874.
119. Falk, L., H. Fredlund, and J.J.S.t.i. Jensen, *Signs and symptoms of urethritis and cervicitis among women with or without Mycoplasma genitalium or Chlamydia trachomatis infection.* 2005. **81**(1): p. 73-78.
120. McGowin, C.L. and C. Anderson-Smits, *Mycoplasma genitalium: An Emerging Cause of Sexually Transmitted Disease in Women.* PLOS Pathogens, 2011. **7**(5): p. e1001324.
121. Ma, L., et al., *Genetic variation in the complete MgPa operon and its repetitive chromosomal elements in clinical strains of Mycoplasma genitalium.* 2010. **5**(12): p. e15660.
122. Taylor-Robinson, D. and W.M.J.N.E.J.o.M. McCormack, *The genital mycoplasmas.* 1980. **302**(18): p. 1003-1010.
123. Taylor-Robinson, D.J.R.i.M., *Mollicutes in vaginal microbiology: Mycoplasma hominis, Ureaplasma urealyticum, Ureaplasma parvum and Mycoplasma genitalium.* 2017. **168**(9-10): p. 875-881.
124. Anagrius, C., B. Lore, and J.J.S.t.i. Jensen, *Mycoplasma genitalium: prevalence, clinical significance, and transmission.* 2005. **81**(6): p. 458-462.

125. Jensen, J.S., et al., *Polymerase chain reaction for detection of Mycoplasma genitalium in clinical samples.* 1991. **29**(1): p. 46-50.
126. Palmer, H., et al., *Detection of Mycoplasma genitalium in the genitourinary tract of women by the polymerase chain reaction.* 1991. **2**(4): p. 261-263.
127. Falk, L., H. Fredlund, and J.J.S.t.i. Jensen, *Tetracycline treatment does not eradicate Mycoplasma genitalium.* 2003. **79**(4): p. 318-319.
128. Unemo, M. and J.S.J.N.R.U. Jensen, *Antimicrobial-resistant sexually transmitted infections: gonorrhoea and Mycoplasma genitalium.* 2017. **14**(3): p. 139.
129. Ioannidis, J.P.J.J., *Contradicted and initially stronger effects in highly cited clinical research.* 2005. **294**(2): p. 218-228.
130. Hackam, D.G. and D.A.J.J. Redelmeier, *Translation of research evidence from animals to humans.* 2006. **296**(14): p. 1727-1732.
131. Morrison, R.P., H.D.J.I. Caldwell, and immunity, *Immunity to murine chlamydial genital infection.* 2002. **70**(6): p. 2741-2751.
132. McGowin, C.L., R.A. Spagnuolo, and R.B. Pyles, *Mycoplasma genitalium Rapidly Disseminates to the Upper Reproductive Tracts and Knees of Female Mice following Vaginal Inoculation.* 2010. **78**(2): p. 726-736.
133. Rice, P.A., et al., *Neisseria gonorrhoeae: Drug Resistance, Mouse Models, and Vaccine Development.* Annual Review of Microbiology, 2017. **71**(1): p. 665-686.
134. Jerse, A., et al., *Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections.* 2011. **2**(107).
135. Murthy, A.K., W. Li, and K.H. Ramsey, *Immunopathogenesis of Chlamydial Infections.* Curr Top Microbiol Immunol, 2018. **412**: p. 183-215.
136. Morrison, S.G., et al., *Murine Chlamydia trachomatis genital infection is unaltered by depletion of CD4+ T cells and diminished adaptive immunity.* J Infect Dis, 2011. **203**(8): p. 1120-8.
137. Lyons, J.M., et al., *Identification of candidate genes using the murine model of female genital tract infection with Chlamydia trachomatis.* Drugs Today (Barc), 2009. **45 Suppl B**: p. 51-9.
138. Pal, S., et al., *Transcervical Inoculation with Chlamydia trachomatis Induces Infertility in HLA-DR4 Transgenic and Wild-Type Mice.* Infect Immun, 2018. **86**(1).
139. Jiang, P., et al., *Evaluation of tandem Chlamydia trachomatis MOMP multi-epitopes vaccine in BALB/c mice model.* Vaccine, 2017. **35**(23): p. 3096-3103.
140. Morrison, S.G., et al., *Murine Chlamydia trachomatis Genital Infection Is Unaltered by Depletion of CD4+ T cells and Diminished Adaptive Immunity.* The Journal of Infectious Diseases, 2011. **203**(8): p. 1120-1128.
141. O'Meara, C., D. Andrew, and K.J.C.m.m. Beagley, *The mouse model of Chlamydia genital tract infection: a review of infection, disease, immunity and vaccine development.* 2014. **14**(3): p. 396-421.
142. Phillips, S., B.L. Quigley, and P. Timms, *Seventy Years of Chlamydia Vaccine Research – Limitations of the Past and Directions for the Future.* 2019. **10**(70).
143. Käser, T., et al., *Contribution of the swine model in the study of human sexually transmitted infections.* 2018. **66**: p. 346-360.
144. de Jonge, M.I., et al., *A novel guinea pig model of Chlamydia trachomatis genital tract infection.* Vaccine, 2011. **29**(35): p. 5994-6001.
145. Neuendorf, E., et al., *Chlamydia caviae infection alters abundance but not composition of the guinea pig vaginal microbiota.* Pathog Dis, 2015. **73**(4).

146. Greaves, E., et al., *A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium.* 2014. **184**(7): p. 1930-1939.
147. Sowerby, E. and J.J.H.F. Parsons, *Prevention of iatrogenic pelvic infection during in vitro fertilization—current practice in the UK.* 2004. **7**(2): p. 135-140.
148. Farley, T.M., et al., *Intrauterine devices and pelvic inflammatory disease: an international perspective.* Lancet, 1992. **339**(8796): p. 785-8.
149. Curry, A., T. Williams, and M.L.J.A.f.p. Penny, *Pelvic Inflammatory Disease: Diagnosis, Management, and Prevention.* 2019. **100**(6): p. 357-364.
150. Lusby, H., et al., *Uncommon cause of pelvic inflammatory disease leading to toxic shock syndrome.* 2018. **2018**: p. bcr-2018-224955.
151. Zhao, W.-H., M.J.G. Hao, and O. Investigation, *Pelvic inflammatory disease: a retrospective clinical analysis of 1,922 cases in North China.* 2014. **77**(3): p. 169-175.
152. Grace, G.A., D.B. Devaleenal, and M.J.T.I.j.o.m.r. Natrajan, *Genital tuberculosis in females.* 2017. **145**(4): p. 425.
153. Eftekhar, M., et al., *Mycobacterium tuberculosis infection in women with unexplained infertility.* 2015. **13**(12): p. 749.
154. Liu, Q., et al., *Abdominopelvic tuberculosis mimicking advanced ovarian cancer and pelvic inflammatory disease: a series of 28 female cases.* 2014. **289**(3): p. 623-629.
155. Marchesi, J.R. and J. Ravel, *The vocabulary of microbiome research: a proposal.* Microbiome, 2015. **3**(1): p. 31.
156. O'Hanlon, D.E., T.R. Moench, and R.A.J.P.o. Cone, *Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota.* 2013. **8**(11): p. e80074.
157. O'Hanlon, D.E., T.R. Moench, and R.A.J.B.i.d. Cone, *In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide.* 2011. **11**(1): p. 1-8.
158. Aldunate, M., et al., *Vaginal concentrations of lactic acid potently inactivate HIV.* 2013. **68**(9): p. 2015-2025.
159. Mirmonsef, P., et al., *Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH.* 2014. **9**(7): p. e102467.
160. Hearps, A., et al., *Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition.* 2017. **10**(6): p. 1480-1490.
161. Tachedjian, G., et al., *The role of lactic acid production by probiotic Lactobacillus species in vaginal health.* 2017. **168**(9-10): p. 782-792.
162. Ravel, J., et al., *Vaginal microbiome of reproductive-age women.* 2011. **108**(Supplement 1): p. 4680-4687.
163. Daniel Johannes Rönnqvist, P., U. Birgitta Forsgren-Brusk, and E.J.A.o.e.g.S. Elisabeth Grahn-Håkansson, *Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH.* 2006. **85**(6): p. 726-735.
164. Breshears, L.M., et al., *Lactobacillus crispatus inhibits growth of Gardnerella vaginalis and Neisseria gonorrhoeae on a porcine vaginal mucosa model.* 2015. **15**(1): p. 1-12.
165. Aldunate, M., et al., *Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis.* 2015. **6**: p. 164.
166. Tachedjian, G., D.E. O'Hanlon, and J.J.M. Ravel, *The implausible "in vivo" role of hydrogen peroxide as an antimicrobial factor produced by vaginal microbiota.* 2018. **6**(1): p. 1-5.

167. Haggerty, C.L., et al., *Identification of novel microbes associated with pelvic inflammatory disease and infertility.* 2016. **92**(6): p. 441-446.
168. Aldunate, M., et al., *Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis.* 2015. **6**(164).
169. Gajer, P., et al., *Temporal dynamics of the human vaginal microbiota.* 2012. **4**(132): p. 132ra52-132ra52.
170. Brotman, R.M., et al., *Association between Trichomonas vaginalis and vaginal bacterial community composition among reproductive-age women.* 2012. **39**(10): p. 807.
171. Ravel, J., et al., *Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis.* 2013. **1**(1): p. 1-6.
172. Srinivasan, S., et al., *Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis.* 2010. **5**(4): p. e10197.
173. Kroon, S.J., et al., *Cervicovaginal microbiota, women's health, and reproductive outcomes.* 2018. **110**(3): p. 327-336.
174. Wang, Y., et al., *Characterization of pelvic and cervical microbiotas from patients with pelvic inflammatory disease.* 2018. **67**(10): p. 1519-1526.
175. Tai, F.-W., et al., *Association of Pelvic Inflammatory Disease with Risk of Endometriosis: A Nationwide Cohort Study Involving 141,460 Individuals.* Journal of clinical medicine, 2018. **7**(11): p. 379.
176. Kiviat, N.B., et al., *Endometrial histopathology in patients with culture-proved upper genital tract infection and laparoscopically diagnosed acute salpingitis.* The American journal of surgical pathology, 1990. **14**(2): p. 167-175.
177. Zheng, X., et al., *Gene Expression Signatures Can Aid Diagnosis of Sexually Transmitted Infection-Induced Endometritis in Women.* 2018. **8**(307).
178. Balamuth, F., et al., *RNA Biosignatures in Adolescent Patients in a Pediatric Emergency Department With Pelvic Inflammatory Disease.* Pediatric emergency care, 2015. **31**(7): p. 465-472.
179. Zheng, X., et al., *Discovery of blood transcriptional endotypes in women with pelvic inflammatory disease.* 2018. **200**(8): p. 2941-2956.
180. Debattista, J., P. Timms, and J.J.I.l. Allan, *Reduced levels of gamma-interferon secretion in response to chlamydial 60 kDa heat shock protein amongst women with pelvic inflammatory disease and a history of repeated Chlamydia trachomatis infections.* 2002. **81**(3): p. 205-210.
181. Darville, T. and T.J.J.T.J.o.i.d. Hiltke, *Pathogenesis of genital tract disease due to Chlamydia trachomatis.* 2010. **201**(Supplement_2): p. S114-S125.
182. Leung-Tack, J., et al., *Modulation of phagocytosis-associated respiratory burst by human cystatin C: Role of the N-terminal tetrapeptide Lys□Pro□Pro□Arg.* 1990. **188**(1): p. 16-22.
183. Tsai, H.-T., et al., *Imbalanced serum concentration between cathepsin B and cystatin C in patients with pelvic inflammatory disease.* 2009. **91**(2): p. 549-555.
184. Ho, T.-c., et al., *Increased plasma soluble CD40 ligand concentration in pelvic inflammatory disease.* 2015. **438**: p. 236-240.
185. Ma, B., et al., *O05. 4 Association Between Chlamydia Trachomatis Genital Infection and the Vaginal Microbiome.* 2013. **89**(Suppl 1): p. A35-A35.
186. Tamarelle, J., et al., *The vaginal microbiota and its association with human papillomavirus, Chlamydia trachomatis, Neisseria gonorrhoeae and Mycoplasma genitalium infections: a systematic review and meta-analysis.* 2019. **25**(1): p. 35-47.

187. Yilmaz, B., et al., *Gut microbiota elicits a protective immune response against malaria transmission.* 2014. **159**(6): p. 1277-1289.
188. Chiu, L., et al., *Protective microbiota: from localized to long-reaching co-immunity.* 2017. **8**: p. 1678.
189. Bohm, M.K., et al., *Pelvic inflammatory disease among privately insured women, United States, 2001-2005.* 2010. **37**(3): p. 131-136.
190. Ness, R.B., et al., *Hormonal and barrier contraception and risk of upper genital tract disease in the PID Evaluation and Clinical Health (PEACH) study.* 2001. **185**(1): p. 121-127.
191. Simms, I., et al., *Risk factors associated with pelvic inflammatory disease.* 2006. **82**(6): p. 452-457.
192. Scholes, D., et al., *Vaginal douching as a risk factor for acute pelvic inflammatory disease.* 1993. **81**(4): p. 601-606.
193. Ness, R.B., et al., *Design of the PID evaluation and clinical health (PEACH) study.* 1998. **19**(5): p. 499-514.
194. Sanders, S.A. and J.M.J.J. Reinisch, *Would you say you had sex if...?* 1999. **281**(3): p. 275-277.
195. Spencer, L., A. Faulkner, and J. Keegan, *Talking about sex: Asking the public about sexual behaviour and attitudes*. 1988: Social and Community Planning Research London.
196. Goyal, M.K., et al., *Development of a sexual health screening tool for adolescent emergency department patients.* 2016. **23**(7): p. 809-815.
197. Wayal, S., et al., *The acceptability and feasibility of implementing a Bio-Behavioral enhanced surveillance tool for sexually transmitted infections in England: mixed-methods study.* 2018. **4**(2): p. e52.
198. Ivaz, S., et al., *Lessons learned from recruiting young female students to a randomised controlled trial of chlamydia screening.* Family Practice, 2006. **23**(2): p. 188-191.
199. Wiesenfeld, H.C., et al., *Subclinical Pelvic Inflammatory Disease and Infertility.* Obstetrics & Gynecology, 2012. **120**(1).
200. Health, A.G.D.o., *Fourth National Sexually Transmissible Infections Strategy 2018-2022*. 2018: Canberra, ACT.
201. Coleman, A., et al., *Young Australians experiences of sexual healthcare provision.* Australian Journal for General Practitioners, 2019. **48**: p. 411-414.
202. Reekie, J., et al., *Risk of pelvic inflammatory disease in relation to chlamydia and gonorrhea testing, repeat testing, and positivity: a population-based cohort study.* 2018. **66**(3): p. 437-443.
203. Prism, G., *Graphpad Software, LA Jolla, California, USA*. 2019.
204. Mann, H.B. and D.R.J.T.a.o.m.s. Whitney, *On a test of whether one of two random variables is stochastically larger than the other.* 1947: p. 50-60.
205. Sweet, R.L.J.C.i.d.r., *Pelvic inflammatory disease: current concepts of diagnosis and management.* 2012. **14**(2): p. 194-203.
206. Soper, D.E.J.O. and Gynecology, *Pelvic inflammatory disease.* 2010. **116**(2): p. 419-428.
207. Sweet, R.L.J.I.d.i.o. and gynecology, *Treatment of acute pelvic inflammatory disease.* 2011. **2011**.
208. Birgisson, N.E., et al., *Positive testing for Neisseria gonorrhoeae and Chlamydia trachomatis and the risk of pelvic inflammatory disease in IUD users.* 2015. **24**(5): p. 354-359.

209. Eastman, A.J., et al., *Impact of the levonorgestrel-releasing intrauterine system on the progression of chlamydia trachomatis infection to pelvic inflammatory disease in a baboon model.* 2018. **217**(4): p. 656-666.
210. Farley, T.M., et al., *Intrauterine devices and pelvic inflammatory disease: an international perspective.* 1992. **339**(8796): p. 785-788.
211. Washington, A.E., et al., *Assessing Risk for Pelvic Inflammatory Disease and Its Sequelae.* JAMA, 1991. **266**(18): p. 2581-2586.
212. Ault, K.A. and S.J.P.m. Faro, *Pelvic inflammatory disease: current diagnostic criteria and treatment guidelines.* 1993. **93**(2): p. 85-91.
213. Harriott, M.M. and M.C. Noverr, *Importance of Candida-bacterial polymicrobial biofilms in disease.* Trends Microbiol, 2011. **19**(11): p. 557-63.
214. Zarrinpar, A., et al., *Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism.* 2018. **9**(1): p. 2872.
215. Den Heijer, C.D., et al., *Chlamydia trachomatis and the Risk of Pelvic Inflammatory Disease, Ectopic Pregnancy, and Female Infertility: A Retrospective Cohort Study Among Primary Care Patients.* 2019. **69**(9): p. 1517-1525.
216. Edwards, V.L., et al., *The Cervicovaginal Microbiota-Host Interaction Modulates Chlamydia trachomatis Infection.* 2019. **10**(4): p. e01548-19.
217. McLaws, M.L., et al., *Sexual behaviour in aids-related research: Reliability and validity of recall and diary measures.* 1990. **27**(2): p. 265-281.
218. Oakeshott, P., et al., *Randomised controlled trial of screening for Chlamydia trachomatis to prevent pelvic inflammatory disease: the POPI (prevention of pelvic infection) trial.* 2010. **340**.
219. Bachmann, L.H., et al., *Patterns ofChlamydia trachomatisTesting and Follow-Up at a University Hospital Medical Center.* 1999. **26**(9): p. 496-499.
220. Geisler, W.M., et al., *The natural history of untreated Chlamydia trachomatis infection in the interval between screening and returning for treatment.* 2008. **35**(2): p. 119-123.
221. Haggerty, C.L., et al., *Risk of sequelae after Chlamydia trachomatis genital infection in women.* 2010. **201**(Supplement_2): p. S134-S155.
222. Wiesenfeld, H.C., et al., *Subclinical pelvic inflammatory disease and infertility.* 2012. **120**(1): p. 37-43.
223. Simms, I. and J.J.S.t.i. Stephenson, *Pelvic inflammatory disease epidemiology: what do we know and what do we need to know?* 2000. **76**(2): p. 80-87.
224. Goller, J.L., et al., *Rates of pelvic inflammatory disease and ectopic pregnancy in Australia, 2009–2014: ecological analysis of hospital data.* 2018. **94**(7): p. 534-541.
225. Curry, A., T. Williams, and M.L. Penny, *Pelvic Inflammatory Disease: Diagnosis, Management, and Prevention.* Am Fam Physician, 2019. **100**(6): p. 357-364.
226. Jennings, L.K. and D.M. Krywko, *Pelvic Inflammatory Disease (PID).* 2019.
227. Ness, R.B., et al., *Bacterial vaginosis and risk of pelvic inflammatory disease.* 2004. **104**(4): p. 761-769.
228. Taylor, B.D., et al., *Microbial correlates of delayed care for pelvic inflammatory disease.* 2011. **38**(5): p. 434.
229. Doxanakis, A., et al., *Missing pelvic inflammatory disease? Substantial differences in the rate at which doctors diagnose PID.* 2008. **84**(7): p. 518-523.
230. French, C.E., et al., *Estimation of the rate of pelvic inflammatory disease diagnoses: trends in England, 2000–2008.* 2011. **38**(3): p. 158-162.
231. Wiesenfeld, H.C., et al., *Comparison of Acute and Subclinical Pelvic Inflammatory Disease.* 2005. **32**(7): p. 400-405.

232. Wiesenfeld, H.C., et al., *Subclinical Pelvic Inflammatory Disease and Infertility.* 2012. **120**(1): p. 37-43.
233. Cui, L., et al., *Chronic pelvic inflammation diminished ovarian reserve as indicated by serum anti Mülerrian hormone.* 2016. **11**(6): p. e0156130.
234. Yosef, A., et al., *Multifactorial contributors to the severity of chronic pelvic pain in women.* 2016. **215**(6): p. 760. e1-760. e14.
235. Foddy, W. and W.H. Foddy, *Constructing questions for interviews and questionnaires: Theory and practice in social research*. 1994: Cambridge university press.
236. Sackett, D.L., *Bias in analytic research.* J Chronic Dis, 1979. **32**(1-2): p. 51-63.
237. Choi, B.C.K. and A.W.P. Pak, *A catalog of biases in questionnaires.* Preventing chronic disease, 2005. **2**(1): p. A13-A13.
238. Wee, B.A., et al., *A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women.* 2018. **58**(3): p. 341-348.
239. Zheng, X., et al., *Discovery of Blood Transcriptional Endotypes in Women with Pelvic Inflammatory Disease.* 2018. **200**(8): p. 2941-2956.
240. Weinstein, L., J.H.J.T.Y.J.o.B. Howard, and Medicine, *The incidence of the Döderlein vaginal bacillus during the post-climacterium.* 1937. **10**(2): p. 185.
241. Weinstein, L.J.T.Y.J.o.B. and Medicine, *The bacterial flora of the human vagina.* 1938. **10**(3): p. 247.
242. Holmes, K.K., et al., *Nonspecific vaginosis.* Scand J Infect Dis Suppl, 1981. **26**: p. 110-4.
243. Spiegel, C.A., et al., *Gardnerella vaginalis and anaerobic bacteria in the etiology of bacterial (nonspecific) vaginosis.* Scand J Infect Dis Suppl, 1983. **40**: p. 41-6.
244. Bradley, W.G., I. Karlsson, and C.J.B.m.j. Rassol, *Metronidazole neuropathy.* 1977. **2**(6087): p. 610.
245. Redondo-Lopez, V., R.L. Cook, and J.D.J.R.o.i.d. Sobel, *Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora.* 1990. **12**(5): p. 856-872.
246. Nugent, R.P., M.A. Krohn, and S.L.J.J.o.c.m. Hillier, *Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation.* 1991. **29**(2): p. 297-301.
247. Eschenbach, D.A., et al., *Prevalence of hydrogen peroxide-producing Lactobacillus species in normal women and women with bacterial vaginosis.* J Clin Microbiol, 1989. **27**(2): p. 251-6.
248. Nagy, E., M. Petterson, and P.A.J.A. MÅRdh, *Antibiosis between bacteria isolated from the vagina of women with and without signs of bacterial vaginosis.* 1991. **99**(7-12): p. 739-744.
249. Johnson, J., et al., *Taxonomy of the Lactobacillus acidophilus group.* 1980. **30**(1): p. 53-68.
250. Collins, M.D., et al., *Deoxyribonucleic acid homology studies of Lactobacillus casei, Lactobacillus paracasei sp. nov., subsp. paracasei and subsp. tolerans, and Lactobacillus rhamnosus sp. nov., comb. nov.* 1989. **39**(2): p. 105-108.
251. Antonio, M.A., S.E. Hawes, and S.L.J.J.o.I.D. Hillier, *The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species.* 1999. **180**(6): p. 1950-1956.
252. Burton, J.P. and G.J.T.J.o.i.d. Reid, *Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques.* 2002. **186**(12): p. 1770-1780.

253. Brotman, R.M., et al., *Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection.* 2010. **202**(12): p. 1907-1915.
254. Wiesenfeld, H.C., et al., *Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection.* Clin Infect Dis, 2003. **36**(5): p. 663-8.
255. Champer, M., et al., *The role of the vaginal microbiome in gynaecological cancer.* 2018. **125**(3): p. 309-315.
256. Gao, W., et al., *Comparison of the vaginal microbiota diversity of women with and without human papillomavirus infection: a cross-sectional study.* 2013. **13**(1): p. 271.
257. Cone, R.A.J.T.J.o.i.d., *Vaginal microbiota and sexually transmitted infections that may influence transmission of cell-associated HIV.* 2014. **210**(suppl_3): p. S616-S621.
258. Balkus, J.E., et al., *Detection of hydrogen peroxide-producing Lactobacillus species in the vagina: a comparison of culture and quantitative PCR among HIV-1 seropositive women.* 2012. **12**(1): p. 188.
259. Liu, Y., et al., *Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method.* 2019. **112**(4): p. 707-717. e1.
260. Hillier, S.L., et al., *Role of bacterial vaginosis–associated microorganisms in endometritis.* 1996. **175**(2): p. 435-441.
261. Korn, A.P., et al., *Plasma cell endometritis in women with symptomatic bacterial vaginosis.* 1995. **85**(3): p. 387-390.
262. Peipert, J.F., et al., *Bacterial vaginosis as a risk factor for upper genital tract infection.* 1997. **177**(5): p. 1184-1187.
263. Yudin, M.H., et al., *Vaginal polymorphonuclear leukocytes and bacterial vaginosis as markers for histologic endometritis among women without symptoms of pelvic inflammatory disease.* 2003. **188**(2): p. 318-323.
264. Ness, R.B., et al., *A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease.* Am J Epidemiol, 2005. **162**(6): p. 585-90.
265. Simms, I., et al., *Associations between Mycoplasma genitalium, Chlamydia trachomatis and pelvic inflammatory disease.* 2003. **56**(8): p. 616-618.
266. Abele-Horn, M., et al., *Association of Ureaplasma urealyticum biovars with clinical outcome for neonates, obstetric patients, and gynecological patients with pelvic inflammatory disease.* 1997. **35**(5): p. 1199-1202.
267. Salter, S.J., et al., *Reagent and laboratory contamination can critically impact sequence-based microbiome analyses.* 2014. **12**(1): p. 87.
268. Gohl, D.M., et al., *Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies.* 2016. **34**(9): p. 942-949.
269. Nakazato, T., T. Ohta, and H.J.P.O. Bono, *Experimental design-based functional mining and characterization of high-throughput sequencing data in the sequence read archive.* 2013. **8**(10).
270. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nature Biotechnology, 2019. **37**(8): p. 852-857.
271. Bokulich, N.A., et al., *Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing.* Nature Methods, 2013. **10**(1): p. 57-59.
272. Rognes, T., et al., *VSEARCH: a versatile open source tool for metagenomics.* 2016. **4**: p. e2584.
273. Price, M.N., P.S. Dehal, and A.P.J.P.o. Arkin, *FastTree 2–approximately maximum-likelihood trees for large alignments.* 2010. **5**(3): p. e9490.

274. Fettweis, J.M., et al., *Species-level classification of the vaginal microbiome.* 2012. **13**(S8): p. S17.
275. McMurdie, P.J. and S.J.P.o. Holmes, *phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.* 2013. **8**(4): p. e61217.
276. Team, R.C.J.w.R.-p.c.o., *R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http s.* 2017.
277. Gu, Z., R. Eils, and M.J.B. Schlesner, *Complex heatmaps reveal patterns and correlations in multidimensional genomic data.* 2016. **32**(18): p. 2847-2849.
278. Kusters, J., et al., *A multiplex real-time PCR assay for routine diagnosis of bacterial vaginosis.* 2015. **34**(9): p. 1779-1785.
279. Ling, Z., et al., *Diversity of cervicovaginal microbiota associated with female lower genital tract infections.* 2011. **61**(3): p. 704-714.
280. Jensen, J.S., et al., *Use of TaqMan 5' nuclease real-time PCR for quantitative detection of Mycoplasma genitalium DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic.* 2004. **42**(2): p. 683-692.
281. Eischeid, A.C.J.B.r.n., *SYTO dyes and EvaGreen outperform SYBR Green in real-time PCR.* 2011. **4**(1): p. 263.
282. Apprill, A., et al., *Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton.* Aquatic Microbial Ecology, 2015. **75**(2): p. 129-137.
283. Totten, P.J.I.J.o.S. and Aids, *The identification of novel organisms associated with idiopathic STD syndromes.* 2001. **12**.
284. Møller, B. and E.J.S.T.D. Freundt, *Monkey animal model for study of mycoplasmal infections of the urogenital tract.* 1983. **10**(4 Suppl): p. 359-362.
285. Taylor-Robinson, D. and Y.L.J.I.j.o.e.p. Boustouller, *Damage to oviduct organ cultures by Gardnerella vaginalis.* 2011. **92**(4): p. 260-265.
286. Geißdörfer, W., et al., *Tuboovarian abscess caused by Atopobium vaginae following transvaginal oocyte recovery.* 2003. **41**(6): p. 2788-2790.
287. Yamagishi, Y., et al., *A case of uterine endometritis caused by Atopobium vaginae.* 2011. **17**(1): p. 119-121.
288. Hsu, G.-j., et al., *Chest wall abscess due to Prevotella bivia.* 2009. **10**(3): p. 233.
289. Sweet, R.L.J.C.I.D., *Role of bacterial vaginosis in pelvic inflammatory disease.* 1995. **20**(Supplement_2): p. S271-S275.
290. Wong, W.-W., et al., *Molecular epidemiology of gonorrhea identified clonal clusters with distinct susceptibilities associated with specific high-risk groups.* 2008.
291. Graspeuntner, S., et al., *Selection of validated hypervariable regions is crucial in 16S-based microbiota studies of the female genital tract.* 2018. **8**(1): p. 9678.
292. Jain, M., et al., *The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community.* 2016. **17**(1): p. 239.
293. Gloor, G.B., et al., *Microbiome Datasets Are Compositional: And This Is Not Optional.* 2017. **8**(2224).
294. Virtanen, S., et al., *Comparative analysis of vaginal microbiota sampling using 16S rRNA gene analysis.* 2017. **12**(7).
295. Kyrgiou, M., A. Mitra, and A.-B.J.T.R. Moscicki, *Does the vaginal microbiota play a role in the development of cervical cancer?* 2017. **179**: p. 168-182.
296. Meade, J.C. and D.C. Cornelius, *Sexually transmitted infections in the tropics*, in *Current Topics in Tropical Medicine*. 2012, IntechOpen.
297. Poulsen, M., R. Johnston, and J.J.A.g.s. Forrest, *Is Sydney a divided city ethnically?* 2004. **42**(3): p. 356-377.

298. Wira, C.R. and J.V. Fahey, *The innate immune system: gatekeeper to the female reproductive tract.* Immunology, 2004. **111**(1): p. 13-15.
299. Mestecky, J., Z. Moldoveanu, and M.W.J.A.j.o.r.i. Russell, *Immunologic uniqueness of the genital tract: challenge for vaccine development.* 2005. **53**(5): p. 208-214.
300. Ganz, T., *Defensins and host defense.* Science, 1999. **286**(5439): p. 420-1.
301. Brotman, R.M., et al., *Microbiome, sex hormones, and immune responses in the reproductive tract: challenges for vaccine development against sexually transmitted infections.* 2014. **32**(14): p. 1543-1552.
302. Pudney, J., A.J. Quayle, and D.J. Anderson, *Immunological Microenvironments in the Human Vagina and Cervix: Mediators of Cellular Immunity Are Concentrated in the Cervical Transformation Zone1.* Biology of Reproduction, 2005. **73**(6): p. 1253-1263.
303. Nguyen, P.V., et al., *Innate and adaptive immune responses in male and female reproductive tracts in homeostasis and following HIV infection.* 2014. **11**(5): p. 410-427.
304. Chang, J.J., et al., *Higher expression of several interferon-stimulated genes in HIV-1-infected females after adjusting for the level of viral replication.* 2013. **208**(5): p. 830-838.
305. Trifonova, R.T., J. Lieberman, and D.J.A.j.o.r.i. van Baarle, *Distribution of immune cells in the human cervix and implications for HIV transmission.* 2014. **71**(3): p. 252-264.
306. Pudney, J., A.J. Quayle, and D.J.J.B.o.r. Anderson, *Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone.* 2005. **73**(6): p. 1253-1263.
307. Wira, C.R., et al., *Innate and adaptive immunity in female genital tract: cellular responses and interactions.* 2005. **206**(1): p. 306-335.
308. Givan, A.L., et al., *Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina.* 1997. **38**(5): p. 350-359.
309. Anne Croy, B., et al., *Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones.* 2006. **214**(1): p. 161-185.
310. Robertson, S.J.J.o.A.S., *Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs.* 2007. **85**(suppl_13): p. E36-E44.
311. Geijtenbeek, T.B., et al., *DC-SIGN, a dendritic cell–specific HIV-1-binding protein that enhances trans-infection of T cells.* 2000. **100**(5): p. 587-597.
312. Kutteh, W., et al., *Concept of mucosal immunology.* 1996: p. 28-51.
313. Robertson, S.A., et al., *Seminal fluid and the generation of regulatory T cells for embryo implantation.* 2013. **69**(4): p. 315-330.
314. Russell, M.W., J.J.M. Mestecky, and infection, *Humoral immune responses to microbial infections in the genital tract.* 2002. **4**(6): p. 667-677.
315. Brown, A.B., K.M.J.A.J.o.O. Crocker, and Gynecology, *Culdoscopy.* 1960. **80**(1): p. 25-29.
316. Tauber, W.F.J.A.J.o.O. and Gynecology, *Symptomatology and treatment of pelvic inflammatory disease: Halter: p. 505.* 1956. **72**(2): p. 462.
317. de ALVAREZ, R.R., D.C.J.O. FIGGE, and Gynecology, *Influence of antibiotics on pelvic inflammatory disease.* 1955. **5**(6): p. 765-772.
318. DECKER, A.J.J.o.t.A.M.A., *Culdoscopy: its diagnostic value in pelvic disease.* 1949. **140**(4): p. 378-385.
319. Ross, J.D.C., *What is endometritis and does it require treatment?* Sexually Transmitted Infections, 2004. **80**(4): p. 252.
320. Workowski, K.A. and G. Bolan, *Sexually transmitted diseases treatment guidelines, 2015.* MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports, 2015. **64**(3).

321. Østergaard, L., et al., *Home sampling versus conventional swab sampling for screening of Chlamydia trachomatis in women: a cluster-randomized 1-year follow-up study.* 2000. **31**(4): p. 951-957.
322. Stelzer, G., et al., *The GeneCards suite: from gene data mining to disease genome sequence analyses.* 2016. **54**(1): p. 1.30. 1-1.30. 33.
323. Schmittgen, T.D. and K.J.J.N.p. Livak, *Analyzing real-time PCR data by the comparative C T method.* 2008. **3**(6): p. 1101.
324. Sanz, J., et al., *Genetic and evolutionary determinants of human population variation in immune responses.* 2018. **53**: p. 28-35.
325. Pritchard, J.K., J.K. Pickrell, and G.J.C.b. Coop, *The genetics of human adaptation: hard sweeps, soft sweeps, and polygenic adaptation.* 2010. **20**(4): p. R208-R215.
326. Fidel Jr, P.L.J.A.j.o.r.i., *History and update on host defense against vaginal candidiasis.* 2007. **57**(1): p. 2-12.
327. Thellin, O., et al., *Lysozyme as a cotreatment during antibiotics use against vaginal infections: an in vitro study on Gardnerella vaginalis biofilm models.* 2016. **19**(2): p. 101-107.
328. Valore, E.V., et al., *Antimicrobial components of vaginal fluid.* 2002. **187**(3): p. 561-568.
329. Cole, A.M., *Innate Host Defense of Human Vaginal and CervicalMucosae*, in *Antimicrobial Peptides and Human Disease*. 2006, Springer. p. 199-230.
330. Shaw, J.L. and E.P.J.B.c. Diamandis, *A potential role for tissue kallikrein-related peptidases in human cervico-vaginal physiology.* 2008. **389**(6): p. 681-688.
331. Adnane, M., K.G. Meade, and C.J.V.r.c. O'Farrelly, *Cervico-vaginal mucus (CVM)–an accessible source of immunologically informative biomolecules.* 2018. **42**(4): p. 255-263.
332. Verstraelen, H., et al., *Gene polymorphisms of Toll-like and related recognition receptors in relation to the vaginal carriage of Gardnerella vaginalis and Atopobium vaginae.* 2009. **79**(2): p. 163-173.
333. Doerflinger, S.Y., A.L. Throop, and M.M. Herbst-Kralovetz, *Bacteria in the Vaginal Microbiome Alter the Innate Immune Response and Barrier Properties of the Human Vaginal Epithelia in a Species-Specific Manner.* The Journal of Infectious Diseases, 2014. **209**(12): p. 1989-1999.
334. Chang, C.-C., et al., *Significant elevation of plasma pentraxin 3 in patients with pelvic inflammatory disease.* 2011. **49**(10): p. 1655-1660.
335. Wang, P.-H., et al., *Significant elevation of plasma matrix metalloproteinase-9 level and its ratio to matrix metalloproteinase-2 in patients with pelvic inflammatory disease.* 2009. **92**(5): p. 1679-1684.
336. Nelson, A.R., et al., *Matrix metalloproteinases: biologic activity and clinical implications.* 2000. **18**(5): p. 1135-1135.
337. Mishra, J., et al., *Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery.* 2005. **365**(9466): p. 1231-1238.
338. Tsai, H.-T., et al., *Significant elevation and correlation of plasma neutrophil gelatinase associated lipocalin and its complex with matrix metalloproteinase-9 in patients with pelvic inflammatory disease.* 2011. **412**(13-14): p. 1252-1256.
339. Zheng, X., et al., *Gene expression signatures can aid diagnosis of sexually transmitted infection-induced endometritis in women.* 2018. **8**: p. 307.
340. Kulbe, H., et al., *Discovery and validation of novel biomarkers for detection of epithelial ovarian cancer.* 2019. **8**(7): p. 713.

341. Guo, R., et al., *New serum biomarkers for detection of esophageal carcinoma using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.* J Cancer Res Clin Oncol, 2011. **137**(3): p. 513-9.
342. Cheng, W., et al., *Caspase-1 Contributes to Chlamydia trachomatis-Induced Upper Urogenital Tract Inflammatory Pathologies without Affecting the Course of Infection.* 2008. **76**(2): p. 515-522.
343. Lu, H., C. Shen, and R.C.J.T.J.o.I. Brunham, *Chlamydia trachomatis infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18.* 2000. **165**(3): p. 1463-1469.
344. Ojcius, D.M., et al., *Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen Chlamydia psittaci.* 1998. **161**(8): p. 4220-4226.
345. Chen, Y., et al., *The role of monocytes and SLC11A1 polymorphisms in the pathogenesis of Chlamydia-induced reactive arthritis.* 2013. **42**(2): p. 146-149.
346. Meilang, Q., et al., *Polymorphisms in the SLC11A1 gene and tuberculosis risk: a meta-analysis update.* 2012. **16**(4): p. 437-446.
347. Kayukova, S., et al., *Changes in Microbiota and Development of Nonspecific Inflammation of Genitals in Female C57Bl/6 Mice after Aerosol Infection with Mycobacterium tuberculosis.* 2019. **167**(5): p. 645-649.
348. Abtin, A., et al., *Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking antimicrobial activities of RNase 5 and RNase 7.* 2009. **129**(9): p. 2193-2201.
349. Spear, G.T., et al., *Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ß and lactoferrin.* 2011. **6**(5).
350. Bedoya, V.I., et al., *Ribonucleases in HIV type 1 inhibition: effect of recombinant RNases on infection of primary T cells and immune activation-induced RNase gene and protein expression.* 2006. **22**(9): p. 897-907.
351. Hooper, L.V., et al., *Angiogenins: a new class of microbicidal proteins involved in innate immunity.* 2003. **4**(3): p. 269-273.
352. Alves, M.G.O., et al., *Comparison of RNA Extraction Methods for Molecular Analysis of Oral Cytology.* Acta stomatologica Croatica, 2016. **50**(2): p. 108-115.
353. Workowski, K.A. and S.M. Berman, *Sexually transmitted diseases treatment guidelines, 2006.* 2006.
354. Park, S.T., et al., *Clinical characteristics of genital chlamydia infection in pelvic inflammatory disease.* BMC Women's Health, 2017. **17**(1): p. 5.
355. Wang, S.-P. and J.T.J.A.j.o.o. Grayston, *Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test.* 1970. **70**(3): p. 367-374.
356. Stephens, R.S., et al., *Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis.* 1998. **282**(5389): p. 754-759.
357. O'Connell, C.M. and M.E. Ferone, *Chlamydia trachomatis Genital Infections.* Microbial cell (Graz, Austria), 2016. **3**(9): p. 390-403.
358. Bastidas, R.J. and R.H. Valdivia, *Emancipating Chlamydia: Advances in the Genetic Manipulation of a Recalcitrant Intracellular Pathogen.* 2016. **80**(2): p. 411-427.
359. O'Meara, C.P., et al., *Induction of partial immunity in both males and females is sufficient to protect females against sexual transmission of Chlamydia.* 2016. **9**(4): p. 1076-1088.

360. Darville, T., et al., *Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response.* 1997. **65**(8): p. 3065-3073.
361. de la Maza, L.M., G. Zhong, and R.C. Brunham, *Update on Chlamydia trachomatis Vaccinology.* Clin Vaccine Immunol, 2017. **24**(4).
362. Sturdevant, G.L., et al., *Infectivity of urogenital Chlamydia trachomatis plasmid-deficient, CT135-null, and double-deficient strains in female mice.* Pathog Dis, 2014. **71**(1): p. 90-2.
363. Olsen, A.W., P. Andersen, and F. Follmann, *Characterization of protective immune responses promoted by human antigen targets in a urogenital Chlamydia trachomatis mouse model.* Vaccine, 2014. **32**(6): p. 685-92.
364. Ramsey, K.H., et al., *Disease Outcome Subsequent to Primary and Secondary Urogenital Infection with Murine or Human Biovars ofChlamydia trachomatis.* 2000. **68**(12): p. 7186-7189.
365. O'Meara, C.P., *The development of an effective vaccine against Chlamydia: utilisation of a non-toxic mucosal adjuvant to generate a protective mucosal response*. 2012, Queensland University of Technology.
366. Tuffrey, M., et al., *Salpingitis in mice induced by human strains of Chlamydia trachomatis.* 1986. **67**(4): p. 605.
367. Carmichael, J.R., et al., *Differences in infectivity and induction of infertility: a comparative study of Chlamydia trachomatis strains in the murine model.* 2013. **15**(3): p. 219-229.
368. Ito, J., et al., *Variation in virulence among oculogenital serovars of Chlamydia trachomatis in experimental genital tract infection.* 1990. **58**(6): p. 2021-2023.
369. Sturdevant, G.L., et al., *Frameshift mutations in a single novel virulence factor alter the in vivo pathogenicity of Chlamydia trachomatis for the female murine genital tract.* 2010. **78**(9): p. 3660-3668.
370. Nelson, D.E., et al., *Chlamydial IFN-γ immune evasion is linked to host infection tropism.* 2005. **102**(30): p. 10658-10663.
371. Byrne, G.I.J.T.J.o.i.d., *Chlamydia trachomatis strains and virulence: rethinking links to infection prevalence and disease severity.* 2010. **201**(Supplement_2): p. S126-S133.
372. Chen, C., et al., *In vitro passage selects for Chlamydia muridarum with enhanced infectivity in cultured cells but attenuated pathogenicity in mouse upper genital tract.* Infection and immunity, 2015. **83**(5): p. 1881-1892.
373. Liu, Y., et al., *Plasmid-encoded Pgp3 is a major virulence factor for Chlamydia muridarum to induce hydrosalpinx in mice.* 2014. **82**(12): p. 5327-5335.
374. Zhang, T., et al., *The plasmid-encoded pGP3 promotes Chlamydia evasion of acidic barriers in both stomach and vagina.* 2019. **87**(5).
375. Zhong, G.J.T.i.m., *Chlamydial plasmid-dependent pathogenicity.* 2017. **25**(2): p. 141-152.
376. Gloeckl, S., et al., *The active site residue V266 of chlamydial HtrA is critical for substrate binding during both in vitro and in vivo conditions.* 2012. **22**(1): p. 10-16.
377. Gloeckl, S., et al., *Identification of a serine protease inhibitor which causes inclusion vacuole reduction and is lethal to C hlamydia trachomatis.* 2013. **89**(4): p. 676-689.
378. Ong, V.A., *Determination of the effect of JO146, a CtHtrA inhibitor, on the chlamydial developmental cycle and persistence*. 2015, Queensland University of Technology.
379. Lawrence, A., et al., *Chlamydia serine protease inhibitor, targeting HtrA, as a new treatment for koala Chlamydia infection.* 2016. **6**(1): p. 1-12.

380. Kari, L., et al., *Generation of targeted Chlamydia trachomatis null mutants.* 2011. **108**(17): p. 7189-7193.
381. Yao, J., et al., *Chlamydia trachomatis relies on autonomous phospholipid synthesis for membrane biogenesis.* 2015. **290**(31): p. 18874-18888.
382. Yao, J., et al., *Chlamydia trachomatis scavenges host fatty acids for phospholipid synthesis via an acyl-acyl carrier protein synthetase.* 2015. **290**(36): p. 22163-22173.
383. Yao, J., et al., *Type II fatty acid synthesis is essential for the replication of Chlamydia trachomatis.* J Biol Chem, 2014. **289**(32): p. 22365-76.
384. Recuero-Checa, M.A., et al., *Chlamydia trachomatis growth and development requires the activity of host Long-chain Acyl-CoA Synthetases (ACSLs).* Scientific Reports, 2016. **6**(1): p. 23148.
385. Ibarguren, M., D.J. López, and P.V.J.B.e.B.A.-B. Escribá, *The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health.* 2014. **1838**(6): p. 1518-1528.
386. Consortium, T.U., *UniProt: a worldwide hub of protein knowledge.* Nucleic Acids Research, 2018. **47**(D1): p. D506-D515.
387. Clark, K., et al., *GenBank.* Nucleic acids research, 2016. **44**(D1): p. D67-D72.
388. Sasser, M., *Identification of bacteria by gas chromatography of cellular fatty acids*. 1990, MIDI technical note 101. Newark, DE: MIDI inc.
389. Kiernan, J.A.J.S., *Histological and histochemical methods: theory and practice.* 1999. **12**(6): p. 479.
390. Sanadgol, N., et al., *Effect of palmitic acid and linoleic acid on expression of ICAM-1 and VCAM-1 in human bone marrow endothelial cells (HBMECs).* Archives of medical science : AMS, 2012. **8**(2): p. 192-198.
391. Abdelsamed, H., J. Peters, and G.I. Byrne, *Genetic variation in Chlamydia trachomatis and their hosts: impact on disease severity and tissue tropism.* 2013. **8**(9): p. 1129-1146.
392. Iwata, N.G., et al., *Trans fatty acids induce vascular inflammation and reduce vascular nitric oxide production in endothelial cells.* 2011. **6**(12): p. e29600.
393. Rao, Y. and B. Lokesh, *Modulatory effects of α-linolenic acid on generation of reactive oxygen species in elaidic acid enriched peritoneal macrophages in rats.* 2014.
394. Caligiani, A. and V. Lolli, *Cyclic Fatty Acids in Food: An Under-Investigated Class of Fatty Acids*, in *Biochemistry and Health Benefits of Fatty Acids*. 2018, IntechOpen.
395. Sandoz, K.M. and D.D.J.F.m. Rockey, *Antibiotic resistance in Chlamydiae.* 2010. **5**(9): p. 1427-1442.
396. Suchland, R.J., et al., *Horizontal Transfer of Tetracycline Resistance among Chlamydia spp. In Vitro.* 2009. **53**(11): p. 4604-4611.
397. Brothwell, J.A., et al., *Interrogating Genes That Mediate Chlamydia trachomatis Survival in Cell Culture Using Conditional Mutants and Recombination.* 2016. **198**(15): p. 2131-2139.
398. Lyons, J.M., J.I. Ito, Jr., and S.A. Morré, *The influence of vaginally applied imiquimod on the course of Chlamydia trachomatis serovar D infection in a murine model.* Infect Dis Obstet Gynecol, 2005. **13**(1): p. 1-3.
399. Darville, T., et al., *Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response.* Infect Immun, 1997. **65**(8): p. 3065-73.
400. Lyons, J.M., et al., *Acquired homotypic and heterotypic immunity against oculogenital Chlamydia trachomatis serovars following female genital tract infection in mice.* BMC Infect Dis, 2005. **5**: p. 105.

401. Ramsey, K.H., et al., *Prior genital tract infection with a murine or human biovar of Chlamydia trachomatis protects mice against heterotypic challenge infection.* Infect Immun, 1999. **67**(6): p. 3019-25.
402. Rajeeve, K. and R. Sivadasan, *Transcervical Mouse Infections with Chlamydia trachomatis and Determination of Bacterial Burden.*
403. Coers, J., et al., *Compensatory T cell responses in IRG-deficient mice prevent sustained Chlamydia trachomatis infections.* 2011. **7**(6): p. e1001346.
404. Ramsey, K.H., et al., *Chlamydia trachomatis persistence in the female mouse genital tract: inducible nitric oxide synthase and infection outcome.* 2001. **69**(8): p. 5131-5137.
405. Miyairi, I., K.H. Ramsey, and D.L. Patton, *Duration of Untreated Chlamydial Genital Infection and Factors Associated with Clearance: Review of Animal Studies.* The Journal of Infectious Diseases, 2010. **201**(Supplement_2): p. S96-S103.
406. Wali, S., et al., *Guinea pig genital tract lipidome reveals in vivo and in vitro regulation of phosphatidylcholine 16:0/18:1 and contribution to Chlamydia trachomatis serovar D infectivity.* Metabolomics, 2016. **12**(4).
407. Tyssen, D., et al., *Anti-HIV-1 Activity of Lactic Acid in Human Cervicovaginal Fluid.* 2018. **3**(4): p. e00055-18.
408. Jewkes, R., et al., *Gender inequitable masculinity and sexual entitlement in rape perpetration South Africa: findings of a cross-sectional study.* 2011. **6**(12): p. e29590.
409. Shen, R., H.E. Richter, and P.D.J.A.j.o.r.i. Smith, *Early HIV-1 target cells in human vaginal and ectocervical mucosa.* 2011. **65**(3): p. 261-267.
410. Yarbrough, V.L., S. Winkle, and M.M. Herbst-Kralovetz, *Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications.* Human Reproduction Update, 2014. **21**(3): p. 353-377.
411. Wira, C.R., et al., *Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections.* 2011. **65**(3): p. 196-211.
412. Hearps, A.C., et al., *Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition.* Mucosal Immunology, 2017. **10**(6): p. 1480-1490.
413. Borgdorff, H., et al., *Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier.* 2016. **9**(3): p. 621-633.
414. Arnold, K.B., et al., *Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells.* Mucosal Immunology, 2016. **9**(1): p. 194-205.
415. Macklaim, J.M., et al., *Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis.* Microbiome, 2013. **1**(1): p. 12.
416. Borgdorff, H., et al., *Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier.* Mucosal Immunology, 2016. **9**(3): p. 621-633.
417. Brooks, J.P., et al., *Changes in vaginal community state types reflect major shifts in the microbiome.* Microbial ecology in health and disease, 2017. **28**(1): p. 1303265-1303265.

418. Smith, S.B. and J.J.T.J.o.p. Ravel, *The vaginal microbiota, host defence and reproductive physiology.* 2017. **595**(2): p. 451-463.
419. Tamarelle, J., et al., *Vaginal microbiota composition and association with prevalent Chlamydia trachomatis infection: a cross-sectional study of young women attending a STI clinic in France.* 2018. **94**(8): p. 616-618.
420. van Houdt, R., et al., *Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to Chlamydia trachomatis infection in Dutch women: a case–control study.* 2018. **94**(2): p. 117-123.
421. Van Der Veer, C., et al., *The cervicovaginal microbiota in women notified for chlamydia trachomatis infection: a case-control study at the sexually transmitted infection outpatient clinic in Amsterdam, The Netherlands.* 2017. **64**(1): p. 24-31.
422. Hagan, T., et al., *Antibiotics-driven gut microbiome perturbation alters immunity to vaccines in humans.* 2019. **178**(6): p. 1313-1328. e13.
423. van de Wijgert, J.H.H.M., et al., *Intermittent Lactobacilli-containing Vaginal Probiotic or Metronidazole Use to Prevent Bacterial Vaginosis Recurrence: A Pilot Study Incorporating Microscopy and Sequencing.* Scientific Reports, 2020. **10**(1): p. 3884.
424. Hay, P.J.C.o.i.i.d., *Recurrent bacterial vaginosis.* 2009. **22**(1): p. 82-86.
425. Taha, T.E., et al., *Intermittent intravaginal antibiotic treatment of bacterial vaginosis in HIV-uninfected and-infected women: a randomized clinical trial.* 2007. **2**(2): p. e10.
426. McClelland, R.S., et al., *Improvement of vaginal health for Kenyan women at risk for acquisition of human immunodeficiency virus type 1: results of a randomized trial.* 2008. **197**(10): p. 1361-1368.
427. van de Wijgert, J.H., et al., *Intermittent Lactobacilli-containing vaginal probiotic or metronidazole use to prevent bacterial vaginosis recurrence: a pilot study incorporating microscopy and sequencing.* 2020. **10**(1): p. 1-15.
428. Borges, S., et al., *The role of lactobacilli and probiotics in maintaining vaginal health.* 2014. **289**(3): p. 479-489.
429. Mastromarino, P., et al., *Effectiveness of Lactobacillus-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis.* 2009. **15**(1): p. 67-74.
430. Larsson, P.-G., et al., *Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study.* 2008. **8**(1): p. 3.
431. Petricevic, L., A.J.B.A.I.J.o.O. Witt, and Gynaecology, *The role of Lactobacillus casei rhamnosus Lcr35 in restoring the normal vaginal flora after antibiotic treatment of bacterial vaginosis.* 2008. **115**(11): p. 1369-1374.
432. Bradshaw, C.S., et al., *Efficacy of Oral Metronidazole with Vaginal Clindamycin or Vaginal Probiotic for Bacterial Vaginosis: Randomised Placebo-Controlled Double-Blind Trial.* PLOS ONE, 2012. **7**(4): p. e34540.
433. Eriksson, K., et al., *A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules.* 2005. **85**(1): p. 42-46.
434. Anukam, K., et al., *Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial.* 2006. **8**(6): p. 1450-1454.
435. Hemalatha, R., et al., *Effectiveness of vaginal tablets containing lactobacilli versus pH tablets on vaginal health and inflammatory cytokines: a randomized, double-blind study.* 2012. **31**(11): p. 3097-3105.
436. Ling, Z., et al., *The restoration of the vaginal microbiota after treatment for bacterial vaginosis with metronidazole or probiotics.* 2013. **65**(3): p. 773-780.

437. Bisanz, J.E., et al., *A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double blind, placebo-controlled clinical trial of post-menopausal women.* 2014. **9**(8): p. e104511.
438. Verdenelli, M.C., et al., *Impact of Probiotic SYNBIO® Administered by Vaginal Suppositories in Promoting Vaginal Health of Apparently Healthy Women.* Current Microbiology, 2016. **73**(4): p. 483-490.
439. Bohbot, J., et al., *Efficacy and safety of vaginally administered lyophilized Lactobacillus crispatus IP 174178 in the prevention of bacterial vaginosis recurrence.* 2018. **47**(2): p. 81-86.
440. Sanders, M.E., et al., *Probiotics and prebiotics in intestinal health and disease: from biology to the clinic.* Nature Reviews Gastroenterology & Hepatology, 2019. **16**(10): p. 605-616.
441. Poeker, S.A., et al., *Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS).* Scientific Reports, 2018. **8**(1): p. 4318.
442. Holscher, H.D.J.G.m., *Dietary fiber and prebiotics and the gastrointestinal microbiota.* 2017. **8**(2): p. 172-184.
443. Moro, T., H. Rasmussen, and B.J.F.i.n. Hamaker, *Potential of prebiotic butyrogenic fibers in Parkinson's disease.* 2019. **10**: p. 663.
444. Henning, S.M., et al., *Health benefit of vegetable/fruit juice-based diet: Role of microbiome.* 2017. **7**(1): p. 1-9.
445. Gurry, T., et al., *Predictability and persistence of prebiotic dietary supplementation in a healthy human cohort.* 2018. **8**(1): p. 1-13.
446. Shivakoti, R., et al., *Dietary macronutrient intake and molecular-bacterial vaginosis: Role of fiber.* 2020.
447. Al-Ghazzewi, F. and R.J.J.o.a.m. Tester, *Biotherapeutic agents and vaginal health.* 2016. **121**(1): p. 18-27.
448. Rousseau, V., et al., *Prebiotic effects of oligosaccharides on selected vaginal lactobacilli and pathogenic microorganisms.* 2005. **11**(3): p. 145-153.
449. Sutherland, A., et al., *Glucomannan hydrolysate (GMH) inhibition of Candida albicans growth in the presence of Lactobacillus and Lactococcus species.* 2008. **20**(3): p. 127-134.
450. Tester, R. and F.H.J.I.j.o.b.m. Al-Ghazzewi, *Intrinsic and extrinsic carbohydrates in the vagina: a short review on vaginal glycogen.* 2018. **112**: p. 203-206.
451. Caddy, S., et al., *Best practices to minimize risk of infection with intrauterine device insertion.* 2014. **36**(3): p. 266-274.
452. Whitaker, A.K., et al., *Effect of a brief educational intervention on the attitudes of young women toward the intrauterine device.* 2010. **23**(2): p. 116-120.