HPV infection, cervical carcinogenesis and the reproductive impact of local treatment for cervical preinvasive and early invasive disease

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A thesis submitted for the Degree of Doctor of Philosophy

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Declaration of Originality

This thesis presents original work of the titled author. That which is not is referenced accordingly.

London,
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Dr Anita Mitra

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Mitra A, MacIntyre DA, Mahajan V, Lee YS, Smith A, Marchesi JR, Lyons D, Bennett PR, Kyrgiou M. Comparison of vaginal microbiota sampling techniques: cytobrush versus swab *Scientific Reports 2017 Aug 29;7(1):9802.*

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Kyrgiou M, **Mitra A**, Arbyn M, Stasinou SM, Martin-Hirsch P, Bennett P, Paraskevaidis E. Fertility and early pregnancy outcomes after treatment for cervical intra-epithelial neoplasia: a systematic review and meta-analysis. *British Medical Journal, 2014;349:g6192*

Manuscripts in preparation

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Other publications arising from work related to this thesis

Halliwell DE, Morais CLM, Lima KMG, Trevisan J, Siggel-King MRF, Craig T, Ingham J, Martin DS, Heys K, Kyrgiou M, **Mitra A**, Paraskevaidis E, Theophilou G, Martin-Hirsch PL, Cricenti A, Luce M, Weightman P, Martin FL. An imaging dataset of cervical cells using scanning near-field optical microscopy coupled to an infrared free electron laser. *Scientific Data 2017 Jul 11;4:170084.*

Halliwell DE, Kyrgiou M, **Mitra A,** Kalliala I, Paraskevaidis E, Theophilou G, Martin-Hirsch PL, Martin FL. Tracking the Impact of Excisional Cervical Treatment on the Cervix using Biospectroscopy. *Scientific Reports 2016 Dec 15;6:38921*.

Halliwell DE, Morais CL, Lima KM, Trevisan J, Siggel-King MR, Craig T, Ingham J, Martin DS, Heys KA, Kyrgiou M, **Mitra A,** Paraskevaidis E, Theophilou G, Martin-Hirsch PL, Cricenti A, Luce M, Weightman P, Martin FL. Imagine cervical cytology with scanning near-field optical microscopy (SNOM) coupled with an IR-FEL. *Scientific Reports 2016 Jul 12;6:29494.*

Oral Plenary Lectures

The Vaginal Microbiome, Metabonome and Biomarkers

- EUROGIN 2017 International Multidisciplinary Congress, Amsterdam, October 2017

Mechanisms of Reproductive Morbidity after Treatment for Cervical Intraepithelial Neoplasia

- EUROGIN 2017 International Multidisciplinary Congress, Amsterdam, October 2017

Antenatal Management of Women after Treatment for Cervical Intraepithelial Neoplasia

- EUROGIN 2017 International Multidisciplinary Congress, Amsterdam, October 2017

Does the Vaginal Microbiome Play a Role in HPV-related Cervical Pathology?

- Scottish HPV Investigators Network Spring Meeting, Edinburgh, June 2017.
- Hellenic Society of Cervical Pathology and Colposcopy, Annual Meeting, Ioannina, Greece, November 2016.
- British Society of Cervical Pathology and Colposcopy Annual Scientific Meeting, Bradford, April 2016
- Infectious Diseases Symposium, Akershus Universetetssykehus, Oslo, Norway, January 2016.

Oral Presentations

The Vaginal Microbiome After Treatment for Cervical Intraepithelial Neoplasia

- International Federation of Cervical Pathology and Colposcopy, Orlando, USA, April 2017
    - ***British Society of Colposcopy & Cervical Pathology Travel Bursary***
- 64th Annual Scientific Meeting of Society for Reproductive Investigation, Orlando, USA, March 2017

Genome-wide association study (GWAS) of Association Between Cervical Intraepithelial Neoplasia and Preterm Birth

- International Federation of Cervical Pathology and Colposcopy, Orlando, USA, April 2017
- International Meeting of HPV Diseases and Pregnancy, Rome, Italy, March 2016
    - ***Best Oral Presentation - 2nd Prize***

Cervical Antimicrobial Peptides Are Decreased Following Excisional Treatment for Cervical Intraepithelial Neoplasia

- EUROGIN 2016 International Mulitidisciplinary Congress, Salzburg, June 2016
- 63rd Annual Scientific Meeting of Society for Reproductive Investigation, Montreal, Canada, March 2016
- International Meeting of HPV Diseases and Pregnancy, Rome, Italy, March 2016
    - ***Best Oral Presentation - 1st Prize***

The Vaginal Microbiome of Women with Cervical Intraepithelial Neoplasia

- 63rd Annual Scientific Meeting of Society for Reproductive Investigation, Montreal, Canada, March 2016
- 30th International Papillomavirus Conference, Lisbon, Portugal, September 2015
- Imperial College London Academic Trainees Meeting May 2015
    - ***Best oral presentation - 2nd Prize***
- British Society of Cervical Pathology and Colposcopy Annual Scientific Meeting, Nottingham, April 2015
- HPV 2015 – XII International Workshop of Lower Genital Tract Pathology 2015 Rome March 2015
    - ***Best oral presentation - 2nd Prize***

Fertility and Early Pregnancy Outcomes after Treatment for Cervical Intraepithelial Neoplasia: A Systematic Review and Meta-analysis

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The Vaginal Microbiome After Treatment for Cervical Intraepithelial Neoplasia

- ESGO 2017, European Society of Gynaecological Oncology International Meeting, Vienna, Austria, November 2017
- British Gynaecological Cancer Society, Annual Scientific Meeting, Glasgow, June 2017

Cervical Antimicrobial Peptides Are Decreased Following Excisional Treatment for Cervical Intraepithelial Neoplasia

- British Society of Cervical Pathology and Colposcopy Annual Scientific Meeting, Bradford, April 2016

- Blair Bell Research Society Annual Academic Meeting, Royal College of Obstetricians & Gynaecologists, London, March 2016

The Vaginal Microbiome of Women with Cervical Intraepithelial Neoplasia

- Blair Bell Research Society Annual Academic Meeting, Royal College of Obstetricians & Gynaecologists, London, March 2016
- Society of Academic Medicine, Royal College of Physicians, London, February 2016
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- British Gynaecological Cancer Society Annual Scientific Meeting, Newcastle, July 2015
    - ***Best poster presentation - 3rd prize***
- International Federation of Cervical Pathology and Colposcopy, London, April 2014

The risk of preterm birth after treatment for cervical pre-invasive and early invasive disease increases with increasing cone length: a systematic review and meta-analysis

- British Society of Cervical Pathology and Colposcopy Annual Scientific Meeting, Bradford, April 2016
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- Blair Bell Research Society Annual Academic Meeting, Royal College of Obstetricians & Gynaecologists, London, March 2016

Fertility and Early Pregnancy Outcomes after Treatment for Cervical Intraepithelial Neoplasia: A Systematic Review and Meta-analysis

- 30th International Papillomavirus Conference, Lisbon, Portugal, September 2015
- British Gynaecological Cancer Society, Annual Scientific Meeting, Newcastle, July 2015
- British Society of Cervical Pathology and Colposcopy Annual Scientific Meeting, Nottingham, April 2015

Abstract

Background

The human papillomavirus (HPV) infects the majority of sexually-active women, yet it is unclear how this highly-prevalent virus causes cervical intraepithelial neoplasia (CIN) and cervical cancer in only a minority of women. Cervical screening programmes exist to detect and treat high-grade CIN to the reduce risk of invasive cancer development, although may be associated with adverse reproductive and obstetric outcomes.

Aims

Firstly, this thesis aimed to test the hypothesis that the vaginal microbiome and innate immune factors (antimicrobial peptides and cytokines) are altered in CIN compared to healthy controls, and that the microbiome composition plays a role in disease outcomes. Secondly, it aimed to summarise, critically appraise and meta-analyse the current literature on adverse reproductive outcomes after treatment for CIN, and to test the hypothesis that treatment impacts on microbiome structure and innate mucosal immunity.

Results

16s rRNA sequencing was used to characterise the vaginal microbiome of women with CIN and revealed increasing diversity with decreasing *Lactobacillus* spp. levels with increased disease severity compared to healthy controls, which in a longitudinal study of women with untreated CIN2 was associated with slower regression and greater rates of persistence. Levels of proinflammatory cytokines and antimicrobial peptides quantified using enzyme-linked immunosorbent assay were higher in high-grade CIN compared to controls. Meta-analysis demonstrated that treatment did not affect fertility, but was associated with higher rates of second-trimester miscarriage. There was heterogeneity in the literature regarding the link between treatment and preterm birth which was shown to be partly explained by choice of control group. There was a positive correlation between preterm birth risk and amount of tissue excised. The altered vaginal microbiome and proinflammatory environment persisted after successful excision, and levels of antimicrobial peptides were lower than in untreated controls.

Discussion

These results suggest the vaginal microbiome and innate immune factors are altered in CIN and may play a role in disease outcomes. Whilst the degree of cervical structural deficit impacts on risk of adverse reproductive outcomes an altered microbiome structure and innate mucosal immune factors likely also play a role.

CHAPTER 1: Introduction

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1 Introduction

1.1 Cervical intraepithelial neoplasia and cervical cancer

Cervical cancer, the commonest infection-associated neoplasm, and its premalignant precursor cervical intraepithelial neoplasia (CIN), are caused by strains of the Human Papillomavirus (HPV). The vast majority of cases of invasive cancer are preventable through cervical screening and treatment of precancerous CIN lesions. Abnormal cervical screening results occur frequently, with one in twenty women who attended the NHS cervical screening programme in 2016-17 having an abnormal result. Most were classified as borderline or low-grade, but 19.49% of abnormalities in 2016-17 were classified as high-grade or suspicious of invasive disease, and therefore likely to require treatment [1]. Globally, cervical cancer remains the fourth most common female malignancy, with over 528,000 new cases and 266,000 deaths in 2012; 84% of these occurred in low-resource settings [2] (Figure 1.1). In 2014, 3224 women were diagnosed with invasive cervical cancer in the UK, and 890 died as a result. It is the 14th most common cancer in the UK, where the lifetime risk is one in 135 and rates are currently increasing [3]. Around two-thirds of cases of invasive cervical cancer are of squamous cell carcinoma (SCC) type [4], with around 15% being adenocarcinoma or adenosquamous carcinomas [5], and the remainder represented by more unusual histological types such as clear cell, small cell neuroendocrine tumours, sarcomas and lymphomas.

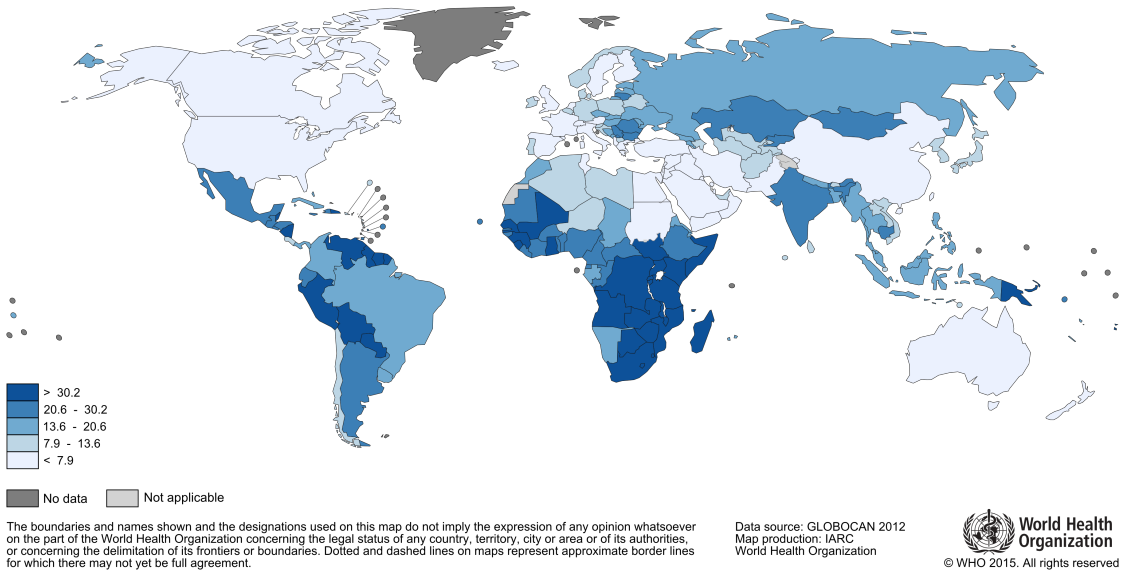


Figure 1.1. Estimated age-standardised rates of cervical cancer per 100,000 women

Area of highest disease prevalence are shown in darkest blue, and lowest prevalence is indicated with lightest blue. Taken from http://globocan.iarc.fr/old/FactSheets/cancers/cervix-new.asp http://globocan.iarc.fr/old/FactSheets/cancers/cervix-new.asp (Key, bottom left).

1.1.1 Human Papillomavirus (HPV)

The human papillomaviruses are a diverse group of over 200 circular double-stranded DNA viruses [6, 7] that are highly prevalent in the human population. Ancient Greek and Roman literature documents the manifestation of sexually-transmitted warts that can likely be attributed to HPV infection [8]. However it was not until the 1970's that a link between these viruses and human cancers was established [9]. In 2008, Harald zur Hausen was awarded the Nobel Prize in Physiology or Medicine for identifying HPV as the causative agent of CIN and cervical cancer. The subtypes causing cervical disease belongs to the alpha-papillomavirus genus of mucosal type and can be further classified as low-risk or high-risk according to their carcinogenic potential. The low-risk subtypes, including HPV-6 and -11 can cause cutaneous and mucosal warts, including in the genital area [10]. The high-risk HPV (hrHPV) subtypes are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 and are causal of cervical cancer in almost 100% of cases [11]. HPV-16 and -18 are the most oncogenic and responsible for around 70% of cases [12].

HPV prevalence peaks around the time of sexual debut [13]. Data from the ARTISTIC trial in the UK revealed prevalence of hrHPV infection prior to the start of the vaccination programme in the UK ranged from 40% at 20-24 years of age, and steadily declined to 6% at 55-64 years [14]. At peak hrHPV prevalence, 12% had HPV-16 and 3% HPV-18 infection [14]. It is estimated that 80% of sexually active women will have been infected at some point by 50 years of age [15]. With more sensitive testing available, studies show that HPV infection is more commonly the rule, not the exception, although the majority of HPV infections do not cause cancer in the hosts that they infect. Since the lifetime risk of developing invasive cervical cancer in the UK is much lower at 0.6% [16], cervical cancer should be regarded as a rare complication of a very common infection by the human papillomavirus. Over 90% of HPV infections are transient, being cleared by an incompletely understood immune response within 6-18 months [17], although reinfection with the same or different HPV subtypes can occur [18]. Persistence is essential for development of high-grade CIN and cervical cancer, and factors that correlate with higher persistence rates include age, immunodeficiency, smoking, oral contraceptives and concomitant bacterial sexually transmitted infections such as *Chlamydia trachomatis*, are discussed later.

1.1.1.1 HPV lifecycle

Transmission of ano-genital HPV infections occur through skin-to-skin contact, and the virus gains access to the basal epithelium through micro-tears arising as a result of sexual contact [19]. The virus preferentially infects the mitotically-active cells of the cervical transformation zone; the area of columnar to squamous metaplasia, where it enters the nucleus of the metaplastic cells, existing in episomal form, and later in the disease process may integrate into the host DNA [20]. HPV is known to cause cancers in other tissues including the vagina and vulva, and outside of the female reproductive tract in the anus, penis and tonsils, the latter three of which also contain a transformation zone. The high-risk alpha HPV genomes contain eight genes, six early and two late, which relates to the timing of their expression as well as their location within the genome [21] (Figure 1.2).

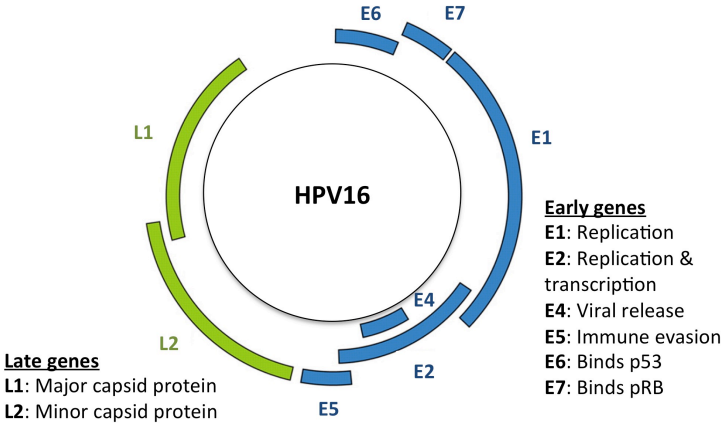


Figure 1.2. Structure of the HPV genome

High risk alpha HPV genomes contain eight genes; six early genes encode proteins involved in viral replication and pathogenesis and two late genes encode structural capsid proteins.

The viral lifecycle lasts between two to three weeks; the same amount of time required for cervical basal cell maturation, surface migration, maturation and apoptosis [22]. During this time, the virus must maintain host cell proliferation and circumvent host cell apoptosis for its own survival, which is the role of the E6 and E7 oncoproteins [23] (Figure 1.2). At the initial phase of infection, viral gene expression is tightly regulated and levels of the oncoproteins E6 and E7 are very low at around 50-100 copies per cell [24]. As the host cell ceases replication and begins to mature and migrate to the tissue surface, there is a massive upregulation of viral replication and control over E6 and E7 expression is lost. E6 targets the tumour-suppressor gene protein p53 for degradation via the ubiquitin pathway, preventing apoptosis and ensuring ongoing cervical cell replication [25]. E7 similarly acts via ubiquitin-mediated degradation of retinoblastoma protein (pRB), another tumour suppressor gene-encoded

protein, resulting in activation of transcription factors and overriding the G1/S checkpoint of the cell cycle, pushing the cells into cell cycle and drive DNA replication [26, 27]. As well as interacting with numerous other crucial cell-signalling pathways [27, 28], E6 and E7 also induce chromosomal instability through interfering with chromosome duplication and segregation during mitosis, which facilitates viral integration into the host chromosomal DNA, found in CIN and the majority of invasive cancer tissues, but not all, suggesting it is not a mandatory step in malignant transformation [20]. As the virus reaches the top layer of epithelium, L1 and L2 gene expression drives capsid protein synthesis, and encaspidated viral particles are released as the keratinocytes undergo programmed cell death resulting in a productive infection.

1.1.1.2 Mechanisms of immune evasion and persistence

The way in which viral replication synergises with keratinocyte replication and maturation is pivotal to HPVs ability to evade the host immune system. The virus does not induce cell death or cytolysis, and the infection is entirely intraepithelial with an absence of viraemia, which would otherwise result in immune activation and subsequent clearance. Cervical keratinocytes express toll-like receptors (TLRs), which are pathogen recognition receptors that recognise pathogen-associated molecular patterns present in various pathogens [29, 30]. Activation of TLRs results in stimulation of a Th1 cytotoxic response, principally through production of antiviral Type 1 interferons. Type 1 interferons are anti-proliferative, anti-angiogenic and have a crucial role in activation of the innate immune system and subsequent the adaptive immune system [24]. High-risk HPV down-regulates interferon-mediated response via the E6 and E7 oncoproteins [31, 32]. Langerhans cells (LCs) are a key antigen-presenting cell (APC) responsible for antigen capture and presentation, resulting in a T-cell-mediated immune response. E6 and E7 interfere with E-cadherin-mediated LC migration and adhesion [33]. A decrease in LCs has been observed in CIN, which correlates with lesion severity [34]. Additionally, decreased expression of LC co-stimulatory molecules and downstream effectors such as TNF-α have been observed in CIN [35].

1.1.1.3 Mechanisms of immune clearance

The mechanisms of clearance of cervical HPV infection are poorly understood although it has been long suggested that a CD4+ cell-mediated Th1 response is responsible [24]. In a small immunohistochemical study of cervical biopsies from 42 women with either regressing CIN1, progressing CIN1, CIN3 or invasive cancer and healthy controls, CD4+ cells predominated in

the stroma and epithelium of women with regressing CIN1. Moreover, this study showed that the CD4+/CD8+ ratio was positively correlated with progressive CIN1, CIN3 and invasive carcinoma [36]. It has also been reported that immunocompromised patients show a higher prevalence of HPV infection with a longer time to clearance and a greater burden of HPV-associated disease [37].

Evidence also suggests that HPV may have a latent phase which can last many years if basal stem cells are infected [38]. Animal models have been used to demonstrate a lack of apoptosis when viral clearance is achieved [39]. Infected cells are replaced by 'apparently normal' cells, which may contain viral genomes as demonstrated in respiratory tissues [40]. The viral life cycle could therefore still become reactivated at times of immune suppression that occur naturally with age [41], which may explain the observed second peak of disease (Figure 1.3).

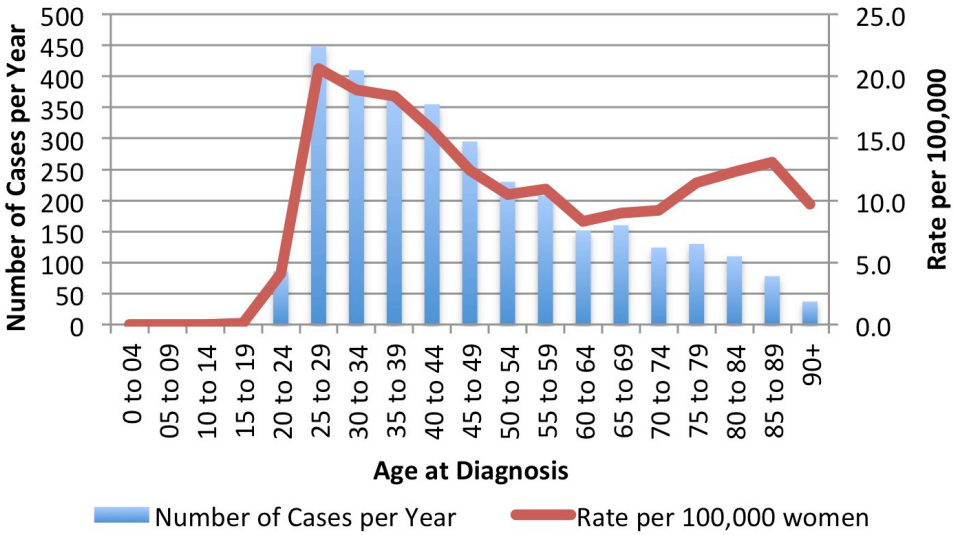
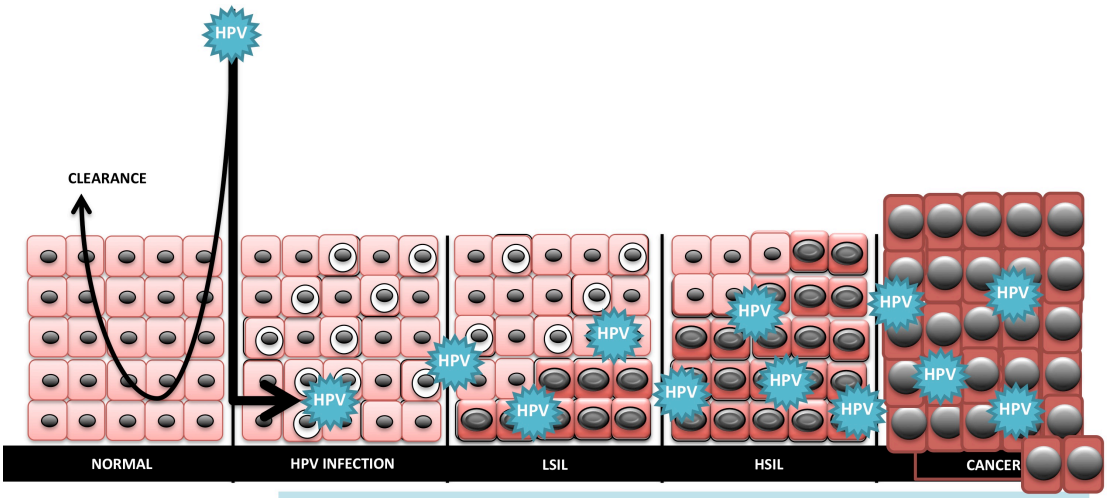


Figure 1.3. Average Number of New Cases Per Year and Age-Specific Incidence Rates, UK, 2012-2014
(Source: cruk.org/cancerstats)

1.1.2 Natural history of CIN and cervical cancer

HPV infection is the first step in development of CIN and cervical cancer. HPV delays nuclear condensation to facilitate viral replication, giving rise to the appearance of koilocytes (Figure 1.4), which are the first histological sign of an HPV infection. CIN is diagnosed histologically on the basis of the thickness of affected epithelium; up to one-third in CIN1, one- to two-thirds in CIN2, and two-thirds to full thickness involvement in CIN, crucially without breach of the basement membrane, which signifies invasive cervical cancer (Figure 1.4). CIN1 is largely appreciated to be a clinically insignificant histological marker of HPV infection, caused by low-

and high-risk HPV subtypes. CIN2 and 3 are regarded as high-grade lesions; CIN2 being a more heterogeneous condition that may also be caused by both low- and high-risk HPV types, whilst CIN3 is considered by some to be true precancer, although regression is still possible [42]. Research into the natural history of CIN has been controversial, largely due to a highly-cited study by McIndoe *et al.* published in 1984, often referred to as 'The Unfortunate Experiment' [43]. The authors observed 948 women with CIN3 for between five and 28 years, without any ethical approval or patient consent, resulting in 43 women (4.3%) being diagnosed with invasive cancer, however the proportion of patients progressing is in fact higher when taking into consideration eight further cancer cases that developed within the first year of follow-up who were subsequently excluded from analysis. Furthermore in a subgroup of 131 with persistent abnormal cytology, almost 40% developed invasive disease within 20 years [44]. These figures should have been no surprise to the authors given their opening sentence; *"It is now generally accepted that carcinoma in situ (CIS) has a significant invasive potential"* [44]. Further observational studies have been conducted with greater ethical consideration, and Östör subsequently published a systematic review of 71 such studies, to gives an indication of the rates of regression, persistence and progression of the different grades of disease severity, displayed in Figure 1.4.



	Borderline	Mild	Moderate	Severe	Dyskaryosis
Regression	57%	43%	32%		
Persistence	32%	35%	56%		
Progression to CIN3	11%	22%	-		
Progression to ICC	1%	1.5%	12%		

Figure 1.4. Natural history of CIN and cervical cancer.

Rates of regression, persistence and regression of CIN are taken from Östör 1993.

Several challenges exist when estimating fate of disease. Firstly, progression or regression may take years and intervention for high-grade prevents determination of disease fate. Loss to follow-up can be a confounding factor. Secondly, a colposcopically-directed biopsy for histological examination is the gold-standard for diagnosing cervical abnormalities, yet the act of taking a biopsy may cause a degree of inflammation, which has been suggested to increase the chance of clearance [45], although is debated by some [46]. Thirdly, diagnosis is dependent on the subjective nature of colposcopic, cytological and histological assessment. Discovery of objective biomarkers could be used to stratify likelihood of disease progression but are also important for the personalised management of women with abnormalities at screening. Overexpression of viral E6/E7 oncogene mRNA and p16-Ki67 are emerging as potential tools for triaging low-grade disease but also for primary screening [47-50]. Currently we are unable to adequately determine which patients will develop invasive disease, therefore UK guidelines recommend treatment of all high-grade disease.

1.1.3 Risk factors for CIN and cervical cancer

Epidemiological studies have shown that several risk factors have been correlated to heightened risk of cervical cancer. The most consistent factors are tobacco smoking, oral contraceptive use, parity and *Chlamydia trachomatis* infection.

1.1.3.1 Smoking

Smoking is an independent risk factor for both CIN and cervical cancer. Epidemiological analysis of 13,541 women with cervical disease and 23,017 controls included in 23 case-control and longitudinal studies, showed that current cigarette smokers have a significantly increased risk of carcinoma *in situ* and cervical cancer (RR = 1.60 (95% CI 1.48-1.73). Furthermore, this risk was proportional to the number of cigarettes smoked daily and was positively correlated to starting smoking at an early age, although duration of smoking was not. A past history of smoking was also associated with an increased risk of disease, but to a lesser extent (RR = 1.12 95% CI 1.01-1.25) [51]. Current smoking has also been associated with a significantly increased risk of squamous cell carcinoma (RR = 1.50, 95% CI: 1.35–1.66) but not adenocarcinoma (RR = 0.86 (0.70–1.05)).

It has been suggested that smoking increases the risk of CIN through increasing the risk of HPV acquisition although there is discrepancy regarding whether it prolongs the duration of HPV infection as this was observed in some cohorts [52, 53], but not in others [54]. The association may also be confounded by associated sexual behaviours of smokers which is also

controvertial, and some studies show a lack of effect of smoking after controlling for sexual behaviour [54] whilst others demonstrate the increased risk of HPV in smokers remains [55].

Whilst there are discrepancies in the epidemiological data, *in vitro* and *in vivo* data support a mechanistic link between smoking and HPV-related cervical disease. Nicotine and its carcinogenic metabolites can be detected in cervical mucous [56] and smoking has been associated with a dampening of local immune markers [57], as well as impaired antibody response to HPV16 in young women under 30 years of age [58]. Smoking has also been correlated with epigenetic changes that could predispose to carcinogenesis [59]. These data point to smoking being a greater risk factor to younger women, which is an important factor to consider when providing health promotion advice in the colposcopy and gynaecology clinics.

1.1.3.2 Hormonal contraception

Use of the combined oral contraceptive pill (COCP) has long been associated with a higher risk of CIN and cervical cancer. A multi-centric case-control study of 3769 women showed an association with duration of use, with an odds ratio of 2.82 (95% CI 1.46-5.42) for five to nine years use, increasing to 4.03 (2.09-8.02) with use of 10 years or longer [60]. More recently, the Royal College of General Practitioners' Oral Contraception study, which followed 46,022 women in the UK over a 44-year period, showed invasive cervical cancer risk was increased in ever-users of the COCP, compared to never-users (Incidence rate ratio (IRR) = 1.31, 99% CI 0.84–2.04). Using these incidence rates the researchers calculated 25.2% of invasive cervical disease may be attributed to COCP use, however it must be noted that this calculation does not take into account any other potentially confounding characteristics such as smoking or sexual behaviour. Their results did however show the risk decreased to the same level as never users by five years after cessation [61]. This connection may be explained by behavioural differences, because women using contraception are more likely to have regular sexual intercourse compared to non-users, resulting in a higher chance of acquiring an HPV infection. Oestrogen and progesterone both increase cell proliferation and hence vulnerability to DNA damage [62, 63], and are also able to increase expression of HPV16 E6 and E7 oncogenes, which enhance HPV-mediated cellular transformation and carcinogenesis [64, 65], which provide biologically plausible explanations for an association between COCP use and HPV-mediated disease. Interestingly the hormone-free copper-containing intrauterine device (IUD) has been inversely associated with cervical cancer risk (OR = 0.41, 95% CI 0.18-0.93) [66],

which supports this theory of a hormone-mediated association rather than confounders associated with sexual behaviour of women using long-term contraception.

1.1.3.3 Parity

Parity is consistently associated with risk of high-grade CIN and invasive cervical cancer. A Danish study, which followed a cohort of 1353 women over a 13-year period showed parous women had a significantly higher risk of CIN3+ compared to nulliparous women (Hazard ratio (HR) = 1.39, 95% CI 1.02–1.91), and was even higher in women with two or more deliveries (HR = 1.90, 95% CI 1.11–3.28). After modelling to adjust for smoking status, length of schooling, and HPV16 positivity, the risk was no longer significant but the trend remained (HR=1.58, 95% CI 0.91–2.74). This observation suggests there may be some behavioural components to the association. Higher parity may however be associated with high levels of hormone exposure and repeated trauma, which could make the cervix more susceptible to viral entry [67]. As with other risk factors, parity also appears to be most strongly associated with squamous cell cancers when compared to adenocarcinoma [68]. It is possible that cervical adenocarcinomas may in fact be more similar histologically to endometrial cancers, which are inversely related to high parity [69]. Obesity is one of the major factors for endometrial cancer, and high body mass index (BMI) has been shown to associated with a higher risk of cervical adenocarcinoma (OR = 2.1, 95% CI 1.1-3.8), but not SCC, in a US case-control study of 124 cases of adenocarcinoma, 139 cases of SCC and 307 matched controls [70], which adds strength to this hypothesis.

1.1.3.4 Chlamydia trachomatis infection

Chlamydia trachomatis is an obligate intracellular, gram-negative, bacterium that can cause infection of the genital region through sexual contact. It is the most common sexually transmitted infection (STI) representing 49% of newly diagnosed STI's in the UK in 2016, and was most common in 15-24 year olds [71]. There is a discrepancy regarding whether *C. trachomatis* is associated with HPV and cervical disease, with some studies reporting a positive association [72], which others failed to replicate [73]. A meta-analysis of 22 studies including 4291 women with cervical cancer and 7628 controls showed that *C. trachomatis* infection is an independent risk factor for cervical cancer (OR = 1.76, 95% CI 1.03–3.01) and that HPV and *C. trachomatis* co-infection is an even greater risk for invasive cancer (OR = 4.03, 95% CI: 3.15–5.16). This was shown to be the case for both SCC (OR = 2.21, 95% CI 2.00–2.45) and adenocarcinoma (OR = 1.61, 95% CI 1.21–2.15). *C. trachomatis* infection has been

demonstrated to promote a pro-inflammatory environment in the cervix and vagina [74]. Not only could this disrupt the immune response to HPV, resulting in a persistent infection leading to future invasive disease, this pro-inflammatory environment could activate a cascade of events resulting in reactive oxygen species and free radical generation and subsequent DNA damage and misrepair. The infection may also facilitate viral entry through epithelial barrier disruption.

1.2 HPV vaccination: primary prevention of cervical cancer

The host response to natural HPV infection is immunoglobulin G (IgG) dominant against L1 only, although is very weak and highly variable within the population [75], and there is little evidence to suggest these natural antibodies provide any long-standing immunity [76].

Prophylactic vaccines have been developed to target the commonest high- and low-risk HPV genotypes, containing papillomavirus virus-like particle (VLP), which stimulates production of antibodies against the viral structural protein L1 [77] (Figure 1.5). Cervarix is a bivalent vaccine against HPV16 and 18 first licensed in the UK in 2008 for girls ages 12-13 years of age. In 2012 this was switched to Gardasil, a quadrivalent preparation with protection against two further HPV types; HPV6 and 11, both low-risk types that cause genital warts. Clinical trials have demonstrated safety of both vaccines and efficacy against the intended HPV types as well as modest cross-protection against some non-vaccine but phylogenetically-related types, most notably HPV31 and 33 for HPV16-related types and HPV45 for 18-related types [78, 79]. Herd immunity has also been demonstrated [78]. Vaccination rates in the UK are currently 84%, which is high compared to other countries including the USA where coverage is currently 40% for females and 22% for males [80]. It has been estimated that the bivalent and quadrivalent vaccines could reduce high-grade CIN by 62.1% and 58.6%, respectively, and prevent 70.5% and 64.8% of cases of squamous cell carcinoma [81]. The bivalent vaccine appears to confer slightly higher cross-protection, hence higher rates of disease prevention.

A nonavalent vaccine, Gardasil 9, had now become available that confers protection against HPV31, 33, 45, 52 and 58 in addition to the 4 types in the original quadrivalent Gardasil. It has been calculated that high coverage with Gardasil 9 could prevent up to 93% of cervical cancer cases worldwide [81]. Whilst various epidemiological studies have shown adenocarcinomas are less frequently predicted by presence of known risk factors of squamous cell carcinoma,

HPV remains to be the main risk factor for development of adenocarcinoma [66], therefore vaccination should still have an impact on its incidence.

In summary, HPV vaccination is an exciting prospect for the future of cervical cancer, however it will be many years before the impact on its incidence, morbidity and mortality are seen and a worldwide increase in vaccine coverage is required for maximum effect.

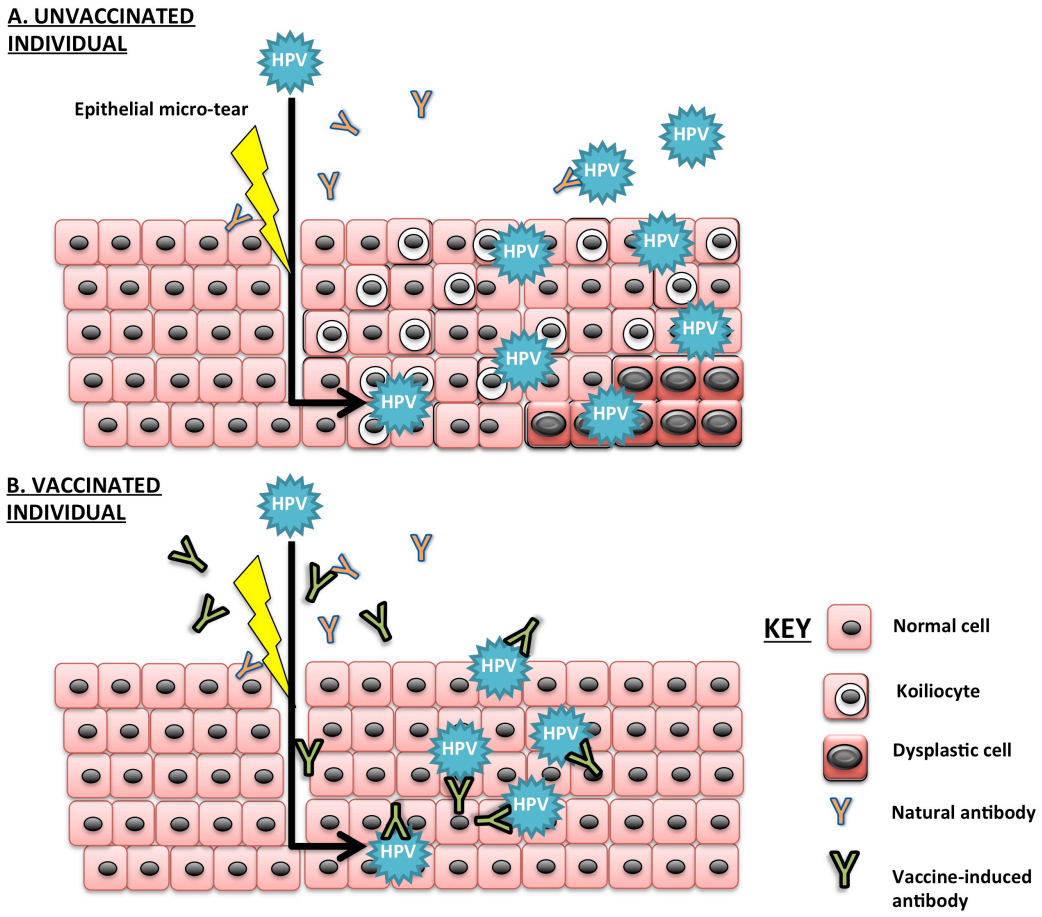


Figure 1.5. HPV vaccination protects against HPV infection

HPV enters the cervical epithelium via microtears and is able to access the basal epithelium where it begins its lifecycle, replicating and producing new virions to maintain an active infection. If the infected individual has previously been infected there may be a small number of natural antibodies which are minimally effective in preventing infection. In vaccinated individuals the presence of vaccine-induced antibodies provides strong protection by binding the HPV virus and preventing infection.

1.3 Cervical screening: secondary prevention of cervical cancer

There are no clinical features of CIN, and it is largely detected through the NHS cervical screening programme introduced in the UK in 1988 for women aged 20-65 years, which is based on cervical cytology. Organised cervical screening programmes have been shown to significantly reduce both incidence and mortality rates of cervical cancer through detection and treatment of the pre-malignant lesions of the uterine cervix; (CIN) [82]. Attendance at screening is in fact one of the most protective factors against development of cervical cancer in the unvaccinated population. A robust epidemiological study of 8,097 women with squamous cell carcinoma and 1,374 women with adenocarcinoma revealed that a history of screening (defined as at least one attendance for cervical screening), was associated with a reduced risk of developing any histological type of cervical cancer, however the risk of squamous cell carcinoma (RR 0.46, 95% CI 0.42-0.50) was significantly less than adenocarcinoma in these screened women (RR 0.68, 95% CI 0.56-0.82) [68].

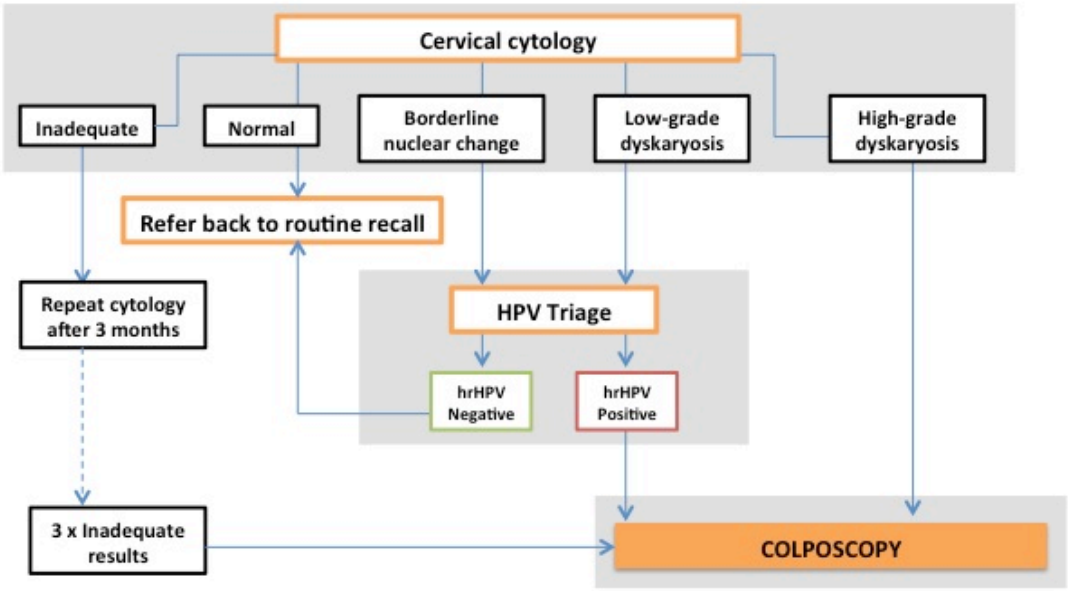


Figure 1.6. Current UK cervical screening algorithm.

Figure adapted from NHS cervical screening - NHS Cancer Screening Programmes (http://www.cancerscreening.nhs.uk/cervical/hpv-triage-test-flowchart-201407.pdf)

Screening begins at 25 years of age in the UK and women are thereafter invited every three years until the age of 50 when the screening interval increases to every five years, with cessation of screening at 64 years. In 2005, the minimum age for screening in England was increased to 25 as screening of 20-24 year olds was found to not reduce rates of invasive cancer [83] and may result in young women undergoing unnecessary treatment that can have

an impact on future obstetric outcomes [84-86]. Cervical screening in the UK is cytology-based, using hrHPV DNA co-testing for triage using the algorithm in Figure 1.6.

A cervical smear, using a liquid-based cytology system has a positive predictive value of 76.7-92.3% for detecting CIN2+ [1], and is classified according to the NHSCSP 2013 system in the UK and the Bethesda system in many other countries (Table 1.1). There is now strong evidence from randomized controlled trials and meta-analysis that screening based on the HPV DNA test reduces the cervical cancer risk in comparison to cytology-based screening. This has been assessed in a few sentinel sites across the UK [87] and will replace the current routine screening within the next few years.

Table 1.1. Classification of cervical cytology and histology

Cytology		**Histology**
BAC/NHSCSP 2013	**Bethesda 2001**	
Squamous epithelium		
Borderline changes in squamous/endocervical cells	ASCUS ASC-H	HPV
Low-grade dyskaryosis	LSIL	CIN1
High-grade dyskaryosis (moderate)	HSIL	CIN2
High-grade dyskaryosis (severe)	HSIL	CIN3
High-grade dyskaryosis/?invasive SCC	HSIL SCC	CIN3 - SCC
Glandular epithelium		
Borderline changes in endocervical cells ? Glandular neoplasia Endocervical Non-cervical	AGC NOS Endocervical Endometrial Glandular AGC favour neoplastic Endocervical Glandular Endocervical AIS Adenocarcinoma Endocervical Endometrial Extrauterine NOS	cGIN/ Adenocarcinoma

AGC: atypical glandular cells; AIS: Adenocarcinoma in situ; ASC-H: Atypical squamous cells, cannot exclude HSIL; ASCUS: Atypical squamous cell of uncertain significance; BAC/NHSCSP: British Association for Cytopathology/NHS Cervical Screening Programme; CIN: Cervical Intraepithelial Neoplasia; cGIN: Cervical Glandular Intraepithelial Neoplasia; HPV: Human Papilloma virus; HSIL: High-grade squamous intraepithelial lesion; LSIL: Low-grade squamous intraepithelial lesion; NOS: Not otherwise specified; SCC: Squamous cell carcinoma.

Coverage of the screening programme in England in 2016-17 was 72.0% amongst all women, and has been dropping slowly over a number of years, and even more concerning is that the attendance rate was lowest in 25-29 year olds at only 62.1%. Of the 3.18 million eligible women who attended screening in this period 184,638 were referred for colposcopy, predominantly due to a borderline or low-grade cytology result. During the same period almost 29,000 excisional treatments and around 990 more ablative procedures for CIN were carried out [1]. Most women who are diagnosed with cervical cancer in the UK will have an incomplete screening history [88]. Cancers may arise in under 25's, however the incidence is incredibly low (Figure 1.3) and these cancers tend to be of an atypical histological subtype [89] and therefore may not be screen-detected even if screening was offered to women under 25.

1.4 Conservative treatment for CIN

Excisional and ablative treatment techniques can be employed for the conservative local treatment of CIN. Both are simple and quick to perform, mostly under local anaesthesia in the outpatient clinic. Neither method has been shown to be superior with regard to recurrent CIN or invasive cancer [90].

1.4.1 Excisional treatment

Excisional treatment should be used if there is any suspicion of invasive or glandular disease, and benefits from the ability to obtain a sample for histological assessment (Figure 1.7) and examination of the margins to confirm whether excision is complete. Large-loop excision of the transformation zone (LLETZ) is the most frequently performed technique in the UK being simple, easy to learn and of low cost. The choice of technique is largely dictated by user preference. NETZ (needle excision of the transformation zone) offers great precision and flexibility but is more challenging than LLETZ. Laser conisation has previously been used widely, but has largely been abandoned due to expense. Cold knife conisation (CKC) does not result in any thermal artifact, and thus offers the best option for accurate assessment of margins however it removes a greater amount of healthy tissue compared to other methods and this technique is rarely used today. Local anaesthesia containing a vasoconstrictor is injected into the cervix for pain relief and to reduce blood loss. A diathermy ball, or Monsel's solution (ferric subsulphate) are used for additional haemostasis.

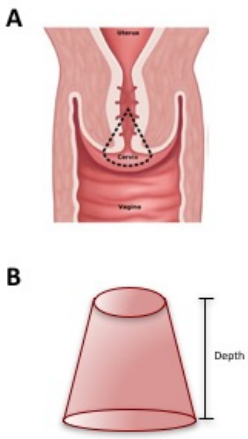


Figure 1.4. A. Site of cervical cone biopsy B. cone of excised tissue

1.4.2 Ablative treatment

Ablative treatments may be used in cases where the cytology, histology and colposcopic appearances are in agreement, when there is no suspicion of invasion, and when the entire lesion and transformation zone can be visualized. These are contraindicated when there is a history of previous treatment. Ablative techniques, apart from laser ablation, are cheap and easy to perform, thus ideal in resource-poor settings. Cold coagulation, uses a thermo-sound heated to 100-120°C to destroy lesions up to a depth of 7mm within 60 seconds, usually without the need for local anaesthesia. Cryocautery is another ablative technique, using a probe on a freeze-thaw-freeze cycle to destroy cells in around two minutes; its efficacy is lower for high-grade lesions compared to excision [90]. Laser ablation/vapourisation using carbon dioxide laser beam to destroy tissue can offer a great deal of precision, although takes slightly longer than the former techniques and again is expensive.

1.4.3 Follow-up after treatment

Following treatment, women remain at higher risk of pre-invasive or invasive disease, compared to the background population, which may be partly related to poor compliance with follow-up [91]. Involved margins, a large lesion, high-grade or glandular disease all increase the risk of recurrence, although repeat treatment is only indicated in women over 50 or those with high-grade disease at the endocervical margin. A negative hrHPV test after treatment may have a greater negative predictive value than cytology alone, although the two together offer the highest degree of reassurance. The current UK practice, introduced in 2012, is to invite women for cervical cytology with HPV 'Test of Cure' at six months, whereby those with normal, borderline or low grade dyskaryosis and negative HPV test are discharged back to 3-year recall. Women treated for CGIN were previously kept under six-monthly colposcopic surveillance for five years, however, since 2014, UK guidelines suggest these women can also return to routine recall after a negative 'Test of Cure'.

1.5 Complications of conservative treatment for CIN

Until relatively recently, local conservative treatment for cervical disease, excisional or ablative, was thought to have minimal, minor complications, mainly related to infection and haemorrhage occurring in around four and five percent of cases, respectively [92]. Several studies published in the last decade documented that treatment for CIN adversely affects reproduction, in particular increasing the risk for preterm birth (PTB), which is delivery between 24 to 37 weeks of gestation [84, 85, 93-96] (Figure 1.8). The mean age for having

conservative treatment for CIN in England is 30 years, which is also the mean age of mothers giving birth in the UK. Increased risk of preterm birth following treatment is understandably a major concern women of women undergoing treatment.

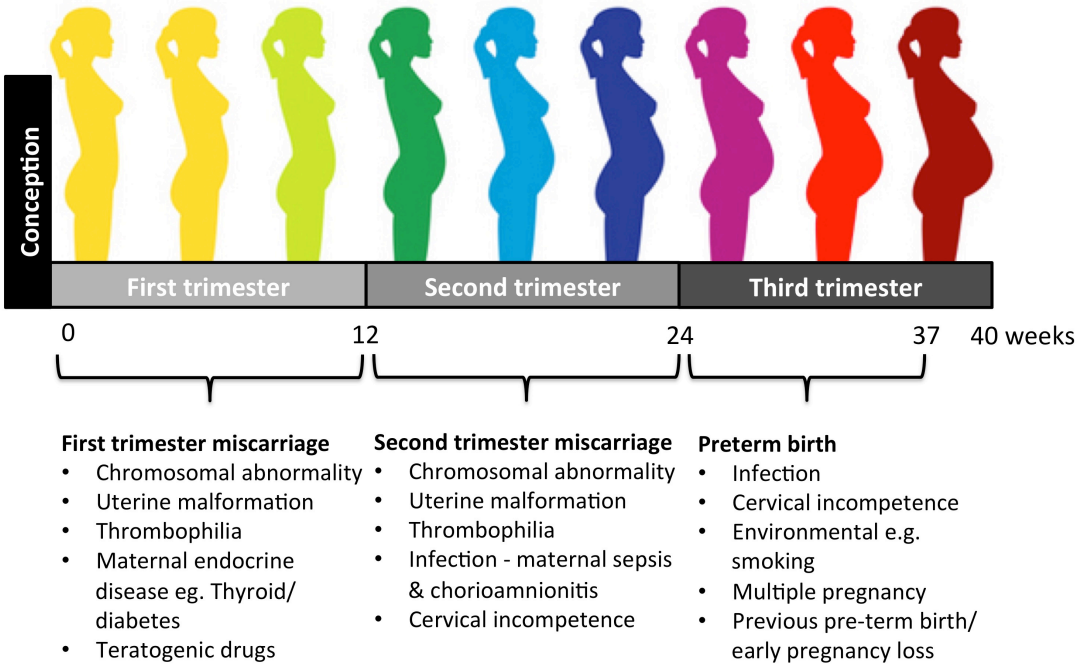


Figure 1.5. Timeline of gestation and causes of pregnancy complications

1.5.1 Late pregnancy complications (24 weeks onwards)

Preterm birth is the leading cause of infant mortality worldwide, and can be sub-classified into extremely preterm (<28 weeks), very preterm (28–32 weeks) and moderate to late PTB (32–36 weeks) [97]. In 2016, PTB accounted for 7.8% of all live births in the UK [98]. Preterm birth may be iatrogenic by means of medical intervention where the risk of the mother or the fetus is thought to outweigh the benefit of prolonging the pregnancy, or can be spontaneous, which accounts for about 75% of PTBs in the UK [99]. Preterm birth is also associated with higher morbidity resulting in a significantly higher chance of admission to the neonatal intensive care unit (NICU), prolonged and recurrent hospital admission, respiratory morbidity, sepsis, necrotizing enterocolitis and neurological disorders [100, 101]. Although much of this may be related to physiological prematurity, there is evidence to suggests that the pathological condition which precipitated preterm labour may both influence and exacerbate the associated morbidity [100]. Preterm pre-labour rupture of membranes (pPROM) in particular, occurs prior to PTB in around one in third of cases [102], and is strongly associated with infection and inflammation, which are likely involved in the pathogenesis of both conditions,

but also with the subsequent neonatal morbidity and mortality [103]. Besides infection, there is a vast array of other causes of spontaneous PTB including antepartum haemorrhage, uterine overdistension, cervical incompetence [104] as shown in Figure 1.8.

Numerous retrospective cohort studies have documented the link between treatment for CIN and spontaneous PTB, which was confirmed in 2006 by the first meta-analysis and closely followed by a subsequent meta-analysis in 2008 demonstrating an association between treatment and other adverse obstetric and neonatal outcomes including pPROM, low-birth weight and neonatal morbidity and mortality [84, 93, 95].

The cervix has both an immunological and structural function during pregnancy, a shortened cervix on antenatal transvaginal ultrasound is widely acknowledged to be a positive predictor of PTB [105]. The risk of PTB appears to correlate with the depth of the cone (Figure 1.7), which poses a dilemma for clinicians aiming for complete excision of affected tissue to ensure good oncological outcomes [106] while balancing limiting any potential impact on future reproductive outcomes of the patient. A nested case-control study of women who underwent treatment for CIN in the UK showed that excision to a depth of 9mm or less does not significantly increase the risk of PTB compared to the general population (9%), but at longer depths however, the risk increased linearly: between 10-14mm, the risk was increased to 9.6% (relative risk (RR) 1.28, 95% CI 0.98 to 1.68), 15-19mm to 15.3% (RR 2.04, 95% CI 1.41 to 2.96) and 20mm or greater to 18.0% (RR 2.40, 95% CI 1.53 to 3.75) [107]. The same study showed a similar pattern with increasing volume of excised cervical tissue with volumes greater than 2.66cm^3 doubling the risk of PTB. This finding has been replicated by other studies [108, 109], yet is still challenged by some [110]. The proportion of excision, rather than absolute depth/volume may play a more important role as the length of the cervix may vary between individuals. Cold knife conisation has been shown to remove a significantly greater volume of healthy stromal tissue compared to LLETZ [111]. It is therefore unsurprising that this technique is associated with the highest risk of PTB whereas ablative treatments, particularly laser ablation, are related to less frequent, if any, adverse complications [84, 93, 95].

There does not appear to be a correlation between time interval from treatment to conception and risk of PTB [107, 108]. Furthermore, the increased risk does not appear to be restricted to the first birth after treatment, but applies to all subsequent pregnancies [112].

The disease itself may be a confounding and contributing factor to PTB after treatment. Bruinsma *et al.* first demonstrated this in a meta-analysis by using internal, untreated controls with CIN and external disease-free controls, where the former group had a smaller, yet still significantly increased risk of PTB compared to the latter [95]. Castanon *et al.* further showed that the risk of PTB in women with CIN without treatment was 9% compared to 6.7% in the general population at the same time [85, 96]. Smoking and lower socioeconomic status are associated with both PTB and CIN, however regression analysis has shown the association between untreated CIN and PTB remains once they are accounted for.

Emerging evidence suggests that women who develop high-grade CIN requiring treatment may have an intrinsic abnormality that predisposes them to CIN [85, 95, 96, 113]. This may be associated with a local deficiency of the cervix enabling persistence of HPV infection and subsequent disease, as well as predisposing them to PTB.

It is now over a decade since the first meta-analysis of risk of adverse pregnancy outcomes was published, and there are now multiple further studies including thousands more patients. The areas of potential confounding discussed above, including depth of excision and choice of comparison group, have led to studies being published documenting both positive and negative associations between treatment and risk of adverse pregnancy outcomes, to varying degrees. This has resulted in confusion amongst clinicians regarding whether treatment does in fact increase the risk of adverse pregnancy outcomes, and if so, to what extent, making it very difficult to counsel patients, and manage them in future pregnancies. Given the large number of new studies available, alongside an opportunity to clarify the confusion by performing subgroup analysis based on the aforementioned potential confounders there is scope to perform a new systematic review and meta-analysis of the data.

1.5.2 Potential mechanisms of PTB after cervical excision

The mechanisms associating PTB with cervical excision are currently unknown and should be the focus of future research to enable clinicians to identify and provide targeted treatment to the women at greatest risk. Cervical regeneration after treatment is reported to be inversely proportional to the percentage of cervix excised [114, 115] and this regenerated tissue may furthermore be of inferior quality due to changes in collagen expression in the regenerated tissue [116]. The inconsistency in the data regarding association with the depth of cone, and

particularly the volume excised, however, indicates that the underlying mechanism is not purely mechanical or anatomical.

Ascending vaginal infection leading to chorioamnionitis may cause up to 40% of cases of PTB and pPROM [117]. Colonisation by pathogenic bacteria activates inflammatory pathways, which result in untimely onset of cervical remodeling and uterine contraction. The glandular cervical tissue provides an important innate immunological barrier during pregnancy, through production of mucus rich in cytokines and antimicrobial peptides. Changes in the properties of cervical mucus may also be associated with preterm birth [118], thus removal of a proportion of the cervix and its mucus-secreting glands may promote these deleterious changes. Many studies assessing the cervico-vaginal immune system have excluded patients with a previous history of cervical pathology and treatment, highlighting the need for research in this area. In animal models, viral infection of the cervix itself was shown to predispose to ascending viral infections in pregnancy [119], which may also account for the increased risk of PTB seen in women with untreated CIN. It is also feasible that these women have an intrinsic immune abnormality, which not only puts them at risk of persistent HPV infection leading to development of CIN, but also results in a higher propensity to deliver preterm.

In order for pathogenic bacteria to ascend, they must first colonise the vagina, which is usually inhibited by the healthy *Lactobacillus* spp. dominant vaginal microbiome. If the cervico-vaginal microenvironment is disturbed, for example by excisional treatment, the vaginal microbiome may also be disturbed, resulting in depletion of *Lactobacillus* spp. and overgrowth of anaerobic, bacterial vaginosis-associated species which has been associated with adverse obstetric outcomes [120].

1.5.3 Fertility and early pregnancy outcomes (<24 weeks of gestation)

Concerns about fertility and early pregnancy-related complications are common amongst women undergoing excisional treatment. However, the published data is often contradictory, which presents a challenge for clinicians with regards to informing patients of the risks and may result in increased anxiety for patients.

1.5.3.1 Fertility

Infertility is defined as the inability to conceive after 1 year of regular, unprotected sexual intercourse. It is expected that within this one year period 84% of women will conceive, with

up to 94% conceiving by two years and 93% by three years [121]. Causes can broadly be divided in female factors, which account for about 45% of infertility, male factors accounting for 30% and the remaining 25% of cases being unexplained [122] (Figure 1.9)

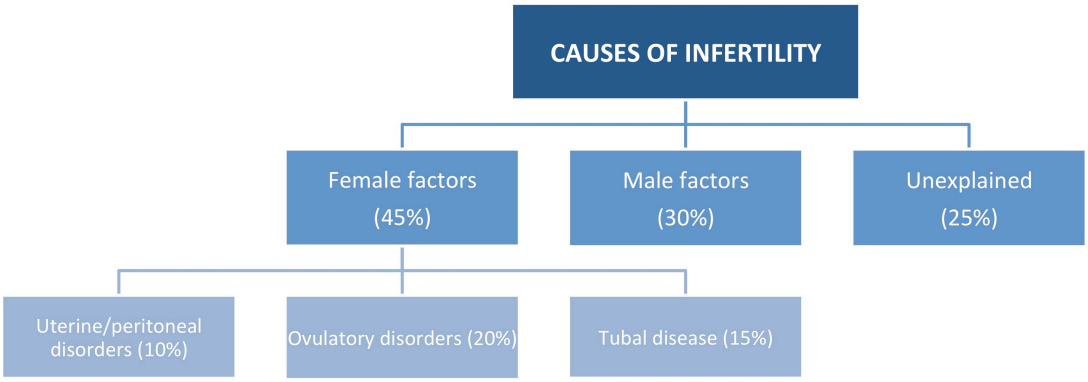


Figure 1.6. Causes of infertility

Whilst 'cervical factors' are rarely, if ever, listed as a cause of infertility there are several plausible mechanisms by which excisional treatment could impair future fertility. Firstly, cervical excision removes part of the endocervical canal and, as a result, mucus-secreting endocervical glands that produce secretions that facilitate sperm penetration and conception, which has been suggested to reduce the chances of a successful conception [123]. Secondly, cervical stenosis is a known complication of treatment, occurring in around 8% of patients following LLETZ [124], the most commonly used technique in the UK. This can arise as a result of the healing process, during regeneration of the crater produced during treatment, and could inhibit penetration of sperm and subsequent conception [125]. Additionally, retrograde menstruation may also occur as a consequence of severe cervical stenosis, resulting in endometriotic deposits, intra-peritoneal adhesions and scarring, causing tubal infertility factors.

A large, registry-based, retrospective cohort of 35,000 women in Finland with a follow-up of over 250,000 women-years assessing total pregnancy and live-birth rates amongst treated and untreated women reported that treated women had a higher pregnancy rate before and after the treatment than the reference population (HR = 1.15 ($p<0.001$) and 1.06 ($p<0.001$) compared to the reference population [126], although pregnancy rates in women with intention to conceive were not reported. The actual time to conception in another recent large series from the USA reported that women after conisation take longer to conceive as compared to untreated normal women or women attending colposcopy but not treated (time

to conception>12 months 16.4% versus 8.4%, adjusted odds ratio (aOR) 2.09, 95%CI 1.26-3.46) [127]. Although this may be attributed to treatment, medical advice may confound this with the suggestion to delaying conception until after the early post-operative period. Two previous small case-series [128, 129] however reported that cervical treatment did not impact time required to conceive.

1.5.3.2 Miscarriage

Miscarriage is defined as pregnancy loss before 24 weeks of gestation and affects up to 30% of biochemical pregnancies (a positive pregnancy test without ultrasound visualisation of the pregnancy) and between 11-20% of clinically-recognised pregnancies (those seen on ultrasound scan) [130]. The vast majority of miscarriages occur in the first trimester (≤12 weeks) [131], and between 50 and 85% are caused by fetal chromosomal abnormalities [132], with inherited thrombophilia, uterine malformation, uncontrolled diabetes and thyroid disease and also teratogenic drugs cited as less common causes [131] (Figure 1.8).

Second trimester miscarriage (loss between 12-24 weeks) is much less common, arising in between 1-4% of pregnancies [133]. Whilst these may represent pregnancies which have failed in the first trimester but are not expelled until the second trimester, the aetiology of a true second trimester miscarriage differs considerably to first trimester losses. Whilst genetic abnormalities may still be a cause, they only represent around 15% of cases [134]. Structural uterine anomalies and thrombophilias, infection (systemic maternal sepsis or chorioamnionitis) and cervical incompetence also play a role [135, 136] (Figure 1.8). It is plausible that this phenomenon, certainly relating to second-trimester miscarriage, is a continuum of the factors causing preterm birth (PTB) in this patient group.

1.5.3.3 Ectopic pregnancy

Ectopic pregnancy is the implantation of a fertilized ovum outside the uterine cavity, which complicates around 1-2% of pregnancies in the first trimester and is associated with a high risk of morbidity and mortality. Around 5% of maternal deaths are related to ectopic pregnancies in the UK [137]. Approximately 90% of cases occur within the fallopian tube, although other sites include the peritoneum, ovary, cervix or Caesarean section scars [138]. Risk factors for ectopic pregnancy including smoking, pelvic inflammatory disease, previous tubal surgery and previous ectopic pregnancy [138].

The association between treatment for CIN and ectopic pregnancy, or lack of, is yet to be investigated in detail. Several studies reporting small numbers, which is expected given the relatively low incidence. If an association were to be found it is likely to be confounded by sexual and behavioural factors, predisposing to both CIN and ectopic pregnancy. It seems unlikely however that treatment would increase the risk of a cervical ectopic pregnancy in particular, because the site of the excisional scar is so low in the cervical cavity, if not completely ectocervical, so that the pregnancy would be lost before it was able to implant in the scar site, as can be seen in Caesarean scar ectopic pregnancy.

1.5.4 A need for further studies assessing reproductive outcomes following cervical excision

The association between CIN and increased risk of PTB may be due to an intrinsic abnormality that links susceptibility to both conditions, or other confounding factors. Having an excisional treatment for CIN significantly increases the risk as compared to having CIN only in a step-wise fashion according to depth of tissue removed and number of treatments performed, which probably arises through damaged cervical architecture and altered cervicovaginal microenvironment, both of which are necessary for maintaining a pregnancy to term although neither have been well-studied in this group of patients, on in association with CIN or HPV itself. The vast majority of published studies are retrospective in nature, and thus subject to varying degrees of bias, along with a degree of heterogeneity in choice of control group. It is therefore important to carry out a prospective study, in order to thoroughly investigate the mechanisms that underlie the increased risk of PTB in this group of women.

1.6 The Human Microbiome

Bacteria account for 50 percent of the cells of the human body, and together with archaea and lower eukaryotes are collectively termed the ‘human microbiome’ [139, 140]. Due to the limitations of culture-based techniques [141], the composition of microbiota in different body sites is increasingly studied through use of next-generation sequencing (NGS) techniques. This can involve shot-gun metagenomic sequencing of all DNA in a biological sample (human and bacterial) but most commonly involves amplifying, sequencing and analysing specific regions of bacterial 16S rRNA genes or other rRNA genes (e.g 18S for eukaryotic microbes). Prokaryotic organisms contain a 70S ribosome, made up of two smaller subunits; 50S and 30S, each of which is formed of several proteins and ribosomal ribonucleic acid (rRNA) (Figure 1.10A). The 16S rRNA gene of the 30S subunit is the most conserved region between all bacteria, with the

hypervariable regions (Figure 1.10B) containing species-specific sequences. A variety of bioinformatics tools and platforms are used to assign resulting sequences to specific microbial taxa at different taxonomic levels as well as for in-depth phylogenetic analysis of microbial community structure [142-144].

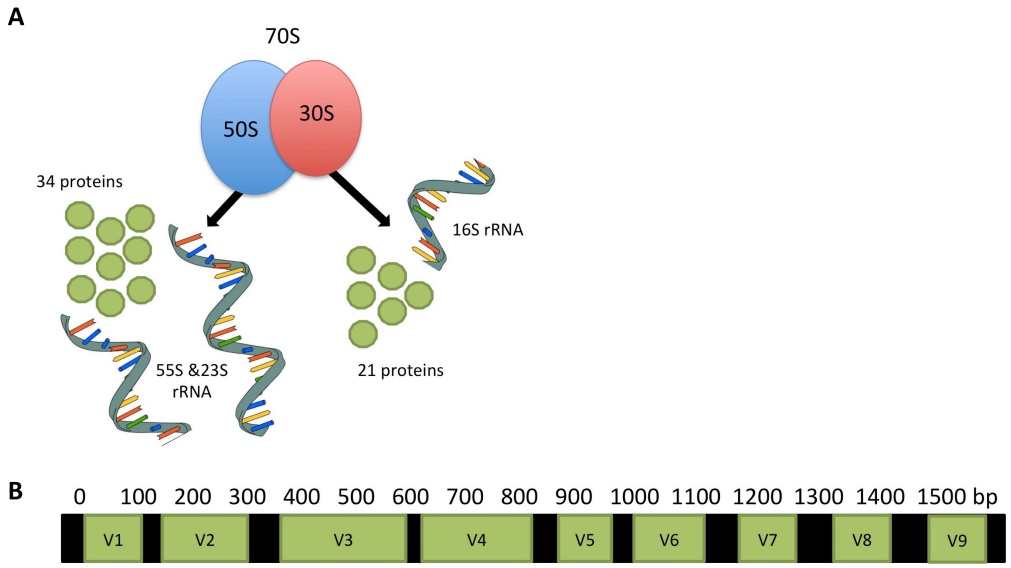


Figure 1.7. The structure of bacterial DNA (A) and the 16S ribosomal RNA gene (B)

A. Bacteria contain a 70s ribosome composed of 2 subunits; the large 50S subunit and a smaller 30S subunit. The 30S subunit contains 21 proteins and a 16S ribosomal RNA (rRNA) gene.
B. The 16S rRNA gene contains many conserved regions as well as 9 hypervariable regions (green) that contain species-specific sequences. It is these variable regions that allow species-level identification through gene sequencing technologies.

The National Institute for health established the Human Microbiome Project in 2008, with the intention to characterise the microbial composition of the human body, and correlate this health and disease states [145]. The link between health, disease, and the human microbiome is a fast-moving and contentious area of research, and an appreciation of the variation in microbiota composition amongst individuals is expanding our understanding of the pathophysiology underlying a variety of diseases affecting many body systems, from colorectal cancer [146, 147] to atopic dermatitis [148].

1.6.1 Bacterial taxonomy

Hierarchical taxonomy is the classification of living organisms according to genetically related phylogenetic groups. Numerous bacterial classification systems have been used since bacterial were first described by Leeuwenhoek in 1676. The current classification was described in 1998 [149] and is shown in Figure 1.11.

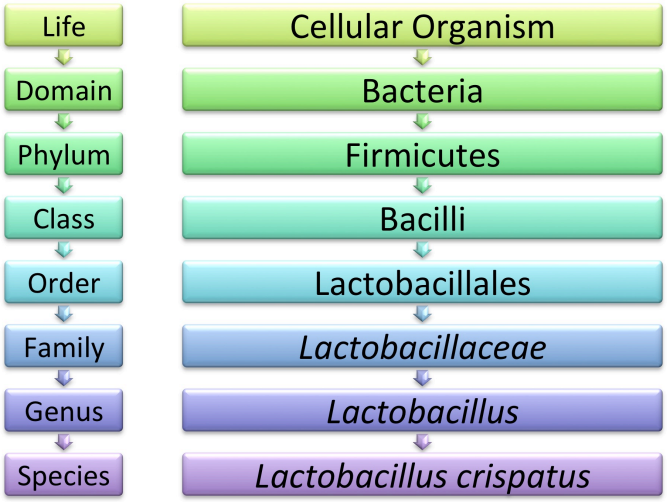


Figure 1.8. Bacterial taxonomy

Lactobacillus crispatus is used as an example.

1.7 The vaginal microbiome

It is a long-established fact that the vagina is not a sterile environment. In 1892 Albert Döderlein, a German Obstetrician and Gynaecologist, first described *Lactobacillus,* which he had isolated from the vagina of a pregnant woman, and subsequently named 'Döderlein's bacillus' [150].

In the majority of human body sites examined to date, highly diverse microbial communities are generally considered a signature of health [145, 151]. However, in the case of the female reproductive tract, health is more commonly associated with low microbial diversity and dominance by only one or a few species of *Lactobacillus* [152-154]. Ravel and co-workers demonstrated that whilst this concept is broadly observed in the majority of reproductive age women, a significant proportion harbour diverse vaginal bacterial communities [153]. In their study, vaginal samples collected from 396 ethnically diverse reproductive-age women were analysed using Roche 454 FLX-based sequencing of bacterial 16S rRNA genes. In total 282 taxa were identified and, using hierarchical taxonomic clustering, the vaginal microbial profile of each woman could be classified into one of five "community state types" (CSTs), which have subsequently been used by numerous other studies [152, 155, 156], although other studies have used alternative classifications still based around the presence or absence of *Lactobacillus*[157]. CST I, II, III and V are characterised by dominance of *Lactobacillus crispatus, L. gasseri, L. iners* and *L. jensenii* respectively and tend to have low species diversity and

evenness. In contrast CST IV is typically devoid of *Lactobacillus* spp. and instead enriched with strict anaerobic species often associated with bacterial vaginosis (BV) including *Gardnerella, Megasphera, Sneathia* and *Prevotella* spp*.* As will be discussed below, the structure of the vaginal microbiota (VMB) is dynamic and transitioning between CSTs can occur. In the vagina, the most common transition observed is from CST III to CST IV [158], which suggests that *L. iners* may be less able to inhibit colonisation of strict anaerobes and pathobionts compared to other *Lactobacillus* spp. [158] or because *L. iners* appears more capable of surviving and adapting to a wide range of pH and other metabolic stress-related conditions due to the constitutive and inducible expression of genes not seen in other lactobacilli [159, 160]. The use of sequencing technologies has had a profound impact on our ability to characterise the human microbiome. Most notably in relation to the VMB, *L.iners*, one of the most common vaginal *Lactobacillus* species was only described in 1999 through the aid of sequencing, because the species is notoriously difficult to culture [161].

1.7.1 Bacterial vaginosis

Bacterial vaginosis (BV) is a polymicrobial disorder characterized by a microbial community structure consistent with CST IV; that is, diminished levels of *Lactobacillus* spp. and overgrowth of anaerobes, particularly *Gardnerella, Prevotella* and *Peptostreptococcus* species [162]. The prevalence of BV is around 9% in the UK [163], and up to 29% in the US [164]. The Hay/Ison criteria used for diagnosis of BV in the UK is largely based on the presence or absence of both *Lactobacillus* and *Gardnerella* or *Mobiluncus* morphotypes [165]. BV has previously been correlated with higher incidence, prevalence and persistence of HPV infection, and with development of CIN [166-169]. However, other studies did not find a positive correlation between CIN and BV [170-172], which may partly be explained by the lack of objective diagnostic tests for BV, the reliance on subjective scoring systems [173], and the heterogeneity of BV itself. The use of sequencing technologies has highlighted that BV cannot be regarded as a single entity, but rather a syndrome of relative *Lactobacillus* spp. depletion associated with a varying bacterial composition associated with equally varied symptomatic and disease outcomes.

1.7.2 Factors affecting vaginal microbiome composition

1.7.2.1 Ethnicity

Ethnicity is a major intrinsic factor known to be significantly associated with variance in vaginal microbiota composition, with Caucasian and Asian women displaying a significantly greater

prevalence of *Lactobacillus* spp. dominant microbiota, compared to Hispanic and Black women [153]. A further study comparing 36 Caucasian and 25 Black women however concluded there was no difference in VMB composition according to ethnicity [174]. However, the investigators excluded women with BV diagnosed according to Nugent criteria from further analysis, which would have significantly altered their results and interpretation of their findings. More recently, a study of 610 women in the Netherlands has confirmed that the vaginal microbiota is indeed associated with race, with African women being significantly more likely to have a polymicrobial *Gardnerella vaginalis* containing VMB or *L.iners* dominance when compared to Dutch Caucasian women and that this relationship is maintains after correction for behavioural factors, such as contraceptive practices, and stability of sexual relationships [157]. Race-associated differences according may be due to genetic factors that influence mucosal immunity or metabolic pathways, which result in preferential conditions for particular species, but could also be explained by variation in hygiene practices (see below), which were not included in the aforementioned Dutch study.

1.7.2.2 Hygiene practices

Menstrual hygiene practices are significantly influenced by cultural and social factors [175]. Vaginal douching was reported by 22% of the 3739 American women sampled in a large representative cohort [164], and is twice as common in Black women, compared to Caucasians [176].

Vaginal douching, particularly after menstruation, has been shown to significantly increase the risk of BV [177], and cessation of the practice reduces the risk of BV [178]. *Lactobacillus* spp. are usually planktonic, whereas biofilms of densely adherent bacteria can be present in the vagina in BV [179]. Douching and other intravaginal practices may therefore wash away the planktonic *Lactobacillus* spp. making way for colonisation by anaerobes that are able to establish themselves in biofilms, which are much more difficult to disrupt. Interestingly, douching is also suggested to increase the risk of CIN and cervical cancer [180].

Tampons are a commonly used menstrual hygiene product in the developed world, which are inserted into the vagina to collect menstrual blood. Whether their use could alter the VMB due to physical or biochemical/metabolic environmental disruption has not been well investigated. Hickey *et al.* studied seven healthy female women using two different types of tampons (one with, and one without calcium citrate malate, a compound used in tampon

production for drying) over the course of three menstrual cycles, and stated that tampons did not have any impact on the composition, abundance or diversity of bacterial populations [181]. The small sample size, lack of a true control group and the fact that all of the study population had a *Lactobacillus* spp. dominant VMB communities makes it difficult to draw any firm conclusions from this study. It is also interesting to note that the study was performed in the facility of a major tampon producer, which could have influenced the published results. Larger, more accurately designed studies on the impact of menstrual hygiene practices, with a greater variety of participants and a true control group are required.

1.7.2.3 Intrinsic female hormones

Female hormones also have a major impact on both the structure and stability of vaginal microbial communities. While the human vagina is thought to be initially sterile at birth, rapid colonization with *Lactobacillus* spp. occurs and is supported by residual maternal oestrogen. The mechanism by which hormones drive vaginal microbial composition is not yet fully elucidated but *Lactobacillus* spp. dominance appears to be strongly influenced by oestrogen-driven maturation of the vaginal epithelium, which leads to the accumulation of glycogen in vaginal epithelia [182]. Host α-amylase, present in vaginal mucosa, metabolise the glycogen to simple sugar products such as maltose, maltotriose, and maltotetraose that appear to preferentially support *Lactobacillus* spp. colonization [183]. Consistent with this, reduced oestrogen levels three to four weeks post-birth correspond with a reduction in vaginal *Lactobacillus* and increased species diversity with enrichment of strict anaerobe and enteric species, which is maintained until puberty [184]. Increased oestrogen and progesterone secretion preceding menarche drives reduced VMB diversity and increased relative abundance of *Lactobacillus* spp. [185]. Throughout a woman's reproductive age fluctuation of VMB composition can be linked to the cyclical secretion of oestrogen and progesterone throughout the menstrual cycle. Highest diversity and instability is observed at the time of menstruation [186, 187] which oestrogen and progesterone levels are at their lowest. The presence of menstrual blood also augments the composition of the vaginal mucosal environment leading to depletion of certain species and enrichment of others. Greatest stability of VMB structure over the menstrual cycle is observed at the time of the oestrogen peak, followed by the progesterone peak a few days later [186]. Following menopause, reduced oestrogen and resulting vaginal atrophy are thought to lead to *Lactobacillus* spp. depletion and increased diversity [155]. Consistent with these findings, it has recently been shown that the postpartum period of pregnancy, which involves a 100-1000-fold decrease in

circulating oestrogen concentrations, is associated with a significant increase in vaginal microbial diversity and richness [152].

1.7.2.4 Extrinsic female hormones

The widespread use of synthetic hormones for contraceptive purposes also has an impact on the composition of vaginal microbiota. Meta-analysis has shown that hormonal contraceptive use is associated with a 31% and 32% reduction in recurrent and prevalent BV, and 18% reduced risk of incidence [188]. The study included both combined hormonal contraceptives (combined oral contracteptive pills and vaginal NuvaRing®) and progesterone-only hormonal contraceptives (progesterone-only pills, depot medroxyprogesterone acetate, Mirena® intrauterine devices and implants). Neither combined nor progesterone-only preparations were shown to be more protective than the other. Besides use of hormonal contraceptives, other environmental factors known to influence VMB composition include smoking [189], and recent intercourse [190], both of which are associated with reduced relative abundance of *L. crispatus* and increase species diversity.

1.7.2.5 Smoking

Nicotine and its metabolites are detectable in cervical mucus [56]. Therefore, it seems plausible that smoking could have an impact on the metabolic environment, and thus microbiome composition. Certainly, tobacco smoking has been associated with altered diversity in the gut, oral cavity and respiratory tract [191, 192]. Bradshaw *et al.* [193] showed that BV (using Nugent Score) was increased in women who smoked more than 30 cigarettes per week. Brotman *et al.* [189] have further used NGS to show that in a small cross-sectional cohort of 20 smokers and 20 non-smokers, smokers had a significantly higher prevalence of CST IV (50% smokers vs 15% non-smokers), and abundance of the genera *Peptostreptococcus* and *Veillonella*. Further studies are required to ascertain whether smoking does indeed have a causal relationship with VM alteration and dysbiosis.

1.7.2.6 Human papillomavirus infection

A strong correlation between vaginal *Lactobacillus* spp. abundance and low pH exists. This acidic environment can inhibit growth of several potentially pathogenic species, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Gardnerella vaginalis* [194-197], yet provides optimal support for cellular metabolic function of the cervix and the vagina [198]. In

a study of 9,165 Costa Rican women, vaginal pH >5 was shown to be significantly associated with a 10-20% increased risk of HPV positivity in premenopausal women [199]. The HPV E5 protein responsible for viral transformation is known to be particularly susceptible to low pH [200], which is one plausible mechanism for this observation.

Lee and colleagues were the first to use next-generation sequencing to examine the impact of HPV infection on VMB composition in a cross-sectional cohort of 912 women participating in a Korean twin study, which included 16 pre-menopausal, monozygotic twin pairs, nine of whom were HPV-discordant. In the latter group, the investigators observed a difference in the VMB structure between twins, with HPV-positive women having higher species diversity and significantly less *Lactobacillus* spp. compared to their uninfected twin [201]. Furthermore, *Sneathia* spp. was identified as a microbiological marker of HPV-infection.

In a longitudinal study, Brotman and co-workers studied a North American cohort of 32 sexually active, premenopausal women over 16 weeks using self-sampling at twice-weekly intervals [202]. From a total of 930 samples, women with CST III and IV were most likely to be HPV-positive (71 and 72% respectively). In addition, CST II, dominated by *L. gasseri* was associated with the most rapid clearance of acute HPV infection, defined as transition from HPV negativity to positivity, and back to negativity, and used continuous time multi-state Markov modeling to calculate adjusted transition rate ratios. Such an observation might point to *L. gasseri* as a potential therapeutic species for maintaining cervical health, however, it is pertinent to note that only two of the 32 women had a predominantly CST II VMB, and that two additional women in the study with CST III and CST IV VMBs, also exhibited the same rapid patterns for acquisition and clearance over the 16-week study period. Further studies are necessary to confirm temporal relationships between vaginal microbiota and HPV infection and to determine whether any difference exists in the dynamics of high- and low-risk HPV subtypes, which is most clinically relevant.

BV has previously been correlated with higher incidence, prevalence and persistence of HPV infection, and with development of CIN [166-169]. However, other studies did not find a positive correlation between CIN and BV [170-172], which as pointed out earlier may partly be explained by the lack of objective diagnostic tests for BV, the reliance on subjective scoring systems [173], and the heterogeneity of BV itself. A recent study of American 1271 women

has also shown douching to increase the risk of HPV infection with high-risk subtypes in particular [203].

1.8 Cervical immune system

The human immune system has two arms; innate and adaptive, which work synergistically to defend the body not only from microbes (bacteria, fungi, and parasites) and viruses but also from toxins and cancer cells. The adaptive immune system is able to recognise and respond to antigen with a gradual and highly specific antibody and/or T cell response, capable of memory in the case of future encounters with the same antigen. The innate immune system in contrast is a highly conserved, non-adaptive system, providing the first rapid-response immunological barrier and consists of epithelial cells, macrophages, granulocytes, pathogen-recognition receptors and secreted molecules including antimicrobial peptides. This innate response is thought to be critical to early control of HPV infection [204], and also plays a significant role both alone, and in conjunction with the adaptive immune system in term and preterm labour [205].

1.8.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small, cationic peptides, secreted by epithelial cells providing mucosal immunity at various sites including the gastrointestinal and respiratory tracts, as well as the female reproductive tract. They are important for health and disease through their role in targeting and destroying microbes, which has afforded them the name of the 'endogenous antibiotics', along with aiding tissue homeostasis through cell signalling, differentiation, and repair. Due to their cationic nature, they are attracted to the anionic phospholipid and lipopolysaccharide (LPS)-containing microbial membranes [206]. They also possess antiviral activity against non-enveloped viruses such as HPV through their ability to inhibit various points in the viral entry process and replication within human cells [207]. AMP expression is both constitutive, to provide prophylactic background protection, and inducible at times of infection, inflammation or tissue injury [208].

The expression of these proteins has not yet been characterised in patients with CIN. Furthermore, these AMPs appear to play an important role in protection against ascending infections, which are implicated in the pathogenesis of preterm birth. Viral infection itself may predispose to ascending viral infections in pregnancy [119], and thus alteration in the innate immune function of the cervix could represent a potential mechanism for CIN alone, without

the added risk of excisional treatment. This treatment for CIN is known to cause a scar on the cervix, and it is unlikely that scarred epithelium is able to produce the same level of AMPs as untouched epithelium, however this hypothesis has not yet been tested.

1.8.1.1 Defensins

Defensins are the largest group of human AMPs, which can be subdivided into the α, β and θ-subgroups according to structure. The α-defensins are expressed by neutrophils [209], β-defensins by epithelial cells and θ-defensins by numerous tissues ranging from bone marrow to skeletal muscle [210]. Human beta-defensin-1 (hBD-1) is constitutively expressed throughout the female reproductive tract [211], and can be upregulated through TLR3 agonist stimulation [212] suggesting an antiviral role. Consistent with this, polymorphisms in the gene DEFB1 that encodes the protein have been associated with increased susceptibility to HPV infection [213] and upregulated hBD-1 expression has been observed in vulvuvaginal condylomata acuminata; low-risk HPV-mediated genital warts [214]. Furthermore hBD-1 exhibits broad spectrum antimicrobial activity, particularly against Gram-negative, preterm birth-associated bacteria including *Ureaplasma* spp. [215] and *Escherichia coli* [216] as well as the yeast *Candida albicans,* which has been associated with intraaminiotic infection [217]. Increased secretion of these defensins is observed at the time of PTB when associated with intrauterine infection [218], suggesting they play a role in protection during pregnancy however an increased innate immune response is insufficient in the presence of overwhelming infection.

1.8.1.2 Secretory leucocyte protease inhibitor

Secretory leucocyte protease inhibitor (SLPI) belongs to the whey acid protein group, and is expressed by both squamous and glandular cervical epithelium [219] in a constitutive and inducible manner [220], and by immune cells including neutrophils and macrophages [220, 221]. Its antiprotease activity plays an important role in prevention of excessive infection and inflammation-induced tissue destruction. SLPI has been shown to possess potent anti-HIV [222] and anti-HSV activity [223] and HPV infection of head and neck tissues is associated with lower levels of SLPI [224], yet up-regulation is seen in cervical cancer [225]. During pregnancy, SLPI is present at high concentration in the mucus plug [226] and cervical expression increases throughout gestation, but has been shown to be significantly higher in women who delivered preterm compared to those with threatened preterm labour who delivered at term, and in normal term deliveries [219]. SLPI also appears to play a role in term parturition, with

concentrations found to be elevated in cervical mucus of women up to seven days prior to uncomplicated term delivery [227]. Bacterial vaginosis is associated with lower SLPI levels [228] and we have recently shown that an increasing prevalence of dysbiosis with *Lactobacillus* spp. depletion is associated with increased CIN severity [229].

1.9 Hypothesis and aims

1.9.1 Rationale

Whilst the association between HPV infection and development of CIN and cervical cancer has been well-established for many years, there are many unanswered questions surrounding how the virus, which infects the majority of sexually-active women, is able to persist and cause significant disease in only a very small proportion of infected women. There is mounting evidence that the cervicovaginal microenvironment plays a role, with particular interest growing in the role of the vaginal microbiome. *Lactobacillus* spp. usually predominate in the vagina and their replacement with other strict anaerobes, as is seen in bacterial vaginosis, has been shown to be associated with higher rates of HPV infection, CIN and cervical cancer. The use of bacterial sequencing techniques will enable a more accurate analysis of the relationship between HPV, the host and the vaginal microbiome than has previously been possible using bacterial culture. It appears that certain bacterial species influence the inflammatory status within the cervix through modulation of local pro-inflammatory cytokines and innate mucosal immune factors resulting in chronic inflammation that is associated with bacterial vaginosis, which may promote chronic HPV infection and cervical carcinogenesis.

There is a current lack of objective tests to determine which patients are at highest risk of progression to invasive cancer, and therefore excisional treatment is the preferred management of all high-grade CIN. If a high-risk microbiological pattern or species is determined, it could be used to identify those women most in need of treatment. It may be possible to manipulate the vaginal microbiome through microbial gene regulation, probiotic and prebiotic treatments in order to establish a healthy environment, combat disease and improve health outcomes.

Whilst excisional treatment is very effective at preventing future development of invasive cervical cancer, it appears to cause iatrogenic preterm delivery in a proportion of women and there is currently a lack of data with which to inform patients regarding possible fertility and early pregnancy complications, which they commonly enquire about.

The mechanisms of these adverse reproductive outcomes following treatment have not yet been investigated. Excisional treatment is associated with shorter post-operative cervical length, however alongside having a structural role in maintaining pregnancy, it also has a functional role in defending against ascending infections and untimely activation of inflammatory pathways that are known to be associated with adverse obstetric outcomes. A better understanding of these factors may enable identification of women at risk of adverse events and development of cause-directed prevention strategies. It has recently been suggested that the vaginal microbiome plays a role in pregnancy outcomes, and we further suggest that the composition may be altered in women with CIN compared to healthy controls, which could impact on the local immune and inflammatory environment predisposing women to both preterm birth and cervical disease. The impact of treatment on vaginal microbiome composition should also be investigated as a potential factor in pregnancy outcomes after treatment, which would also be modifiable through microbiome manipulation.

1.9.2 Hypothesis

The first hypothesis to be tested by the work presented in this thesis is that the vaginal microbiome plays a role in cervical carcinogenesis, as suggested by previous studies using largely culture-based techniques. We hypothesise that 16s ribosomal RNA (rRNA) sequencing will show an altered vaginal microbiome composition in women with cervical pre-invasive and invasive disease compared to healthy controls, and that the composition also influences chance of regression, progression or persistence of CIN in a serially-sampled population. Disruption of a healthy vaginal microbiome may induce a chronic inflammatory reaction and immune dysregulation, which is known to promote malignant transformation and carcinogenesis. Furthermore, excisional treatments to remove CIN may result in alteration of the vaginal microbiome promoting a proinflammatory environment and altered innate immune function, which could predispose a treated woman to adverse pregnancy outcomes.

Excisional treatment is known to result in a shorter cervix compared to before treatment, which likely contributes to adverse reproductive outcomes due to a mechanical deficiency. We hypothesise that treatment does not influence fertility or early pregnancy outcomes when cervical length is unlikely to play a role, but may be associated with a higher risk of second trimester miscarriage. However, we hypothesise that treatment increases the risk of late pregnancy complications, such as preterm rupture of membranes and preterm labour, in a

dose-dependent manner. It is unlikely that all women have the same cervical length prior to treatment, and it is therefore hypothesised that the proportion of excision correlates more accurately with the degree of cervical regeneration than absolute depth of excision.

1.9.3 Aims

The first part of this thesis will investigate the role of the vaginal microbiome in relation to cervical carcinogenesis and aims to

1) Characterize the vaginal microbiome in pre-invasive or invasive cervical disease and in healthy controls

2) Determine whether vagina microbiome composition associates with cervical pre-invasive disease progression using serial cytology samples collected from a cohort of untreated women with CIN.

3) Explore the impact of the microbiome on inflammation and innate immune response

The second part of this thesis will focus on the reproductive morbidity associated with CIN and its treatment and aims to

1) Perform a systematic review and meta-analysis to assess impact of treatment for CIN on a) fertility outcomes, b) early (<24 weeks gestation) and c) late (>24 weeks gestation) pregnancy outcomes

2) Assess the impact of local cervical treatment on cervical length and volume

3) Characterise the impact of local CIN treatment on the a) vaginal microbiome and b) inflammation and innate immune response

CHAPTER 2: Materials & Methods

2 Materials and Methods

2.1 Materials

Experimental process	Chemical/Kit/Buffer	Supplier
General	Ethanol	VWR
	Methanol	VWR
	Phosphate buffered saline (PBS)	In house
	Tris/Borate/EDTA (TBE)	In house
	Sodium Citrate	Sigma
	0.2ml PCR tubes	Starlabs
	0.5ml eppendorf tubes	Starlabs
	1.5ml eppendorf tubes	Starlabs
	2ml eppendorf tubes	Starlabs
	96-well optical plate	Starlabs
Sample collection	Amber eppendorf tubes	VWR
	Cervical cytobrush	Hologic
	BBL™ CultureSwab™ cont. liquid Amies	Becton Dickinson
	Protease inhibitor cocktail	Sigma
	Speculum	Pelican
	ThinPrep® PreservCyt®	Hologic
	Voluson E6	GE Healthcare
	5-9 MHz (RIC5-9-RS) transvaginal probe	GE Healthcare
16s rRNA extraction	DNA Mini Kit	Qiagen
	Lysostaphin	Sigma
	Lysozyme	Sigma
	Mutanolysin	Sigma
	QiAMP *Cador* Pathogen Kit	Qiagen
	TE (10mM Tris, 1mM EDTA at pH7.4)	In house
	Tissue Lyser LT	Qiagen
	0.1mm zircona/silica beads	BioSpec
PCR	Agarose	Sigma
	AmpliTaq Gold DNA Polymerase	ThermoFisher Scientific
	BioRad Tetra 2 machine	BioRad
	Deoxynucleotide mix (dNTP) 10mM	ThermoFisher Scientific
	$MgCl_2$ 25µM	ThermoFisher Scientific
	PCR-grade sterile water	Sigma
	SYBR® Safe DNA gel stain	ThermoFisher Scientific
	6x Blue/Orange Loading Dye	Promega
	16s rRNA forward & reverse primers	Invitrogen

Real-time PCR	BactQUANT 16S rRNA primers	Sigma
	BactQUANT probe (FAM labeled)	Sigma
	Gardnerella vaginalis primers	Invitrogen
	Fast SYBR® Green Master Mix	ThermoFisher Scientific
	Diethylpyrocarbonate (DEPC) water	ThermoFisher Scientific
	Lyophilized Genomic *E.Coli* DNA Strain B	Sigma
	Platinum Supermix UDG (including ROX)	ThermoFisher Scientific
	StepOnePlus real-time PCR system	ThermoFisher Scientific
Protein quantification	OPTImax plate reader	Molecular diagnostics
	Quick Start™ Bradford Protein Assay	BioRad
Enzyme linked immunosorbent assays (ELISA)	Human BD-1 ELISA Development Kit	Peprotech
	Human SLPI ELISA Kit	Hycult Biotech
	Luminex Screening Assay multiplex kit	R&D Systems
	Luminex MAGPIX Analyzer	R&D Systems
	Magnetic plate holding device	Bio-Rad
	Tween-20	Sigma
Immunohistochemistry	Anti-human BD-1	Peprotech
	Anti-human SLPI	Hycult Biotech
	Rabbit HRP-DAB Staining Kit	R&D Systems
	Goat HRP-DAB Staining Kit	R&D Systems
	Mayer's Haematoxylin	Sigma
	Xylene	Sigma
Software	Excel	Microsoft
	PRISM	GraphPad
	R	Lucent Technologies
	Revman	Cochrane
	Statistical Analysis of Metagenomic Profiles (STAMP)	Oximetrics
	4D View	GE Healthcare
	MicrobiomeAnalyst	www.microbiomeanalyst.ca

2.2 Methods for systematic review and meta-analysis

Two systematic review and meta-analysis titles are included in this thesis. The methods for each are detailed below.

2.2.1 Search methods

A search of two electronic databases (MEDLINE and EMBASE) was conducted targeting using the search criteria detailed in Appendix 1 & 2. There was no language restriction. The references of the retrieved articles and meta-analyses, and the proceedings of the relevant conferences were hand-searched in an attempt to identify any articles missed by the initial search or any unpublished data. Two independent investigators performed the literature search, the assessment of the eligible reports according to the inclusion and exclusion criteria and the extraction of the data. The results of the search and data extraction were then compared; in the cases of discrepancies amongst the two investigators, a consensus was reached with the involvement of a third investigator if necessary.

2.2.1.1 Types of studies and interventions, inclusion and exclusion criteria

All studies that reported the outcomes described in sections 2.2.1.2 and 2.2.1.3 (see below) in women with or without cervical treatment for CIN were included. All treatment modalities were included, both ablative [laser ablation (LA), cold coagulation (CC), cryotherapy (CT) or radical diathermy (RD)] and excisional [cold knife conisation (CKC), large loop excision of the transformation zone (LLETZ) = loop electrosurgical excisional procedure (LEEP), laser conisation (LC), needle excision of the transformation zone (NETZ) = straight wire excision of the transformation zone (SWETZ)]. Studies reporting multiple treatment methods were included, even if they did not report outcomes separately. Furthermore, where outcomes were not reported separately for each technique, we analysed the intervention under broader terms, i.e. excisional treatment not otherwise specified (NOS), ablative treatment NOS and treatment NOS. Studies were included irrespective of the type of untreated reference population - i.e. matched or unmatched untreated women, internal self-matching with the pregnancies of the same women before treatment, women attending colposcopy but not receiving treatment. Studies were excluded if there was no untreated reference population, and where treatments were performed during pregnancy.

2.2.1.2 Types of outcome measures – Fertility and Early Pregnancy outcomes

All outcome measures related to fertility and early pregnancy outcomes that were included are described in Table 2.1 Early pregnancy was defined as less than 24 weeks gestation.

Table 2.1. Primary and secondary outcome measures for fertility and early pregnancy outcomes

Primary outcomes	Secondary outcomes
• Total pregnancy rates	*Fertility outcomes* • Pregnancy rates in women with an intention to conceive in an unspecified period • Conception rates within a given period: 0 to 3 months (m), 0 to 6m, 0 to 9m, 0 to 12m, 0 to 24m, > 12m, > 36m
	Early pregnancy outcomes (< 24 weeks of gestation) • Total miscarriage rates (< 24 weeks of gestation) • 1st trimester miscarriage rates (< 12 weeks of gestation) • 2nd trimester miscarriage rates (between 12 and 24 weeks of gestation) • Ectopic pregnancy rates • Molar pregnancy rates • Termination of pregnancy rates

2.2.1.3 Types of outcome measures – Cone depth and obstetric outcomes

All outcome measures described in Table 2.2 were included in pregnancies progressing beyond 24 weeks gestation.

Table 2.2. Maternal and neonatal outcomes assessed according to cone depth

Maternal outcomes	Neonatal outcomes
• Preterm birth (spontaneous and threatened) • Premature rupture of the membranes • Chorioamnionitis • Mode of delivery • Length of labour • Induction of delivery • Oxytocin use • Haemorrhage • Analgesia • Cervical cerclage • Cervical stenosis	• Birth weight • Neonatal intensive care unit admission • Stillbirth • APGAR scores • Perinatal mortality

2.2.2 Data Extraction & Statistical Analysis

The number of events in treated and untreated women for each outcome of interest was retrieved from each study. The data were analysed separately for each treatment modality, grouped into ablative and excisional techniques and as a whole, irrespective of the type of method used. We distinguished the different untreated reference populations across studies (matched to factors in the treated group, women with CIN but no treatment, self-matching/internal controls=before vs after treatment). In studies with multiple treatment groups, we proportionally divided the 'shared' comparison group into the number of treatment groups. We treated comparisons between each treatment group and the split comparison group as independent comparisons. If a study presented data for more than one comparison group, the external comparison group of women with or without disease was used in preference to internal controls. The data were pooled using a random effects model [230]; relative risks (RR) and 95% confidence intervals (95%CI) were calculated using the Cochrane Revman 5 software. Heterogeneity among studies for each outcome was assessed using the Cochrane's Q test and I^2 statistic, which corresponds with the percentage of total variance across studies caused by heterogeneity [231, 232]. We used the Newcastle-Ottawa score to formally assess the risk of bias of non-randomised cohort studies [233], according to the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist [234]. This scoring system assesses a) cohort selection, b) comparability and c) assessment of outcomes, to give a maximum score of 9 (highest quality).

2.3 Study design, population and recruitment – Imperial College London

2.3.1 Patient recruitment

Pre-menopausal, non-pregnant women aged 18-45 years were recruited in the Colposcopy Clinic at St. Mary's Hospital, Imperial College Healthcare NHS Trust. Women were included irrespective of parity, contraceptive practices, hygiene practices (eg. douching) or history of recent sexual intercourse (<48hours). Exclusion criteria were current pregnancy, use of antibiotics or intravaginal pessaries in the last 2 weeks, HIV positive or history of previous cervical treatment. All patients underwent colposcopy on the day of recruitment performed by a British Society of Colposcopy and Cervical Pathology (BSCCP)-accredited colposcopist. Diagnostic cytology and histology samples were collected following current UK guidelines where clinically indicated. The colposcopist was not asked to take extra diagnostic samples by the research team. Extra samples were however obtained solely for research purposes as detailed below in Section 2.3.2, prior to colposcopic examination. Patients recruited into the

treatment cohort were followed up in a dedicated research clinic at the routine 6-month follow-up appointment (Figure 2.1), as per UK guidelines. No patient was asked to attend the hospital for extra visits. Histological classification was used where available, but in its absence the cytology result was used. LSIL included BNC, low grade dyskaryosis (cytology), HPV-related changes and CIN1 (histology). HSIL included high-grade dyskaryosis (cytology), CIN2 and CIN3 (histology). Cancer included squamous cell carcinoma and adenocarcinoma.

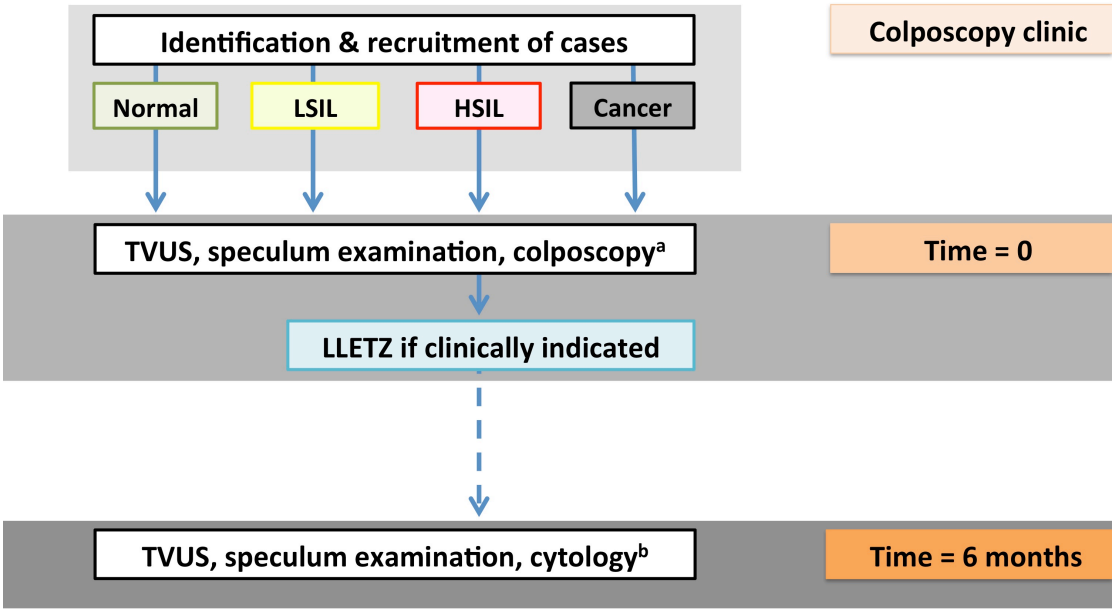


Figure 2.1. Patient recruitment schemata

[a] Colposcopy was performed with cytology +/- histology according to clinical indication and was at the discretion of the performing clinician [b] Cytology was performed as part of the 'Test of Cure' according the current UK guidelines.

2.3.2 Sample collection

A sterile, disposable, plastic speculum (Pelican Feminine Healthcare, Cardiff, UK) was inserted, without the use of any lubricant. The cervix was visualised and samples were collected in the manner and order described in Table 2.3. All samples were collected immediately prior to colposcopy, before the use of acetic acid or iodine.

Table 2.3. Sample collection techniques and immediate processing

	Sample type	Location	Collection technique and immediate storage
1	Cervical smear – cytology & HPV biomarkers	Ectocervix	Cervical cytobrush inserted into cervical os, rotated through 360 degrees five times, and tapped off into a ThinPrep® PreservCyt® vial (Hologic, Crawley, UK) and stored at 4°c.
2	Leucocytes*	Ectocervix	Cervical cytobrush (Hologic) inserted into cervical os, rotated through 360 degrees five times, and tapped off into 0.05% bovine serum albumin (BSA) in PBS.
3	Vaginal microbiome	Posterior vaginal fornix	BBL™ CultureSwab™ containing liquid Amies (Becton Dickinson, Oxford, UK) - stored on ice and transferred to -80°C within one hour of sampling.
4	Vaginal metabonome*	Posterior vaginal fornix	Rayon swab (Medical Wire & Equipment, Bath, UK) placed directly into empty amber eppendorf (VWR) and stored on ice and transferred to -80°c within one hour of sampling.
5	Antimicrobial peptides and cytokines	Posterior vaginal fornix	Rayon swab (Medical Wire & Equipment) inserted into 1.5ml eppendorf containing 750µl PBS with 4µl protease inhibitor cocktail (Sigma, Dorset, UK) and tip pressed against side of tube 20 times and then discarded. The tube was stored on ice until transport to lab and then centrifuged at 3000rpm for 10 minutes. Supernatant aspirated and transferred to a fresh 1.5ml eppendorf and transferred to -80°C within one hour of sampling.
6	High vaginal microscopy and culture*	Posterior vaginal fornix	Sent at room temperature to Imperial Healthcare NHS Trust microbiology lab for routine microscopy, culture and sensitivity analysis.

**The results of these samples are not presented in this thesis.*

2.3.3 Transvaginal ultrasonography

Transvaginal ultrasonography (TVUS) was performed immediately following vaginal sample collection and prior to colposcopy/excisional treatment using a Voluson E6 with a 5-9 MHz (RIC5-9-RS series) transvaginal probe (GE Healthcare, Zipf, Austria) to assess cervical length and volume. Women were examined in the lithotomy position with an empty bladder. The vaginal probe was slowly introduced into the vagina until a satisfactory image was obtained without exerting pressure on the cervix. Three length measurements were taken using a straight-line technique from the internal to external os and the anterior-posterior diameter. The average of three straight-line cervical length measurements and the anterior-posterior diameter, both measured in the sagittal plane (Figure 2.2) were used to calculate a two-dimensional (2D) cervical volume (=(π x AP diameter/2)^2 x average length). The cervix was then centralised in the 3D sector in the sagittal plane as the initial plane of cervical volume acquisition, with the cervix filling approximately 75% of the image capture field. The fast volume acquisition mode was used to minimize movement artifact, with an angle sweep of 90° whilst holding the probe as still as possible. This was repeated, and these two 3D volume files were stored for offline analysis. The file demonstrating the clearest cervical contour was used for subsequent analysis using Virtual Organ Computer-aided AnaLysis (VOCAL) software (4D View, Version 9.0, GE Healthcare).

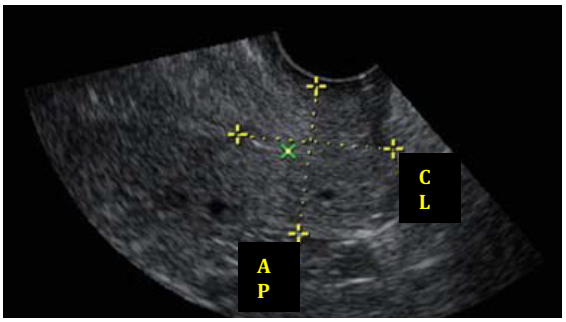


Figure 2.2. Transvaginal ultrasound scan of the uterine cervix in saggital plane demonstrating cervical length (CL) and antero-posterior diameter (AP)

2.3.3.1 Analysis of 3 dimension (3D)-cervical volume files

3D-TVUS image files were analysed using VOCAL software (4D View, Version 9.0, GE Healthcare) to determine 3D cervical volume as described by Papoutsis et al. [114]. Briefly, in the multiplanar display mode, the coronal view of the cervix was manipulated to display the mid-saggital place of the cervix demonstrating the entire cervical canal from the internal to external os. Using the manual mode, the external cervical contour was traced manually, using a rotational angle of 30°, which has been shown to be quicker, and as accurate as using smaller angles (6°, 9°, 15°) [235]. The software subsequently uses these contours to calculate cervical volume in cm^3 (Figure 2.3).

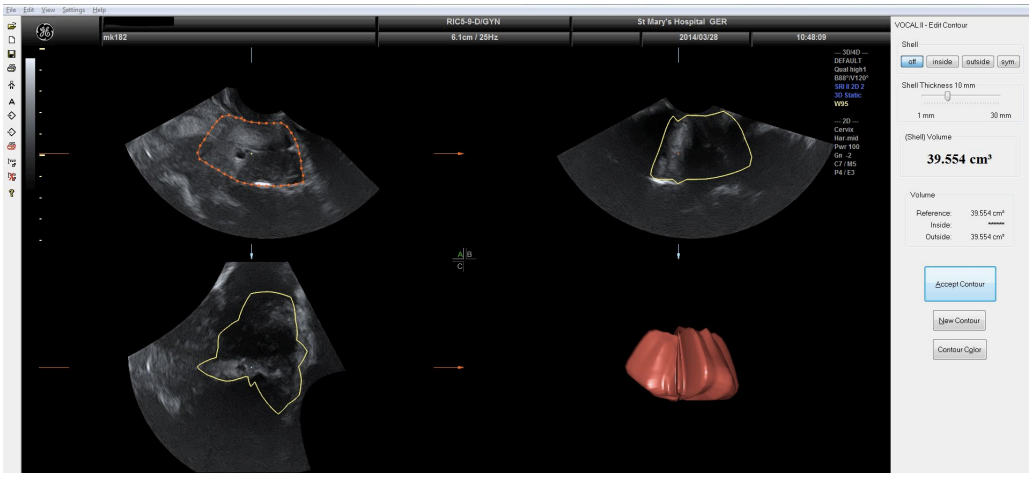


Figure 2.2. 3D transvaginal ultrasound analysis using VOCAL to estimate 3D volume

2.3.4 Calculating the volume of tissue removed

Prior to fixation in formalin and transport to the pathology laboratory for routine histopathological examination, the dimensions of the cone of tissue removed were measured using an electronic digital calliper (Precision Gold). Figure 2.4 shows the formula that was used to calculate the volume from these dimensions. Proportion of excision was calculated using the formula *(length or volume of cone ÷ length or volume of cervix immediately prior to excision) x 100*. Cervical deficit at follow-up was calculated as *(1 – (cervical length or volume at 6 months ÷ cervical length or volume prior to treatment) x 100.* A 50ml syringe was also used to calculate the volume of excised tissue by water displacement.

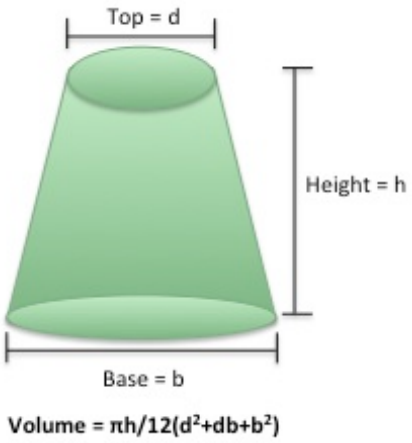


Figure 2.3. Formula for calculating the volume of excised cervical tissue

2.3.5 Study design, population and recruitment for longitudinal CIN2 study – University of California, San Francisco

The samples for the longitudinal CIN2 study (see Chapter six) were obtained as part of a collaboration with Professor Anna-Barbara Moscicki, University of California, Los Angeles. Women between the ages of 13 to 24 years of age who had an abnormal cervical cytology test result at one of 12 clinics in Kaiser Permanente, Northern California, USA between 2002 and 2007 were eligible for recruitment. Exclusion criteria included current pregnancy, previous cervical treatment for CIN, immunosuppression. Ethical approval was obtained from the

Institutional Review Boards of the University of California, San Francisco, and Kaiser Permanente, Northern California. Information regarding demographics, past medical and gynaecological history were obtained by the recruiting clinician. A sterile speculum examination was performed and high vaginal and endocervical swabs were obtained for baseline diagnosis of bacterial vaginosis, trichomonas, yeasts, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* as well as a cytobrush sample for cervical cytology, HPV testing and microbiome analysis. Routine colposcopy was performed with lesions biopsied at the discretion of the clinician, with a biopsy only mandated for entry into the study. An exit biopsy was also performed in all patients who attended their final visit and gave consent to a biopsy regardless of clinical need. In cases where there was no visible lesion at the exit visit, a biopsy was taken from the site of the previous CIN2.

HPV testing was performed using Roche LINEAR ARRAY® HPV Genotyping Test, a qualitative test to detect 37 high- and low-risk human papillomavirus genotypes (6, 11, 16,18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, and 89). Histological classification of cervical biopsies was performed by a single histopathologist.

Women with a minimum of two visits (baseline and at least one follow-up visit) were included for analysis. Progression was defined as biopsy proven CIN3 at any visit following the baseline visit. Regression was defined as two consecutive visits with negative cytology, and negative biopsy on any of these visits, if available. If there was insufficient follow-up (i.e. only one visit with negative cytology), the analysis was censored at the last visit with abnormal cytology or histology. Persistence was defined as continued CIN1 or CIN2 histology, or low- or high-grade cytology at the end of follow-up.

2.3.6 Vaginal microbiome

2.3.6.1 Bacterial DNA extraction

Bacterial DNA was extracted from the BBL™ CultureSwab™ swabs and from PreservCyt liquid-based cytology solution using a QiAmp Mini DNA kit (Qiagen, Venlo, Netherlands). For this, swabs were removed from the -80°C freezer and thawed on ice. Amies transport solution (approx. 500µl/swab) was squeezed from the transport sponge into a 2ml centrifuge tube using a 5ml syringe. For the cytobrush collected samples, PreservCyt solution was vortexed for 10 seconds and 500µl was then transferred into a 2ml centrifuge tube. Samples were

centrifuged at 8000rpm for 10 minutes to form a pellet. The Amies supernatant (swabs)/PreservCyt solution (cytobrush samples) was removed and discarded, and the pellet resuspended in 130µl phosphate buffered saline (PBS). Cells were lysed in a lysis buffer cocktail containing 50µl lysozyme (10mg/ml)(Sigma, Dorset, UK), 6µl mutanolysin (25,000U/ml) (Sigma), 3µl lysostaphin (4,000 U/ml) (Sigma), 41µl TE(10mM Tris, 1mM EDTA at pH7.4) per sample, at 37 °C for one hour. Mechanical lysis was then carried out by bead beating by adding 100mg bleached and rinsed 0.1mm zircona/silica beads to each sample and oscillating for two minutes at 2000rpm in a Tissue Lyser LT (Qiagen). After brief centrifugation to bring down the beads, the supernatant lysate was transferred to fresh 2ml centrifuge tubes and 200µl supernatant aspirated and applied to the Mini DNA kit or QiAmp cador pathogen kit (Qiagen) used according to the manufacturer's protocol. At the final step 100µl elution buffer (supplied with kit) was added to give 100µl template DNA. This was frozen at -20°C until use in further experiments.

2.3.6.2 16S rRNA Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) using 16S rRNA barcoded primer (Invitrogen, Carlsbad, CA, USA) was performed to confirm presence of bacterial DNA, as a quality control step following bacterial DNA extraction. PCR master mix (see Table 2.4) containing 2µl template DNA was used in a total of 50µl per reaction. Negative controls included PCR-grade sterile water (Sigma) and a blank extracted swab whereas purified bacterial DNA was used as a positive control. PCR was run on a BioRad Tetra 2 machine (BioRad) using the conditions detailed in Table 2.5. Eight microliters of 6x Blue/Orange Loading Dye (Promega) was added to each PCR tube and the PCR products were run on a 2% agarose gel, containing SYBR® Safe DNA gel stain (ThermoFisher Scientific, Waltham, MA USA) with 1x Tris buffer supplemented with Borate and EDTA (TBE) used as running buffer. The gel was viewed under ultraviolet (UV) light.

Table 2.4. Bacterial PCR Mastermix (Total 50µl per reaction)

	Volume per reaction
10 x AmpliTaq Gold Buffer (ThermoFisher Scientific)	5µl
$MgCl_2$ 25µM (ThermoFisher Scientific)	5µl
AmpliTaq Gold DNA Polymerase (ThermoFisher Scientific)	0.25µl
16s rRNA forward primer (Invitrogen) Sequence: 3' GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AC	1µl
16s rRNA reverse primer (Invitrogen) Sequence: 5' GCC TCC CTC GCG CCA TCA GCA CTG CAT GCT GCC TCC CGT AGG AGT	1µl
Deoxynucleotide mix (dNTP) 10mM (ThermoFisher Scientific)	1µl
PCR-grade sterile water (Sigma)	34.75µl
Template DNA (from bacterial DNA extraction)	2µl

Table 2.5. PCR conditions for 16S rRNA V1-V2 primers

Step 1. Incubate at 95°C for 5 minutes
Step 2. Incubate at 95°C for 30 Seconds
Step 3. Incubate at 55°C for 30 Seconds
Step 4. Incubate at 72°C for 1 minute 30 Seconds
Step 5. Cycle to step 2 for 30 more times
Step 6. Incubate at 72°C for 7 minutes
Step 7. Incubate at 4°C till removed

2.3.6.3 Sequencing of 16s rRNA gene amplicons

Microbial profiling using a MiSeq platform (Illumina, San Diego, CA, USA) was performed commercially (Research and Testing, Lubbock, TX, USA) [152]. The forward primer consisted of an Illumina i5 adapter (5'-3') (AAT GAT ACG GCG ACC ACC GAG ATC TAC AC), an 8-10bp barcode, a primer pad, and the 28F-GAG TTT GAT CNT GGC TCA G primer [236], whilst the reverse fusion primer was constructed with (5'-3') the Illumina i7 adapter (CAA GCA GAA GAC GGC ATA CGA GAT), an 8-10bp barcode, a primer pad, and the reverse primer (519R-GTN TTA CNG CGG CKG CTG) [237]. PCR amplification was performed in 25 µl reaction volumes with Qiagen HotStar *Taq* master mix (Qiagen Inc, Valencia, California), 1µl of each 5uM primer, and 1µl of template on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) using the following thermal profile: 95°C for 5 min, 35 cycles of 94°C for 30 sec, 54°C for 40 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min and a 4°C hold. Products were visualised with eGels (Life Technologies, Grand Island, New York) and pooled in equimolar amounts. Each pool was size selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) in a 0.7 ratio for both rounds before being quantified using the Qubit

2.0 Fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, San Diego, California) 2x300 flow cell at 10pM.

2.3.6.4 Processing of sequence data

Sequence data was analysed in Mothur using the MiSeq SOP Pipeline [238]. Sequence reads were quality checked and normalised to the lowest number of reads. Singleton operational taxonomic units (OTUs) and OTUs<10 reads in any sample were collated into OTU_singletons and OTU_rare phylotypes respectively, to maintain normalisation and to minimise artefacts. OTUs were defined using a cut off value of 97% and resulting data analysed using the Vegan package in R for assessment of microbial composition and diversity (R Development Core Team 2008). OTU taxonomies (from Phylum to Genus) were determined using the RDP MultiClassifier script to generate the RDP taxonomy [239] while species level taxonomies of the OTUs were determined using the USEARCH algorithm combined with the cultured representatives from the RDP database [240]. Alpha and beta indices were calculated from these datasets with Mothur and R using the Vegan package.

2.3.6.5 Statistical Analysis of Sequencing Data

Analysis of statistical differences between the vaginal microbiome of patient groups was performed using the Statistical Analysis of Metagenomic Profiles (STAMP) package (Version 8, OxMetrics, Timberlake, London, UK) [241]. Data were subjected to multivariate analysis using principal components analysis (PCA) and hierarchical clustering analysis (HCA) by nearest neighbour linkage with a clustering density threshold of 0.75. Richness (species observed and Chao1 [242]) and diversity (Inverse Simpson and non-parametric Shannon) indices were calculated for each patient group and compared using a non-parametric ANOVA test using PRISM (Version 7, GraphPad, LaJolla, CA, USA).

Linear discriminant analysis (LDA) effect size (LEfSe) modeling was used to identify biomarkers based on different clinical classification, according to relative taxonomic abundance through all taxonomic levels [243]. This analysis was performed using taxonomic relative abundance, with per-sample normalisation and default settings for alpha values (0.05) for the factorial Kruskal-Wallis test among classes and pairwise Wilcoxon test between subclasses. A logarithmic LDA score greater than two was used to determine discriminative features.

Multivariate dissimilarity analysis was performed using the Vegan package in R. A Bray-Curtis dissimilarity index was constructed using the *vegdist* function. Non-metric multidimensional scaling (NMDS) was further performed using species assignments, and a PERMANOVA was used to perform multivariate ANOVA based on dissimilarities using the *adonis* function.

2.3.6.6 Quantitative PCR (qPCR)

2.3.6.6.1 16s rRNA qPCR

Quantitative PCR (qPCR) was carried out for quantification of 16S rRNA gene copy number in order to estimate the bacterial load collected by different sampling techniques and to determine the volume of PreservCyt that should be used for bacterial DNA sequencing described in Chapters five and six. Real-time qPCR was performed with universal BactQUANT 16S rRNA primers (Forward primer: 5'-CCT ACG GGA GGC AGC A, Reverse primer: 5'-GGA CTA CCG GGT ATC TAA TC) (Sigma) with the FAM labeled BactQUANT probe ((6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ)) [244] on the Applied Biosciences StepOne machine (Thermo Fisher Scientific, Ashford, UK) with StepOne software (Version 2.3, Life Technologies). Samples were run in duplicate. All apparatus and plasticware were exposed to UV light for 15 minutes prior to use in order to destroy any DNA contaminants.

Lyophilized Genomic DNA from *Escherichia coli (E.Coli)* Strain B (Sigma, Dorset, UK) was serially diluted in diethylpyrocarbonate (DEPC) water to make a ten-fold standard curve to obtain concentrations from $3x10^5$ to $3x10^0$ copies of 16S gene to establish a standard curve from 300,000 to 3 copies (cps) of 16S rRNA according to Table 2.6.

Table 2.6. Dilutions for E.Coli standard curve

Standards (S)	S5 (300,000 cps)	S4 (30,000 cps)	S3 (3000 cps)	S2 (300 cps)	S1 (30 cps)	S0 (3 cps)
Recipe	85.71µl (Stock E.coli) + 14.29µl H_2O	10µl S5 + 90µl H_2O	10µl S4 + 90µl H_2O	10µl S3 + 90µl H_2O	10µl S2 + 90µl H_2O	10µl S1 + 90µl H_2O

Fifteen microliters of Mastermix (Table 2.7) was added into each well of the optical 96-well reaction plate (Starlab), followed by 5µl of standards or sample. DEPC water used as a negative control. The plate was sealed and spun for 20 seconds in a plate centrifuge immediately prior to StepOnePlus real-time PCR system (Applied Biosystems, ThermoFisher Scientific)) using the PCR cycle conditions as detailed in Table 2.8.

Table 2.7. 16s rRNA qPCR mastermix (Total 20µl per reaction)

	Volume per reaction
Diethylpyrocarbonate (DEPC) water (ThermoFisher Scientific)	4.195µl
Platinum Supermix UDG (including ROX) (ThermoFisher Scientific)	0.04µl
BactQuant forward primer 100 µM (0.025 µmol synthesis scale, desalted) (Sigma) Sequence: 5'-CCT ACG GGA GGC AGC A	0.36µl
BactQuant reverse primer 100 µM (0.025 µmol synthesis scale, desalted) (Sigma) Sequence: 5'-GGA CTA CCG GGT ATC TAA TC	0.36µl
BactQuant probe (LifeTech, 6000 pmol scale) Sequence: (6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ)	0.045µl

Table 2.8. qPCR conditions for 16s rRNA primers

Step 1. Incubate at 50°C for 2 minutes
Step 2. Incubate at 95°C for 10seconds
Step 3. Incubate at 95°C for 15 seconds
Step 4. Incubate at 60°C for 60 seconds
Step 5. Cycle to step 3 for 40 more times

2.3.6.6.2 *Gardnerella vaginalis* qPCR

Real-time qPCR was performed with primers specific to *G.vaginalis* (Forward primer: 5'-GGAAACGGGTGGTAATGCTGG, Reverse primer: 5'-CGAAGCCTAGGTGGGCCATT) [245] using a SYBR green-based assay on the Applied Biosciences StepOne machine (Thermo Fisher Scientific) with StepOne software (Version 2.3, Life Technologies). Samples were run in duplicate. All apparatus and plastic ware were exposed to UV light for 15 minutes prior to use in order to destroy any DNA contaminants. Pure cultured *G.vaginalis* (GVI) was serially diluted in diethylpyrocarbonate (DEPC) water to make a ten-fold standard curve to obtain concentrations from $3x10^5$ to $3x10^1$ copies of 16S gene to establish a standard curve from 300,000 to 3 copies (cps) of 16S rRNA according to Table 2.9.

Table 2.9. Dilutions for G.vaginalis standard curve

Standards (S)	**S5 (300,000 cps)**	**S4 (30,000 cps)**	**S3 (3000 cps)**	**S2 (300 cps)**	**S1 (30 cps)**
Recipe	21.43µl (Stock GVI) + 3.58µl H_2O	2.5µl S5 + 22.5µl H_2O	2.5µl S4 + 22.5µl H_2O	2.5µl S3 + 22.5µl H_2O	2.5µl S2 + 22.5µl H_2O

Fifteen microliters of Mastermix (Table 2.10) was added into each well of the optical 96-well reaction plate (Starlab), followed by 5µl of standards or sample. DEPC water used as a negative control. The plate was sealed and spun for 20 seconds in a plate centrifuge immediately prior to StepOnePlus real-time PCR system (Applied Biosystems, ThermoFisher Scientific)) using the PCR cycle conditions as detailed in Table 2.11.

Table 2.10. G.vaginalis qPCR mastermix (Total 20µl per reaction)

	Volume per reaction
Diethylpyrocarbonate (DEPC) water (ThermoFisher Scientific)	4.48µl
SYBR mix	10µl
ROX	0.2µl
G. vaginalis forward primer Sequence: 5'-GGA AAC GGG TGG TAA TGC TGG (Invitrogen)	0.16µl
G. vaginalis reverse primer Sequence: 5'-CGA AGC CTA GGT GGG CCA TT (Invitrogen)	0.16µl

Table 2.11. qPCR conditions for G.vaginalis primers

Step 1. Incubate at 95°C for 2 minutes Step 2. Incubate at 95°C for 60 seconds Step 3. Incubate at 65°C for 60 seconds Step 4. Cycle to step 2 for 40 more times Step 5. Incubate at 72°C for 60 seconds

2.3.6.7 Human papilloma virus (HPV) testing

Human papilloma virus testing was performed by the Edinburgh HPV Research laboratory, using the Abbott RealTime High Risk (HR) HPV assay on Abbott M2000 platform; a clinically validated *in vitro* polymerase chain reaction (PCR) assay with identification of HPV-16, -18 and 12 other HR HPV subtypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) [246].

2.3.6.8 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunsorbent assays (ELISAs) were used to quantify the amount of target protein in a solution, through comparison with a standard curve created from samples containing known concentrations. The assays performed in this project were all sandwich ELISAs, which uses plates coated with an antibody raised against one epitope of the substance to be measured. The sample or the standard is then added, and the substance binds to the antibody. A detection antibody is then added, which is raised against a second epitope and labelled with biotin. Streptavidin peroxidase is then added. Streptavidin complexes strongly

with the biotin and the peroxidase can be detected by addition of substrate (Tetramethyl benzidine, urea hydrogen peroxide, sodium acetate). The reaction is stopped with sulphuric acid solution and absorbance measured by spectrophotometry.

For the analysis, supernatants derived from swabs were thawed on ice and centrifuged at 700 x G for one minute and passed through a sterile 0.2µM filter to remove debris. Total protein content was determined using *DC* Protein Assay Kit (Bio-Rad, Hemel Hempstead, UK); an alkaline copper tartrate and folin-based colourimetric assay, according to the manufacturer's instructions. Absorbance was read at 405nm.

Human β Defensin-1 (HBD-1) levels were determined using a Human BD-1 enzyme-linked immunosorbent assay (ELISA) Development Kit (Peprotech, London, UK) according to the manufacturer's protocol. Briefly, 96-well plates were coated with goat polyclonal anti-HBD1 (100µl/well, 25µg/ml) diluted in PBS, and incubated at room temperature overnight. Non-specific binding was blocked by the addition of 200µl per well of blocking buffer (0.01% Tween-20, 0.1%BSA) and a further incubation for one hour at room temperature. Plates were washed four times with wash buffer. Standards of recombinant human HBD1 were serially diluted in ELISA buffer containing Tween-20, from 1000pg/ml to 1.95pg/ml. 100µl of standard or sample were applied in duplicate, and incubated at room temperature for two hours. The plates were washed four times, before application of biotin-labelled detection antibody diluted in ELISA buffer containing Tween-20 (100µl/well, 5µg/ml). Plates were incubated for two hours at room temperature, before being washed another four times. Streptavidin peroxidase at 0.02IU was added to each well (100µl/well), and incubated for 15 minutes. Plates were thoroughly washed four times, and 200µl per well of substrate added. Colour was allowed to develop for approximately five minutes, at which point plates were read at an optical density of 450nm within 30 minutes of quenching.

Secretory leukocyte proteinase inhibitor (SLPI) levels were determined using a human SLPI ELISA kit (Hycult Biotech, Uden, The Netherlands) according to the manufacturer's protocol at 150x dilution. Briefly samples and standards (100µl, range 5000 to 20pg/ml) were diluted in provided diluent, applied to the pre-coated supplied plate and incubated at 37°C for one hour. Plates were rinsed with wash buffer, and detection antibody (100µl) added. Plates were incubated for two hours on an orbital plate shaker at room temperature, and then washed again. Streptavidin peroxidase was applied to each well (100µl, 0.02IU) and incubated for 15

minutes. 100µl of the provided substrate was then added after a further wash, and the reaction was quenched with stop solution (100µl).

2.3.6.9 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was used to demonstrate the localization of HBD-1 and SLPI in cervical excisional biopsies. Twelve women who underwent repeated conisation from the prospective cohort were identified. Slides suitable for IHC were cut from the diagnostic paraffin-embedded blocks from the histological specimen from the first and second excisional treatment.

Slides were deparaffinised using the following wash steps for three minutes in each solution: xylene, xylene, 100% ethanol, 100% ethanol, 95% ethanol, 70% ethanol, distilled water. Slides were then rinsed in running tap water. Methylene bridges may form during tissue fixation which causing protein cross-linking and can mask antigenic sites. Heat-induced antigen retrieval was performed using sodium citrate at pH six. Slides were placed into a plastic dish and submerged in sodium citrate buffer. This was placed in an 850W domestic microwave on full-power and brought to the boil, and boiled for 20 minutes. The slides were then slowly cooled in running tap water for 10 minutes. A horseradish peroxidase (HRP) – 3,3' Diaminobenzidine (DAB) Cell and Tissue Staining Kit (R & D Systems) designed to detect primary IgG antibodies was used for staining and detection. The principle of this kit is firstly antigen detection based on formation of an Avidin-Biotin Complex (ABC) with primary antibodies followed by visualization based on enzymatic conversion of a DAB, a chromogenic substrate into a coloured brown precipitate by HRP which is then be viewed using light microscopy. This kit was used according to the manufacturer's protocol. Briefly, following antigen retrieval samples were covered with three drops of the Peroxidase Blocking Reagent for five minutes and rinsed in PBS for five minutes. Slides were incubated with three drops of Serum Blocking Reagent for 15 minutes, and excess reagent was drained before application of Avidin Blocking Reagent for 15 minutes. PBS was used to rinse and wash slides for five minutes. Three drops of Biotin Blocking Reagent were applied to slides for 15 minutes which were then rinsed and washed for five minutes in PBS. Primary antibodies were applied and incubated at 4°C overnight. HBD-1 was used at 1:10 dilution and SLPI at 1:5 dilution.

The following day the slides were washed three times in PBS for 15 minutes per wash. Excess PBS was removed and three drops of Biotinylated Secondary Antibody was applied for 60

minutes. Adjust the incubation time depending on the thickness of the section (the thicker the section, the longer the incubation time). Again slides were washed three times in PBS before three drops HSS-HRP were added for 30 minutes. Slides were then washed three times in PBS for two minutes per wash. Excess PBS was removed and DAB Chromogen in DAB Chromogen buffer was applied for 20 minutes. Slides were washed in distilled water and counterstained using Meyer's Haematoxylin, and mounted using Permount. A cover slip was applied and once dry the samples were visualised underneath a light microscope.

2.3.6.10 Cytokines

Cytokine levels in cervicovaginal secretions were determined using the Magnetic Luminex Screening Assay multiplex kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The kit uses colour-coded magnetic microparticles, which are pre-coated with the analyte of interest; interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10 and tumour necrosis factor-alpha (TNF-α).

Sample supernatant was thawed slowly on ice and centrifuged at 3000rpm for one minute and passed through a sterile 0.2µM filter to remove debris. Fifty microliters of diluted microparticle cocktail was added to each well of the supplied 96 well plate, followed by 50µl standard or sample. A standard curve was constructed using the supplied Human Standard Cocktail, serially diluted in the supplied Calibrator Diluent RD6-52. The plate was covered with a foil plate sealer to prevent photobleaching, and incubated at room temperature for two hours on a horizontal orbital microplate shaker set to 800rpm, to allow analyte binding by the antibody on the microparticles. Using a magnetic plate holding device (Bio-Rad), the plate was washed three times using 100µl wash buffer per well and 50µl biotinylated antibody cocktail was added to each well. The plate was incubated for one hour at room temperature on the microplate shaker, washed three times using 100µl wash buffer to wash away unbound antibody, and 50µl streptavidin-phycoerthyrin (-PE) conjugate added to each well to bind the biotinylated antibody for detection. After 30 mins incubation at room temperature on the microplate shaker, the plate was washed three times using 100µl wash buffer per well to remove unbound streptavidin-PE and 100µl wash buffer was added to each well to resuspend the microparticles for analysis using the MAGPIX Analyzer (Luminex Corporation, s-Hertogenbosch, Netherlands). This machine contains a magnet to capture the microparticles in a monolayer and two light emitting diodes (LEDs) to illuminate the beads; one of which

identifies the analyte of interest and the second determines the magnitude of PE-derived signal.

2.3.6.11 Statistical analysis

Statistical analysis was performed with Graph-Pad Prism v5 (GraphPad Software, San Diego, CA). The correct statistical test was carried out depending on normality and the number of groups analysed. P value <0.05 was considered significant.

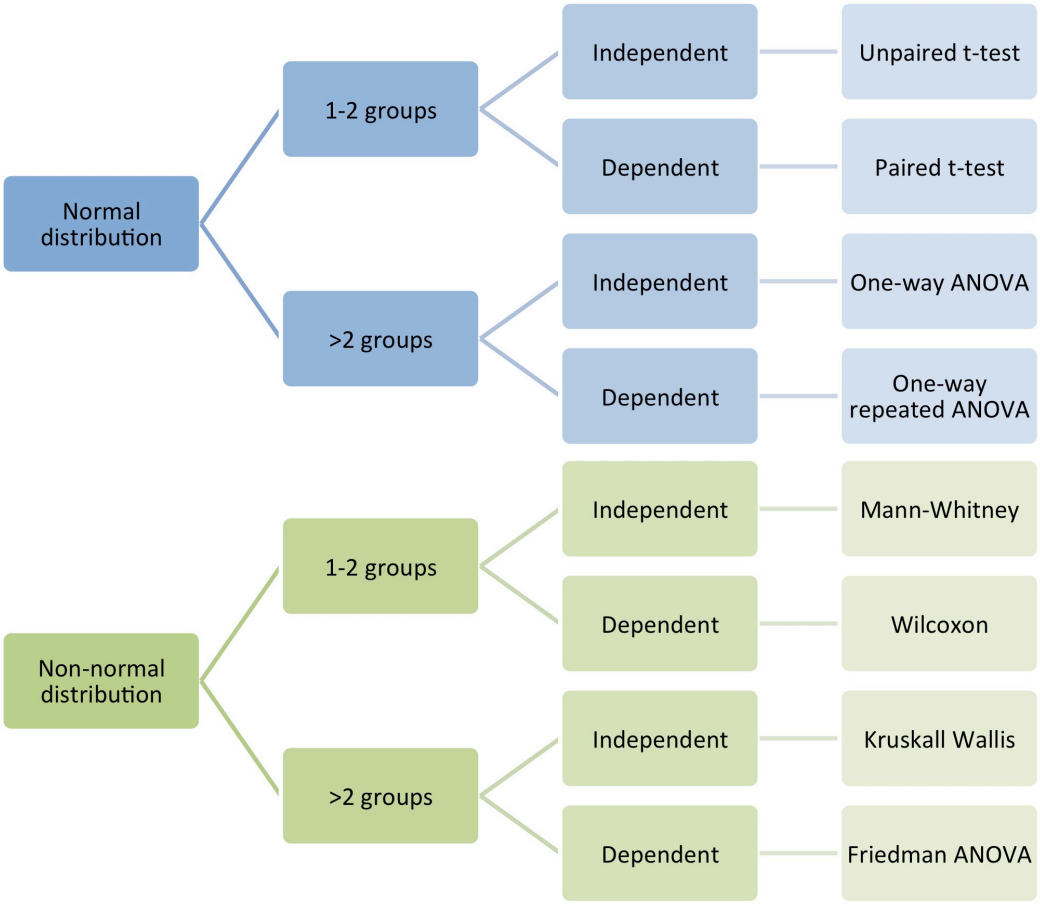


Figure 2.4. Statistical analysis

CHAPTER 3. Cervical intraepithelial neoplasia disease severity is associated with increased vaginal microbiome diversity

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3 Cervical intraepithelial disease severity is associated with increased vaginal microbiome diversity

3.1 Background

Persistent infection with a high-risk oncogenic Human Papillomavirus (HPV) subtypes, most commonly 16 and 18, is a necessary, although not sufficient, condition for development of invasive cervical cancer (ICC) and its precancerous precursor; cervical intra-epithelial neoplasia (CIN) [247]. Although HPV infection is very common in sexually-active women [248], the majority of infections are transient [249]. Only a small proportion of women infected with the virus goes on to develop clinically significant pre-invasive lesions and, if not treated, invasive malignant disease. Mechanisms of persistence of HPV infection are not well understood.

The vaginal microenvironment plays an important role in reproductive health. Commensal vaginal *Lactobacillus* spp. are thought to defend against pathogens and sexually transmitted infections [250] through maintenance of a hostile pH [182], production of species-specific metabolites, bacteriocins and through adherence to mucous and disruption of biofilms [251-254]. Next generation sequencing (NGS) based studies have facilitated detailed characterisation of the "healthy" vaginal microbiome and shown that 5 major community-state types (CSTs) exist; CST I, II, III and V are dominated by *Lactobacillus crispatus, L. gasseri, L. iners* and *L. jensenii* respectively, whereas CST IV has characteristically low numbers of *Lactobacillus* spp. and increased diversity of anaerobic bacteria [153]. Longitudinal studies of the vaginal microbiome using NGS indicates that bacterial community structure is dynamic and hormonally influenced with a propensity to become less stable during menstruation [186] and conversely more stable and less diverse during normal pregnancy [152, 158]. The stability and composition of the vaginal microbiome may play an important role in determining host innate immune response and susceptibility to infection. Bacterial vaginosis (BV), a condition characterised by *Lactobacillus* spp. depletion, overgrowth of anaerobic species, and higher vaginal pH has been associated with increased transmission rates of sexually-transmitted infections [255] and human immunodeficiency virus (HIV) [256]. Conversely, it has recently been reported that viral infection of the cervix during murine pregnancy increases susceptibility of ascending vaginal bacterial infection through sensitisation and priming of the host innate immune system [119].

Relatively little is known about the mechanisms associated with clearance or persistence of HPV infection. Along with higher rates of HPV infection, BV has been associated with delayed clearance of the virus and with CIN, suggesting that a diverse, *Lactobacillus*-species depleted microbiome may play a mechanistic role [166-169]. A recent study of 68 HPV-discordant monozygotic female Korean twins using NGS showed that HPV-positive twins had lower levels of *Lactobacillus* spp. and increased counts of *Fusobacteria* and *Sneathia* spp. compared to their HPV-negative twins [201]. Consistent with these findings, analysis of vaginal swabs collected longitudinally for 16 weeks from 32 sexually active women found that a *Lactobacillus* spp.-depleted, *Atopobium* spp. enriched (CST IV) community structure is associated with slowest regression of HPV whereas a *Lactobacillus gasseri*-dominated microbiome (CST II) is associated with the most rapid regression rates for HPV [202].

While there is evidence of an association between vaginal microbiome structure and HPV infection, the potential relationship between the vaginal microbiota and CIN disease progression has yet to be investigated.

3.2 Aims

- Characterise the vaginal microbiome structure and diversity in women with HPV infection and/or CIN to assess any correlation with disease presence and severity and compare to healthy controls
- Identify possible microbiological markers of high-grade CIN or HPV infection.

3.3 Results

One hundred and sixty-nine women were included in the study carried out at Imperial College London and were classified into 4 groups; normal (n=20), low-grade squamous intraepithelial lesion (LSIL) (n=52), high-grade squamous intraepithelial lesion (HSIL) (n=92) and invasive cervical cancer (ICC) (n=5) according to the criteria in section 2.3.1.

Table 3.1. Patient metadata

	Normal (n=20)	**LSIL (n=52)**	**HSIL (n=92)**	**Cancer (n=5)**	**Total (n=169)**	***P* value**	**Q value**
Age, years						NS	NS
Mean (SD, range)	31 (5.19, 24-43)	32 (5.66, 23-45)	30 (4.65, 25-43)	34 (5.29, 27-41)	31 (5.08, 23-45)		
Ethnicity , n/N (%)						0.04	0.20
Caucasian	12/20 (60)	46/52 (88)	78/92 (85)	4/5 (80)	140/169 (83)		
Asian	2/20 (10)	3/52 (6)	9/92 (10)	0/5 (0)	14/169 (8)		
Black	6/20 (30)	3/52 (6)	5/92 (5)	1/5 (20)	15/169 (9)		
Parity, n/N (%)						0.83	1.00
Nulliparous	16/20 (80)	38/52 (73)	68/92 (74)	3/5 (60)	125/169 (74)		
Parous	4/20 (20)	14/52 (27)	24/92 (26)	2/5 (40)	44/169 (26)		
Smoking status, n/N (%)						0.07	0.37
Current smoker	1/20 (5)	11/52 (21)	28/92 (30)	1/5 (20)	41/169 (24)		
Non-smoker	19/20 (95)	41/52 (79)	64/92 (70)	4/5 (80)	128/169 (76)		
Phase of menstrual cycle, n/N (%)						0.13	0.52
Luteal	7/20 (35)	27/52 (52)	42/92 (46)	5/5 (100)	81/169 (48)		
Follicular	11/20 (55)	21/52 (40)	47/92 (51)	0/5 (0)	79/169 (47)		
Unknown	2/20 (10)	3/52 (8)	3/92 (3)	0/5 (0)	8/169 (5)		
Contraception, n/N (%)						0.02	0.12
Nil	15/20 (75)	18/52 (35)	29/92 (32)	1/5 (20)	63/169 (37)		
Condoms	1/20 (5)	10/52 (19)	16/92 (17)	0/5 (0)	27/169 (16)		
COCP	3/20 (15)	11/52 (21)	35/92 (38)	2/5 (40)	51/169 (30)		
POP	0/20 (0)	4/52 (8)	4/92 (4)	0/5 (0)	8/169 (5)		
Copper IUD	0/20 (0)	2/52 (4)	2/92 (2)	1/5 (20)	5/169 (3)		
Mirena IUS	1/20 (5)	1/52 (2)	3/92 (3)	1/5 (20)	6/169 (4)		
Vaginal Ring	0/20 (0)	0/52 (0)	2/92 (2)	0/5 (0)	2/169 (1)		
Contraceptive implant	0/20 (0)	4/52 (8)	1/92 (1)	0/5 (0)	5/169 (3)		
Contraceptive injection	0/20 (0)	2/52 (4)	0/92 (0)	0/5 (0)	2/169 (1)		
Time since last intercourse, n/N (%)						0.20	0.60
>48 hours	16/20 (80)	49/52 (94)	77/92 (84)	5/5 (100)	147/169 (87)		
<48 hours	4/20 (20)	3/52 (6)	14/92 (16)	0/5 (0)	21/169 (13)		

Classification was based on histology where available; in all HSIL and cancer cases (100%), and for 69% (36/52) and 5% (1/20) of LSIL and normal cases, respectively. Cytology was used in the remainder of cases. Patient metadata were analysed (age, ethnicity, parity, smoking status, phase of menstrual cycle, contraception and time since last intercourse) and showed no difference in key parameters across the four disease-groups after adjustment for multiple hypothesis testing (Table 3.1). There was equal distribution in the samples collected at the follicular or luteal phase of the cycle and the rate of women that had intercourse within 48 hours from sample collection was comparable.

3.3.1 The structure of the vaginal microbiome correlates to the disease severity

In total 8,409,192 reads were obtained from 169 samples with an average number of reads per sample of 49,759 and the mean and median read lengths of 513 and 520 bp respectively. To avoid sequencing bias, operational taxonomic units (OTUs) were randomly sub-sampled to the lowest read count of 183, which retained 97% of OTU counts and still provided coverage of >96% for all samples. Following removal of singletons and rare OTUs, a total of 49 taxa were identified in the vaginal microbiome of the study cohort.

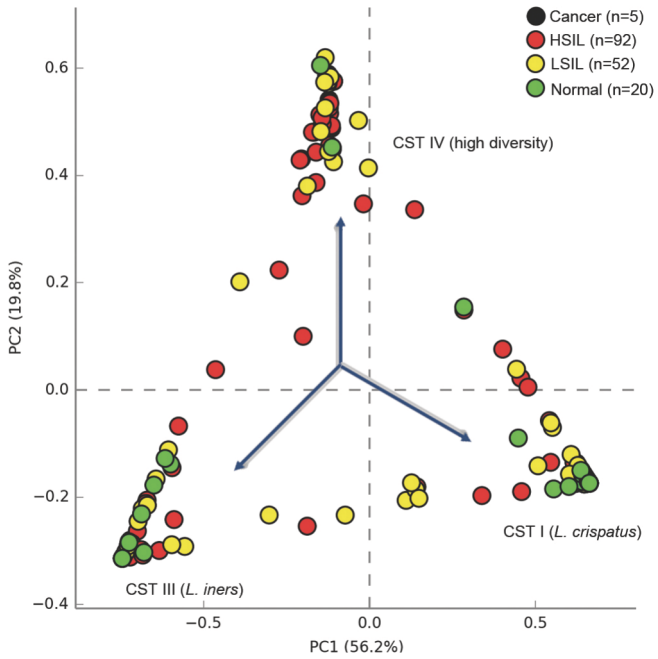


Figure 3.1. Bacterial species diversity in study cohort and controls

Cancer: Black; High-grade squamous intra-epithelial lesions (HSIL): Red; Low-grade squamous intra-epithelial lesions (LSIL): Yellow; Normal: Green.

Initial assessment of vaginal community structure was performed using principal component analysis (PCA) of species sequence data in the context of disease grade (normal, LSIL, HSIL and ICC) (Figure 3.1). Three major clusters were identified which represented samples dominated by either *L.crispatus, L. iners* or samples depleted of *Lactobacillus* spp. with higher diversity.

Hierarchical clustering analysis (HCA) of the sequence data using nearest neighbour linkage at

species level (Figure 3.2) identified 5 major clusters that exhibited bacterial community structure consistent with previously described vaginal microbiome community state types (CSTs); CST I: *L. crispatus*-dominated, CST II: *L. gasseri*-dominated, CST III: *L. iners*-dominated, CST IV: *Lactobacillus*-depleted and CST V: *L. jensenii*-dominated [153].

The rates and frequency of the different CSTs (I, II, III, IV and V) were compared between CIN disease severities and healthy controls (Figure 3.2, Table 3.2). CST I was the most frequent CST in the study cohort (70/169, 41% of all patients), followed by CST III (47/169, 28%), CST IV (40/169, 24%), CST V (7/169, 4%) and CST II (5/169, 3%). Higher rates of CST IV (*Lactobacillus*-depleted, high diversity) were associated with increasing disease severity with CST IV observed twice as frequently in women with LSIL, three times as frequently in women with HSIL and four times as frequently in women with ICC when compared to disease free controls (normal= 2/20, 10%; LSIL= 11/52, 21%; HSIL= 25/92, 27%; ICC = 2/5, 40%). Conversely, frequency of CST I (*Lactobacillus crispatus*-dominant) was lower with increasing disease severity (normal=10/20, 50%; LSIL=22/52, 42%; HSIL=37/92, 40%; cancer=1/5, 20%). The number of ICC cases was small for any valid conclusion. Although the results of the analysis did not attain significance given the modest sample size, there appears to be a correlation of CST IV and increasing disease severity.

A similar distribution of CST IV was observed when women with HPV/Atypical squamous cells of undetermined significance (ASCUS) and LSIL changes were analysed as two separate groups (normal= 2/20, 10%; ASCUS= 5/26, 19%; LSIL= 6/26, 23%; HSIL= 25/92, 27%; cancer= 2/5, 40%) (Figure 3.2, Table 3.2). These analyses are suggestive of association between CST IV and increasing disease severity, and are consistent with the expected direction of effect, but do not attain significance due to modest sample-size.

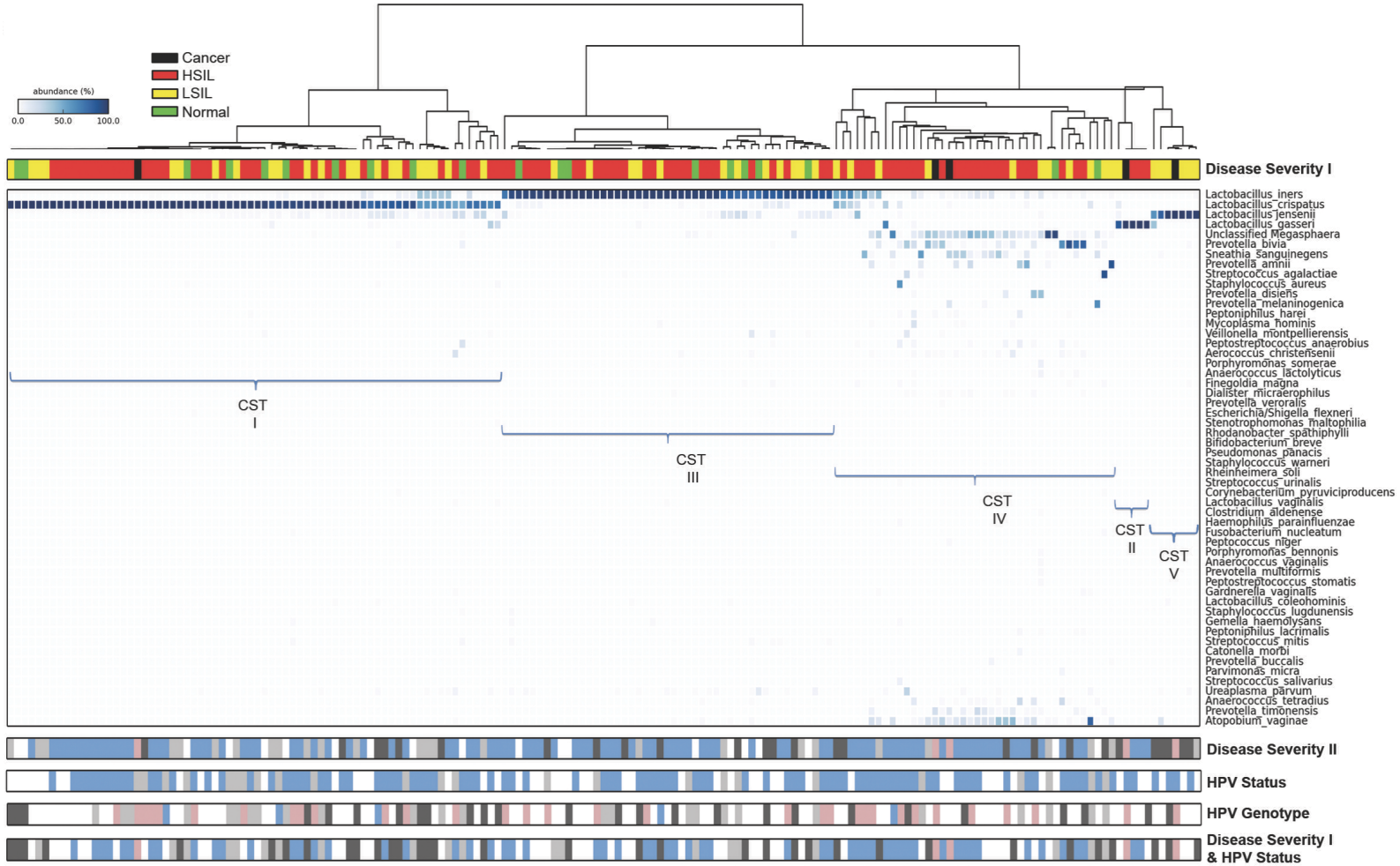


Figure 3.2. Vaginal microbiome composition according to disease and HPV status

DISEASE SEVERITY I - Normal: Green; Low-grade squamous intra-epithelial lesions (LSIL): Yellow; High-grade squamous intra-epithelial lesions (HSIL): Red; Cancer: Black

DISEASE SEVERITY II - Normal: white; ASCUS: light grey; LSIL: dark grey; HSIL: blue; Cancer: pink

HPV STATUS - HPV negative: light grey; HPV positive: blue; Unknown: white

HPV GENOTYPE - HPV negative: light grey; HPV16: pink; HPV18: blue; Other HR-HPV: dark grey; Unknown: white

DISEASE SEVERITY I & HPV STATUS - LSIL/Normal HPV negative: light grey; LSIL/Normal HPV positive: dark grey; HSIL/HPV positive: blue; Cancer: pink; unknown/other: white

ASCUS: atypical squamous cells of undetermined significance; CST: community state type; HPV: Human Papillomavirus; HR-HPV: high-risk HPV; HSIL: High-grade squamous intra-epithelial lesion; ICC: invasive cervical cancer; LSIL: Low-grade squamous intra-epithelial lesion

Table 3.2. Rates of each CST according to disease severity and HPV status/genotype

	CST I *L. crispatus* **n/N (%)**	**CST II** *L. gasseri* **n/N (%)**	**CST III** *L. iners* **n/N (%)**	**CST IV** *Mixed* **n/N (%)**	**CST V** *L. jensenii* **n/N (%)**	**TOTAL n/N (%)**
DISEASE SEVERITY (A)						
Normal	10/20 (50)	0/20 (0)	8/20 (40)	2/20 (10)	0/20 (0)	20/20 (100)
LSIL	22/52 (42)	1/52 (2)	12/52 (23)	11/52 (21)	6/52 (12)	52/52 (100)
HSIL	37/92 (40)	3/92 (3)	27/92 (29)	25/92 (27)	0/92 (0)	92/92 (100)
Cancer	1/5 (20)	1/5 (20)	0/5 (0)	2/5 (40)	1/5 (20)	5/55 (100)
Total	*70/169 (41)*	*5/169 (3)*	*47/169 (28)*	*40/169 (24)*	*7/169 (4)*	*169/169* (100)
***P* value**[1]	0.30	0.12	0.38	0.06	0.47	-
Q value[1]	0.47	0.46	0.47	0.32	0.47	-
DISEASE SEVERITY (B) (ASCUS/LSIL separate)[2]						
Normal	10/20 (50)	0/20 (0)	8/20 (40)	2/20 (10)	0/20 (0)	20/20 (100)
ASCUS	16/26 (62)	0/26 (0)	4/26 (15)	5/26 (19)	1/26 (4)	26/26 (100)
LSIL	6/26 (23)	1/26 (4)	8/26 (31)	6/26 (23)	5/26 (19)	26/26 (100)
HSIL	37/92 (40)	3/92 (3)	27/92 (29)	25/92 (27)	0/92 (0)	92/92 (100)
Cancer	1/5 (20)	1/5 (20)	0/5 (0)	2/5 (40)	1/5 (20)	5/5 (100)
Total	*70/169 (41)*	*5/169 (3)*	*47/169 (28)*	*40/169 (24)*	*7/169 (4)*	*169/169* (100)
***P* value**[1]	0.10	0.11	0.65	0.06	0.87	-
Q value[1]	0.33	0.33	0.87	0.32	0.87	-
HPV STATUS						
Negative	13/24 (54)	0/24 (0)	6/24 (25)	5/24 (21)	0/24 (0)	24/24 (100)
Positive	38/93 (41)	2/93 (2)	25/93 (27)	26/93 (28)	2/93 (2)	93/93 (100)
Total	*51/117 (44)*	*2/117 (2)*	*31/117 (26)*	*31/117 (26)*	*2/117 (2)*	*117/117* (100)
***P* value**[3]	0.26	1.00	1.00	0.61	1.00	-
Q value[3]	1.00	1.00	1.00	1.00	1.00	-
HPV GENOTYPE						
HPV-16	13/31 (42)	1/31 (3)	8/31 (26)	9/31 (29)	0/31 (0)	31/31 (100)
HPV-18	3/5 (60)	0/5 (0)	1/5 (20)	1/5 (20)	0/5 (0)	5/5 (100)
Other HR-HPV subtype	12/26 (46)	1/26 (4)	7/26 (27)	5/26 (19)	1/26 (4)	26/26 (100)
Total	*28/62 (45)*	*2/62 (3)*	*16/62 (26)*	*15/62 (24)*	*1/62 (1)*	*62/62* (100)
***P* value**[3]	0.74	1.00	1.00	0.81	0.50	-
Q value[3]	1.00	1.00	1.00	1.00	1.00	-
DISEASE SEVERITY and HPV STATUS						
Normal/LSIL, HPV neg	11/20 (55)	0/20 (0)	6/20 (30)	3/20 (15)	0/20 (0)	20/20 (100)
Normal/LSIL, HPV pos	17/34 (50)	0/34 (0)	7/34 (21)	7/34 (21)	3/34 (8)	334/4 (100)
HSIL, HPV pos	21/58 (36)	2/58 (3)	16/58 (28)	19/58 (33)	0/58 (0)	58/58 (100)
Cancer	1/5 (20)	1/5 (20)	0/5 (0)	2/5 (40)	1/5 (20)	5/5 (100)
Total	*52/117 (44)*	*3/117 (3)*	*29/117 (24)*	*31/117 (26)*	*3/117 (3)*	*117/117* (100)
***P* value**[1]	0.08	0.05	0.69	0.15	0.57	-
Q value[1]	0.34	0.24	0.69	0.46	0.69	-

Consistent with increased rates of CST IV in high grade disease, vaginal microbiota richness and diversity were also found to be higher in women with high-grade disease (25/92, 29%), compared to low-grade disease (11/52, 21%), and lowest in normal women (2/20, 10%) but this was not statistically significant (p=0.2204, one-way ANOVA) (Figure 3.3).

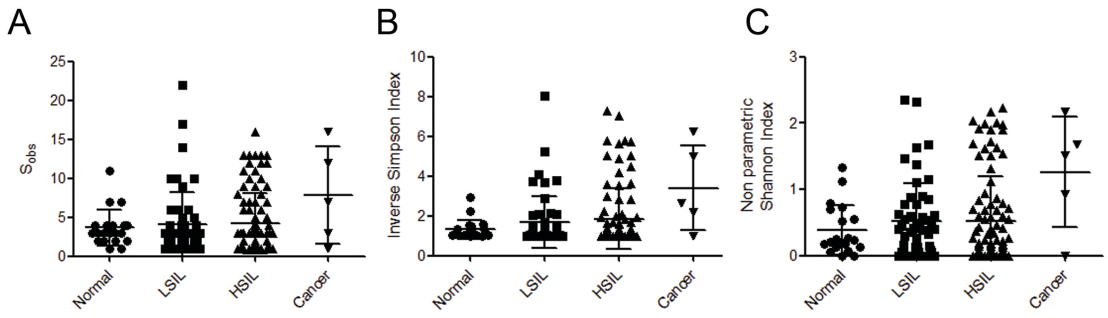


Figure 3.3. Vaginal microbiome richness (A) and diversity (B&C) indices associated with disease status

HSIL: High-grade squamous intra-epithelial lesion; ICC: invasive cervical cancer; LSIL: Low-grade squamous intra-epithelial lesion; S_{obs}: Species observed.

3.3.2 The structure of the vaginal microbiome correlates with HPV status and genotype

HPV status was available for 117 women in our cohort. CST IV was most frequently observed in high-risk HPV (HR-HPV) positive as compared to HR-HPV negative women (26/93, 28% versus 5/24, 21%, p=0.2522, unpaired t-test). HPV negative women were most likely to have CST I (13/24, 54%) as opposed to HPV positive women (38/93, 41%, p=0.0564, unpaired t-test). The rates for the other CSTs were comparable between HR-HPV positive and negative subjects (Figure 3.2, Table 3.2).

Of 93 women who were HPV positive, genotyping was available for 62 subjects. The rate of CST IV was higher for women infected with HPV16 (9/31, 29%) when compared to HPV18 (1/5, 20%, p=0.1907, unpaired t-test) or women with other high-risk oncogenic types (5/26, 19%, p=0.8310, unpaired t-test) although did not reach significance, likely due to sample size (Figure 3.2, Table 3.2).

The rate of CST IV was no different for Normal/LSIL HPV negative versus HPV positive individuals (3/20, 15% versus 7/34, 21%, p=0.6242, unpaired t-test), but substantially higher for HSIL or worse (HSIL= 19/58, 33%; ICC= 2/5, 40%, p=0.1180, unpaired t-test), suggesting that the presence of a high diversity Lactobacillus-depleted microbiome may be more strongly correlated to the presence of clinically significant pre- or invasive disease rather than the

presence of the virus itself (Figure 3.2, Table 3.2). Again, the results did not reach statistical significance.

3.3.3 Identification of vaginal microbiota composition markers of CIN disease severity

LEfSe modeling was used to identify differences in microbiota composition that may be related to increasing disease severity (Figure 3.4). Due to sample size restrictions, the comparison was limited to LSIL versus HSIL patients. In the LSIL group, significant over-representation of *Lactobacillus jensenii* ($P<0.01$) (Figure 3.4A, B & F) and *Lactobacillus coleohominis* ($P<0.05$) (Figure 3.4B) were observed. In contrast, HSIL samples were found to have significantly higher levels of *Peptostreptococcus anaerobius* ($P<0.05$), and *Anaerococcus tetradius* ($P<0.05$) (Figure 3.4A, B, C, D). HSIL samples were also found to have significant overrepresentation of *Fusobacteria*- primarily *Sneathia sanguinegens* ($P<0.01$) (Figure 3.4A, B & E).

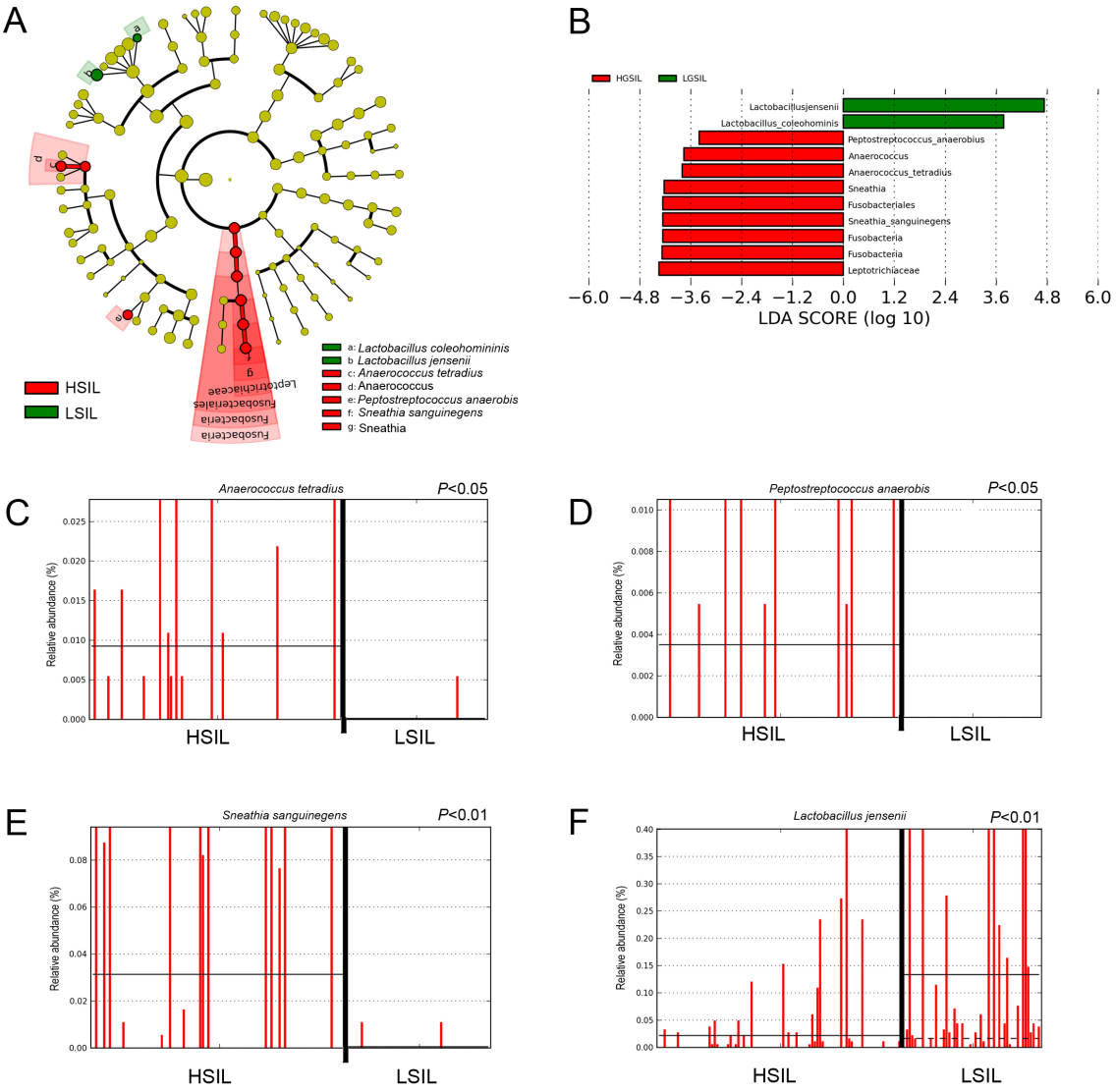


Figure 3.4. Identification of vaginal microbiota biomarkers of LSIL vs. HSIL by LEfSe analysis

(A) Cladogram representing taxa with different abundance according to disease severity. Size of circle is proportionate to abundance of taxon. (B) Histogram of the LDA scores computed for features differentially abundant between LSIL and HSIL disease states. Relative abundance counts of *Anaerococcus tetradius* (C), *Peptostreptococcus anaerobis* (D) and *Sneathia sanguinegens* (E), which were significantly over-represented in HSIL whereas Lactobacillus jensenii (F) was enriched in LSIL samples (Welch's t-test).

3.4 Discussion

This study detected a two-fold increase in the rate of a CST IV vaginal microbiome in those women with LSIL, a three-fold increase in women with HSIL and a four-fold increase in women with invasive cancer compared to controls. Increasing disease severity was also associated with decreasing relative abundance of *Lactobacillus* spp. A recent longitudinal study by Brotman *et al.,* reported that women with a high diversity, *Lactobacillus* spp. depleted (CST IV) vaginal microbiome were most likely to become HPV-positive, and to have persistent HPV infection [202]. The findings of this study suggest that vaginal microbial diversity is associated not only with HPV infection, but also with advancing CIN severity, but does not attain significance due to a lack of statistical power.

It is currently unclear if a CST IV microbiome is a causal factor in progression of CIN or a consequence of it. BV, a condition diagnosed using traditional culture techniquesand characterized by *Lactobacillus* spp. depletion and increased diversity of potentially pathogenic gram-negative bacteria, is associated with significantly higher rates of HPV infection and CIN [168, 169]. In this cohort, *Sneathia sanguinegens* is identified as a biomarker of HSIL, which has previously been shown to associate with HPV infection [201]. Two other BV-associated bacteria; *Peptostreptococcus anaerobius* and *Anaerococcus tetradius* were also found to be markers of HSIL in this cohort. It is possible that specific anaerobic species may play a role in disease progression, rather than simply signifying presence of HPV infection.

Bacteria are increasingly appreciated as a key player in the initiation and progression of other malignancies including colorectal cancer [146, 147, 257] where *Fusobacteria* has been identified as a potential pro-carcinogenic bacterial class [257, 258]. These data show that this class, and specifically *Sneathia sanguinegens*, is discriminatory of HSIL suggesting similar mechanisms, likely involving activation of inflammatory pathways, may be involved in the cervix. Approximately one third of premalignant lesions go on to develop invasive cervical disease, if untreated. It is possible that the women in this cohort with CST IV microbiomes are those at highest risk of progression to clinically significant invasive lesions, yet these findings only demonstrate association, not causality, between cervical pre-cancer, persistent HPV infection and the structure of the vaginal microbiota. Whilst women with BV display higher rates of HPV infection [168, 255] the virus has been shown to induce a pro-inflammatory environment to facilitate integration of viral DNA [259-263]. Thus, HPV infection itself may adversely impact on the host's immune defences and mucosal metabolism leading to aberration of vaginal microbiota, thus promoting viral persistence and disease progression.

Lactobacillus spp. are classically regarded as 'protective', yet this study supports other previous reports which suggest the clinical picture may be dictated by the specific species present, and that the genus as a whole may not be regarded as protective. Brotman and colleagues reported that an *L. iners* dominated vaginal microbiome (CST III) was associated with HPV-infection whereas vaginal microbiomes dominated by *L. gasseri* (CST II) exhibited the most rapid clearance of HPV infection [202]. While over-representation of CST II or III in this CIN cohort was not observed, the reduced prevalence of *L. iners* (CST III) concurrent with increased rates of CST IV in CIN, compared to controls may represent a shift from *L. iners* (CST III) towards a CST IV type microbiome with the acquisition of CIN. Unlike the majority of *Lactobacillus* spp., *L. iners* does not produce H_2O_2, which has been shown to have antibacterial and antiviral properties [264-266]. Consistent with the possibility that H_2O_2-producing *Lactobacillus* spp. are protective against CIN progression, higher prevalence of *L. jensenii* and *L. coleohominis,* both H_2O_2-producing lactobacilli, was observed in women with LSIL compared to HSIL, suggests this species may be particularly protective in preventing progression of the dysplastic and ultimately carcinogenic process. Furthermore *Lactobacilli* spp., have been shown to be cytotoxic when co-cultured with cervical cancer cells *in vitro*, but not normal cells, independent of lactic acid concentrations, highlighting interactions amongst cervical cells, the microbiota and the mucosal metabolic milieu [267].

Environmental and hormonal factors are also known to modulate the vaginal microbiome. Smoking has been previously correlated to HPV persistence and CIN as well as *Lactobacillus* spp. depletion and dysbiosis [189]. Although women in this cohort with high-grade disease were more likely to be smokers, the differences did not reach statistical significance and no correlation between vaginal microbial composition and smoking status was identified. A sub-analysis of smokers showed that the prevalence of CST IV increased with disease severity indicating that a high-diversity microbiome is correlated to disease status rather than smoking as a potential confounder.

Future therapeutic strategies permitting the modulation of the vaginal microbiome with oral or topical regimes to a *Lactobacillus* spp.-dominant microbiome may promote HPV clearance or even reverse the process of tumourigenesis, reducing the morbidity resulting from these conditions and their treatments [84, 86]. Probiotics have been used in a similar manner to reduced recurrence of BV, through accurate, targeted modification of the bacterial community [268]. Further research is required to understand the molecular mechanisms

involved in the complex role that bacterial communities can play in the development of cancer. An understanding of the functional properties of the community state types is required in order to complement what is already known about their structure. Further longitudinal studies are needed to investigate the changes and stability of the microbiome during transition from acute HPV infection, to persistent infection through to development of CIN and cancer.

3.5 Conclusion

In summary, this is the first study to correlate the structure of the vaginal microbiome with presence of CIN. These findings suggest that the presence and prevalence of specific vaginal microbiome CSTs may be involved in the pathogenesis of CIN and cervical cancer. Five bacterial species have been identified that could help to differentiate low- and high-grade disease, and with further research these may improve our understanding of the role of the bacterial microenvironment in HPV persistence, development of CIN and progression to cancer. Future longitudinal studies should aim to elucidate the causality between HPV infection, CIN, the immune microenvironment and the vaginal microbiome and increase our understanding of the role that vaginal bacteria in the tumour microenvironment.

In the field of cervical pathology, a number of well-characterised biobanks exist, collected both by individuals, and as part of cervical screening programmes. Such samples are usually collected using cervical cytobrushes, rather than the swabs used in this chapter and most commonly reported in the current literature. A study to compare these two techniques was conducted, as a validation step in the analysis of a longitudinal observational cohort of women with untreated CIN from the biobank of Professor Anna-Barbara Moskicki and these results are presented in the following two chapters.

CHAPTER 4. Comparison of vaginal microbiota sampling techniques: cytobrush versus swab

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4 Comparison of vaginal microbiota sampling techniques: cytobrush versus swab

4.1 Background

Emerging evidence suggests that vaginal microbiota (VM) plays a role in the persistence or regression of HPV infection, and in the natural history of subsequent disease [201, 202, 229, 269]. As described in the previous chapter and elsewhere, CST IV, and to some extent CST III (*L. iners* dominated), have been associated with increased acquisition and persistence of HPV infection [202], as well as with increased severity of CIN disease status [229, 269, 270]. The majority of existing data in the literature is derived from cross-sectional cohorts, limiting reported correlations between vaginal microbiota, HPV infection, cervical dysplasia and carcinogenesis to associations that lack causal inference. Longitudinal samples stored in existing cytology biobanks may provide a unique resource that permits temporal assessment and identification of causal associations between HPV infection and cervical disease.

Rayon swabs are one of the most common devices for sampling mucosal surfaces and have been widely used for next-generation sequencing-based analyses of cervico-vaginal microbial composition [156, 202, 229, 270, 271]. However, in the context of CIN and cervical cancer, biobanked samples are typically collected using a cytobrush, which exfoliate the top layer of cervical epithelial cells for detection of dysplasia by cytological analysis using light microscopy and are specifically designed to sample from the transformation zone of the cervix, which is the area in which HPV infects and causes dysplastic lesions and invasive cancers [272]. For this reason they may be superior to swabs due their ability to have a greater surface area contact with the cervical epithelium, ensuring the bacteria in closest contact with this mucosal surface are collected. Furthermore, biofilms of densely adherent bacteria can often be present in the vagina, particularly in the case of bacterial vaginosis (BV) [179]. A relatively soft-tip swab may be unable to disturb these biofilms and result in sampling of primarily planktonic bacteria not in direct contact with the cervical epithelium. It is also plausible that differences in absorbance and exfoliation between the two sampling devices could lead to variation in the composition of the VM. Previous studies have compared sampling techniques in the nasal sinuses (swab versus biopsy) [273], and ileum (brush versus biopsy) [274] and found no significant difference in relative abundance, richness or diversity of bacterial species. A comparison of swabs and cytobrushes used for vaginal microbiota sampling and subsequent analysis by sequencing has not previously been conducted.

4.2 Aims

- Compare swabs and cytobrushes for vaginal microbiota composition analyses based on
    a. Bacterial load collected using qPCR
    b. Diversity of sequencing data produced using Illumina MiSeq
    c. Community state type classification of sequencing results

4.3 Results

Thirty premenopausal, non-pregnant women with histologically-proven HSIL were recruited in the colposcopy clinic at Imperial College London between July 2014 and April 2015 according to the criteria in section 2.3.1. Patient characteristics are detailed in Table 4.1.

Table 4.1. Patient characteristics

Age, years	
Mean (SD, range)	30.8 (4.9, 26 – 43)
Ethnicity , n/N (%)	
Caucasian	27/30 (90.0)
Asian	3/30 (10.0)
Black	0/30 (0)
Parity, n/N (%)	
Nulliparous	20/30 (66.7)
Parous	10/30 (33.3)
Smoking status, n/N (%)	
Current smoker	5/30 (16.7)
Non-smoker	25/30 (83.3)
Phase of menstrual cycle, n/N (%)	
Luteal	8/30 (26.7)
Follicular	15/30 (50.0)
Unknown	7/30 (23.3)
Contraception, n/N (%)	
Nil	13/30 (43.3)
Condoms	5/30 (16.7)
COCP	5/30 (16.7)
POP	2/30 (6.7)
Copper IUD	1/30 (3.2)
Mirena IUS	2/30 (6.7)
Contraceptive implant	2/30 (6.7)
Time since last intercourse, n/N (%)	
>48 hours	26/30 (86.7)
<48 hours	4/30 (13.3)
Histological severity of lesion	
CIN2	20/30 (66.7)
CIN3	10/30 (33.3)

CIN: Cervical intraepithelial neoplasia; COCP: Combined oral contraceptive pill; IUD: Intrauterine device; IUS: Intrauterine system; POP: Progesterone-only pill

Two samples were taken from each woman during the same vaginal examination, providing a total of 60 samples. There was no difference in the mean storage duration from sample collection to DNA extraction between the two sample types (swabs: mean 49 weeks, range 22-62 weeks; cytobrushes: mean 50, range 23-63 weeks, p=0.7015, paired t-test).

4.3.1 Cytobrushes collected a significantly greater bacterial load

As estimated using qPCR, cytobrushes collected a greater total bacterial load when compared to swabs (swabs: mean 4.75 $\log_{10}$ 16s rRNA gene copies, range 2.56 – 6.35 $\log_{10}$; cytobrushes: mean 6.41 $\log_{10}$, range 3.72 – 8.75 $\log_{10}$; p<0.001, paired t-test)(Figure 4a, Table 4.2). However, when bacterial load was corrected for volume of storage media, this difference was no longer significant (swabs: mean 4.75 $\log_{10}$, range 2.56 – 6.35 $\log_{10}$; cytobrushes: mean 4.81 $\log_{10}$, range 2.12 – 7.14 $\log_{10}$; p=0.7361, paired t-test)(Figure 4b, Table 4.2). A total of 500uL was therefore used for further sequencing studies to ensure comparable bacterial DNA loads.

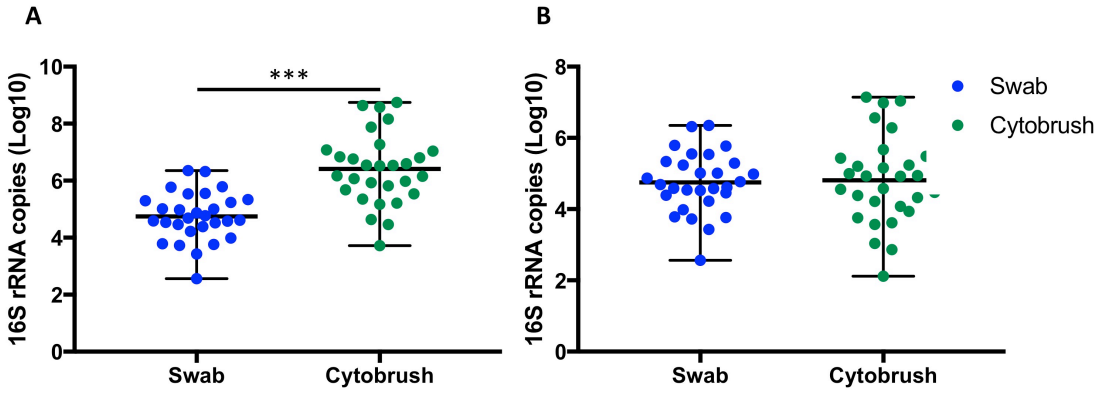


Figure 4.1. 16S rRNA consensus qPCR results

A. Cytobrushes collected a greater total bacterial load compared to swabs (swabs: mean 4.75 $\log_{10}$ 16s rRNA gene copies, range 2.56 – 6.35 $\log_{10}$; cytobrushes: mean 6.41 $\log_{10}$, range 3.72 – 8.75 $\log_{10}$; p<0.001)(paired t-test).
B. When the bacterial load was normalized to 500µl with similar amount of medium from the liquid based cytology and Aimes swab solution for Illumina MiSeq sequencing, there was no longer a significant difference between the two techniques (swabs: mean 4.75 $\log_{10}$, range 2.56 – 6.35 $\log_{10}$; cytobrushes: mean 4.81 $\log_{10}$, range 2.12 – 7.14 $\log_{10}$; p=0.7361) (paired t-test).
***p<0.001

4.3.2 Bacterial richness and diversity are comparable between sampling devices

MiSeq sequencing of V1-V3 hypervariable regions of 16S rRNA genes resulted in a total of 696 582 reads, with an average number of 11 610 reads per sample, and a mean and median read length of 543 and 550 bp respectively. Operational taxonomic units (OTUs) were randomly sub-sampled to the lowest read count of 3942 to avoid sequencing bias, which retained 78% of total OTU counts and >99% coverage for all samples. Following removal of singletons with less than 10 counts, a total of 70 taxa were identified across all 60 samples. Of these, 61 were identified in both samples, eight exclusively in cytobrushes and one exclusively in swabs (Table 4.2).

Table 4.2. Results of qPCR and sequencing data analysis

	Swabs (n=30)	**Cytobrushes** (n=30)	**p-value**
Total bacteria load			
Total bacterial load collected by sampling technique, *Log_{10} 16S rRNA copies (mean, range)*	4.75, 2.56 – 6.35	6.41, 3.72 – 8.75	<0.0001
Total bacterial load used for 16s rRNA sequencing, *Log_{10} 16S rRNA copies (mean, range)*	4.75, 2.56 – 6.35	4.81, 2.12 – 7.14	0.7361
Richness and diversity indices			
Species observed	20, 3.00 – 72.00	14, 3.00 – 64 .00	0.8109
Inverse Simpson index	0.77, 0.01 – 2.39	0.63, 0.01 – 2.28	0.9125
Community state types, *n/N (%)*			
CST I	7/30 (23.3)	7/30 (23.3)	1.000
CST II	3/30 (10.0)	2/30 (6.7)	>0.9999
CST III	14/30 (46.7)	12/30 (40.0)	0.7948
CST IV	5/30 (16.7)	8/30 (26.7)	0.5321
CST V	1/30 (3.3)	1/30 (3.3)	1.000
Taxa unique to sampling technique			
	- Achromobacter denitrificans	*- Sphingopyxis chilensis* *- Comamonas* spp. unclassified *- Brevundimonas diminuta* *- Sphingomonas koreensis* *- Burkholderia fungorum* *- Pseudomonas plecoglossicida* *- Ralstonia insidiosa* *- Arthrobacter oryzae*	-

CST: community state type; rRNA: ribosomal RNA; spp.: species.

There was no significant difference in richness, as determined by number of species observed (p=0.8109 paired t-test), or diversity, quantified by inverse Simpson index (p=0.9125, paired t-test) (Figure 4.2 A & B, Table 4.2) between the two sampling techniques. In most individuals the richness and diversity values were similar in both samples, however they were not consistently higher or lower where different (Figure 4.2C & D).

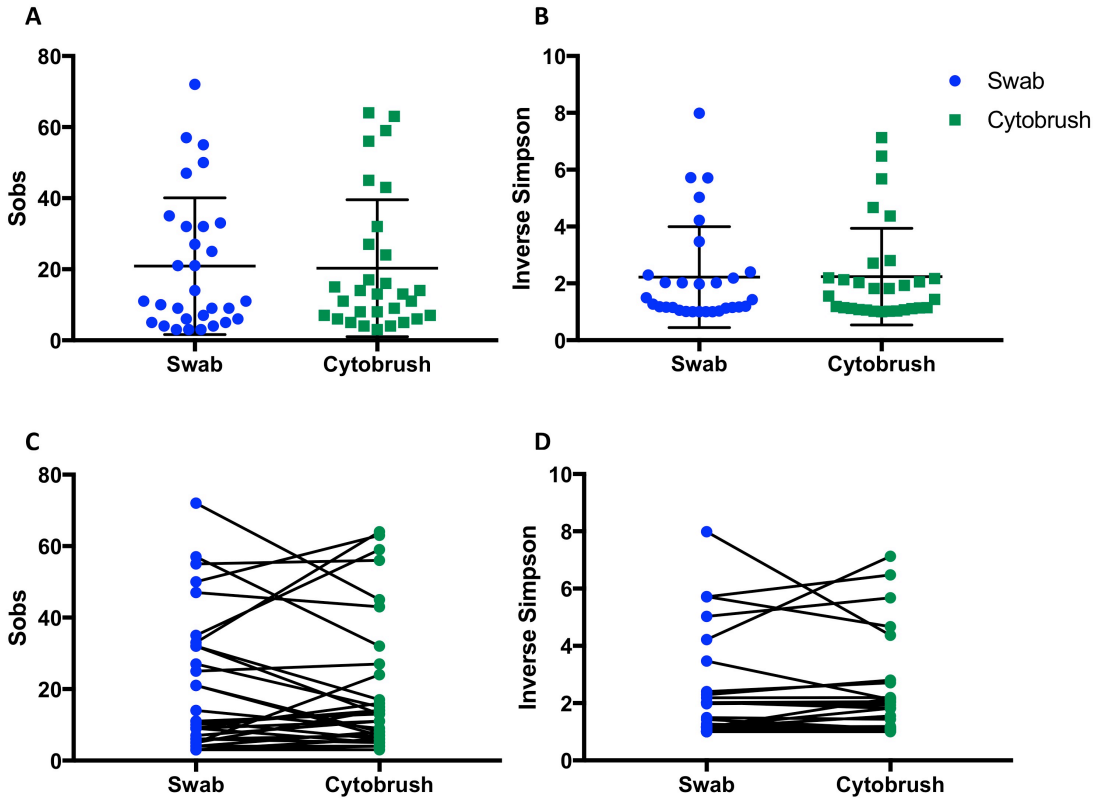


Figure 4.2. Species richness and diversity indices

Richness, as determined by number of species observed (p=0.8109) (A) and diversity, quantified by inverse Simpson index (p=0.9125) (B) do not differ significantly between the two sampling techniques (paired t-test). Richness (C) and diversity (D) were also similar in most women, however where different, the values were not consistently higher or lower with either technique.
Sobs = Species observed

4.3.3 Community state type at species level is comparable

Ward clustering of relative abundance at species level was performed and demonstrated the presence of four of the five previously described CST's [153] within the dataset, with CST V not observed (Figure 4.3). Concordance in CST between the two sampling techniques was

found in 27 of 30 patients (90%), with discordance in three of 30 (10%). Of these, two patients displayed a *Lactobacillus iners*-dominant (CST III) structure on the swab and high-diversity *Lactobacillus*-spp. deplete CST IV on the cytobrush-collected sample. The remaining discordant sample set also showed CST IV using the cytobrush, but the *Lactobacillus gasseri*-dominant CST II on the swab. When comparing the entire dataset, this discrepancy between CST's in the swab and cytobrush-collected samples was not statistically significant (Table 4.2).

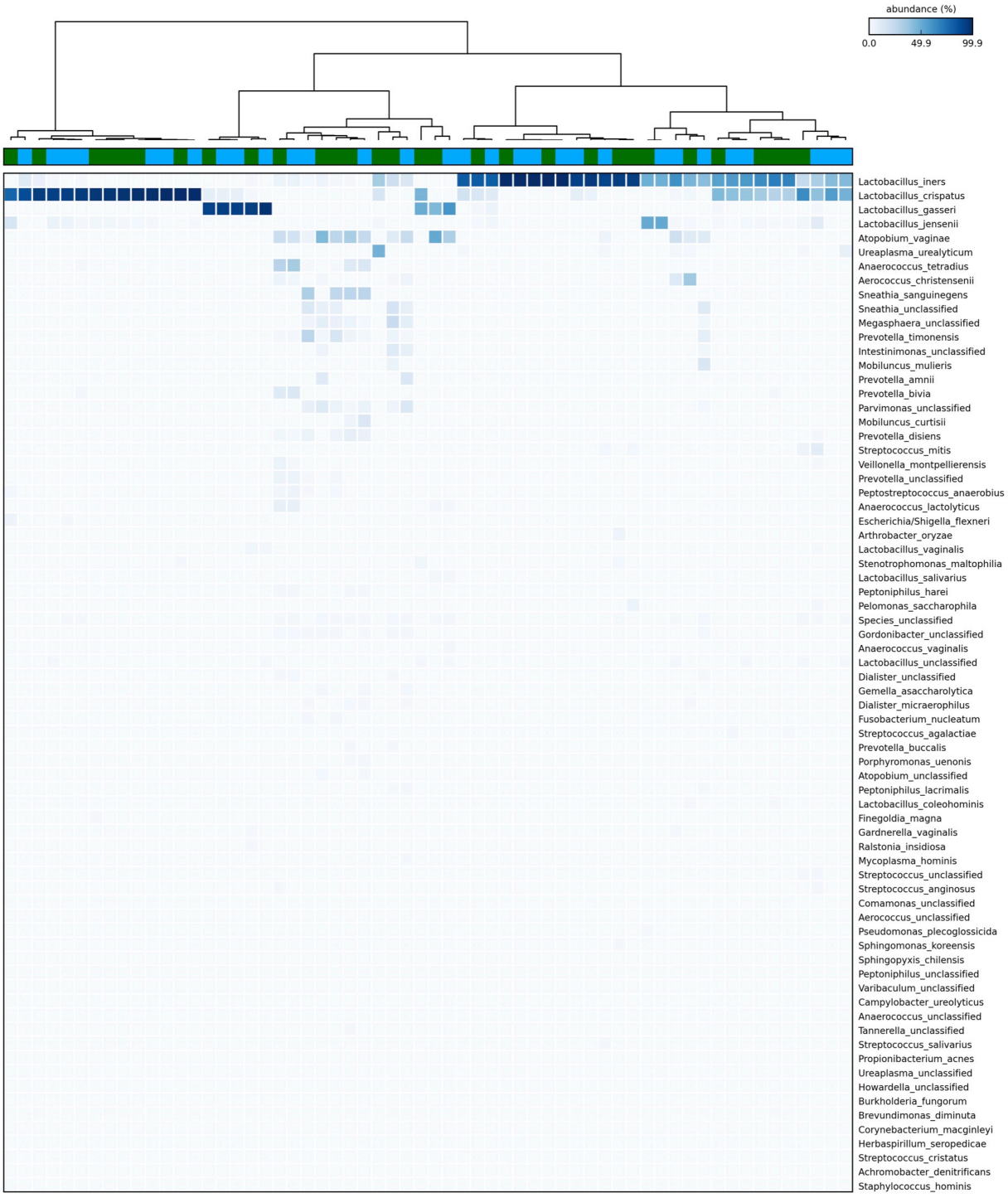


Figure 4.3. Vaginal microbiota composition according to sampling device

Hierarchical clustering analysis using ward clustering was used to classify samples into community state types (CSTs). There was a 90% concordance in CST classification (27/30 patients) between swab (blue) and brush (green) sampling. Three patients with a *Lactobacillus*-spp. dominant vaginal microbiota on swab sampling were subsequently found to have a high-diversity CST IV on cytobrush sampling.

Bray-Curtis index of dissimilarity was used to compare the microbial community structure of the samples collected via the two different techniques (Figure 4.4). Visualisation of the dissimilarity matrix using NMDS revealed no difference in the overall community structure between sampling devices (p=0.99, PERMANOVA test).

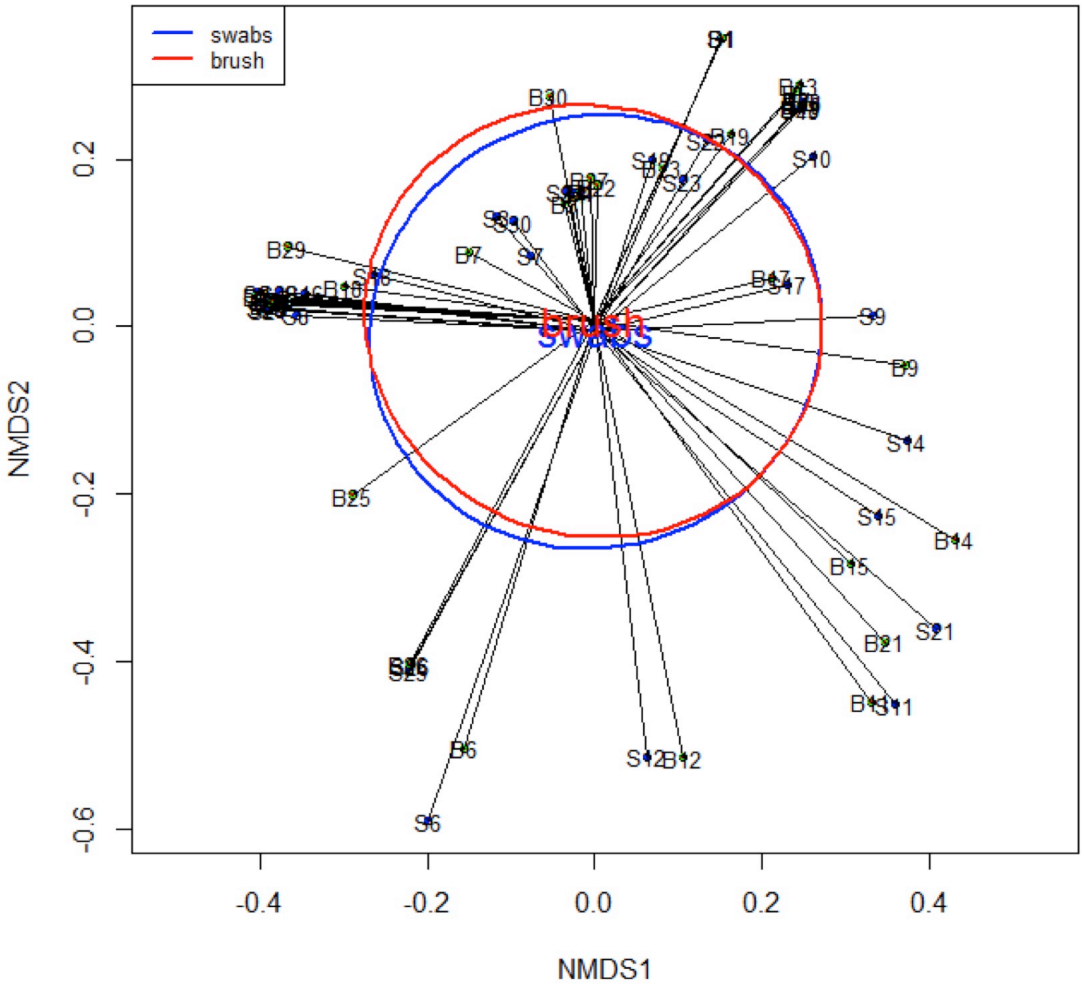


Figure 4.4. Non-metric multidimensional scaling (NMDS) analysis for paired brush and swab samples.

NMDS analysis of the Bray-Curtis dissimilarity matrix revealed no significant difference in community composition between cytobrush- and swab-collected samples. Ellipses represent standard error.

A two-group comparison of the different sampling techniques was also performed showing that relative abundance of bacterial taxa was highly comparable between all swabs and cytobrushes (R^2 = 0.998-0.999 from phylum to genus level, R^2 = 0.993 at species level) (Figure 4.5A). Similarly, high correlation was observed when a paired two-sample comparison was

performed as to examine individual patient correlation of swab and cytobrush-collected samples (R^2=0.908; range 0.408 – 1.00). When comparing intra-patient variability between the two sampling techniques, significantly less correlation of species abundance was observed between the two samples in women with CST IV compared to women with *Lactobacillus* species- dominant VM (*Lactobacillus*-dominant CST mean R^2=0.982 vs. CST IV mean R^2= 0.706, p=0.0049, Mann-Whitney U test) (Figure 4.5). The mean R^2 values for the individual *Lactobacillus*-dominant CST's were 0.995 (CST I), 1.00 (CST II) and 0.971 (CST III).

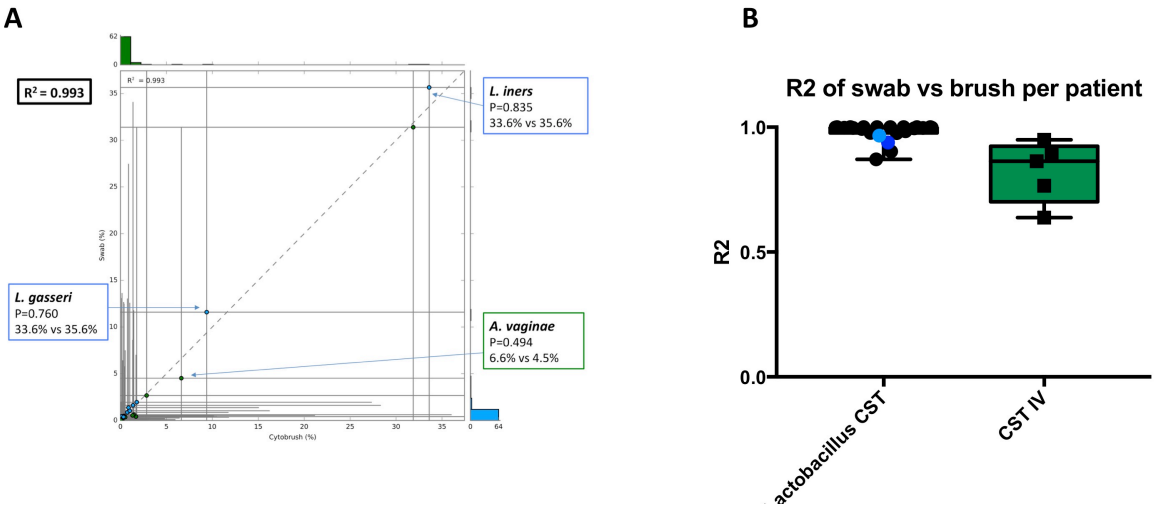


Figure 4.5. Correlation between sample composition at species level

A. Using a 2-group comparison the correlation between composition at species level was found to be 0.993 (Welch's t-test). B. Using 2-sample comparison, the correlation between swab and cytobrush samples was significantly less in women with CST IV, compared to those with a *Lactobacillus*-spp. dominant vaginal microbiota (p=0.0049, Mann-Whitney U test).

LEfSe analysis identified 5 taxa belonging to the same clade to be significantly over-represented in the cytobrush samples (*Proteobacteria, Betaproteobacteria, Burkholderiales, Burksholderiaceae* and *Comamondaceae*; Figure 4.6), although the relative abundance of these taxa was low overall. Taxa attributed to unclassified *Lactobacillus* spp. was over-represented in swabs. Further LEfSe analysis performed on the subgroup of patients with at least one CST IV sample (n= 8) failed to identify any differentially abundant features.

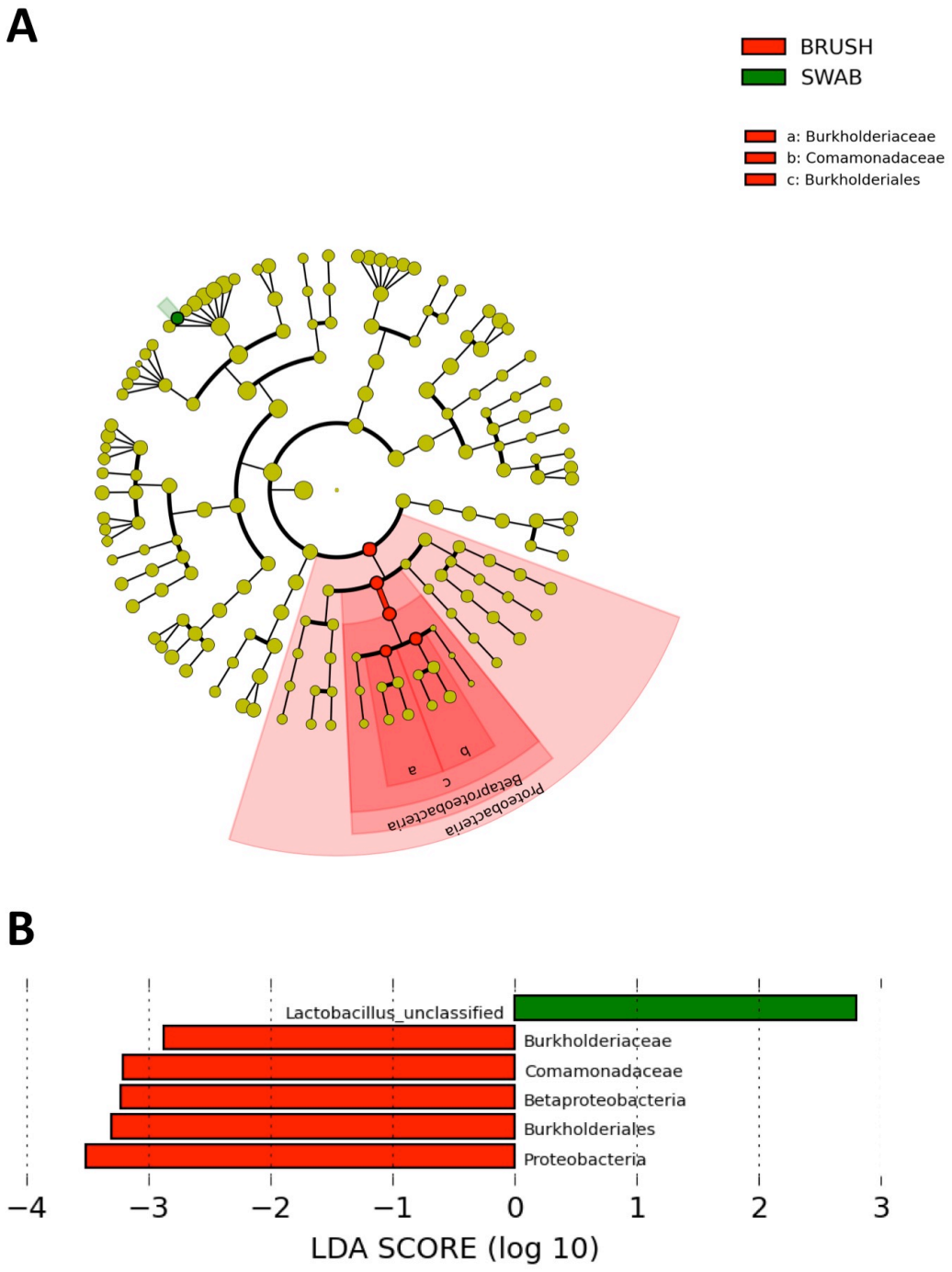


Figure 4.6. Identification of differentially abundant taxa between swabs and cytobrushes

A. Cladogram representing taxa with different relative abundance according to sampling technique. Size of circle is proportional to relative abundance of taxon. B. Histogram of the LDA scores computed for features differentially abundant between swab and cytobrush-collected samples (Welch's t-test). *LDA score: Linear discriminant analysis score*

Gardnerella vaginalis qPCR was used to determine whether the choice of 16s rRNA sequencing primers may have influenced the comparison between the two techniques. When comparing 500ul swab to 500ul LBC fluid, the volume with which comparable bacterial counts are seen (Figure 4.1) there is no significant difference in levels of *G. vaginalis*. When a difference between swabs and brushes was observed it was neither consistently higher nor lower (Figure 4.7).

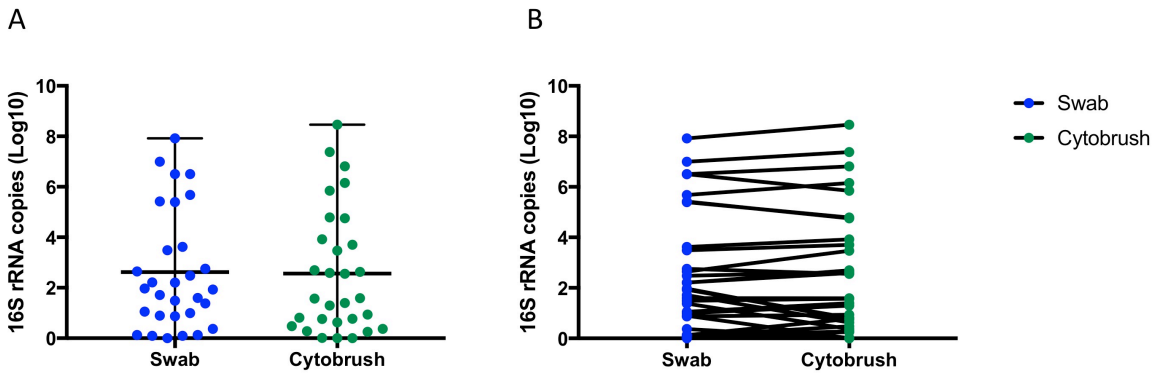


Figure 4.7. Comparison of Gardnerella vaginalis gene copies using qPCR

A. There was no significant difference between the number of *G.vaginalis* gene copes present in paired swabs and cytobrush collected samples, normalised for total bacterial load (Figure 4.1) (p=0.6313) (Paired t-test).
B. Where different the values were not consistently higher or lower with either technique.

4.4 Discussion

Cross-sectional studies exploring the associations between the VM, HPV infection and cervical pre-invasive and invasive disease have shown that a high-diversity VM, and to a lesser extent *L. iners*-dominant VM's correlate with increasing cervical disease severity [229, 269, 270], and in acquisition and persistence of its causative agent HPV [202]. Longitudinal studies are required to infer causality with regards to the role of the human microbiota in oncogenesis [275]. However, the change from a normal healthy cervix, through HPV acquisition, chronic infection resulting in dysplasia and onward neoplastic transformation to invasive cancer takes at least a decade [276]. Biobanks, largely collected as part of cervical screening programmes, contain liquid-based cytology samples collected using cytobrushes, which provide a unique resource of serial samples required to more deeply explore the associations between VM and cervical carcinogenesis. Several techniques have been described in the literature for obtaining samples for the purpose of sequencing bacterial DNA to study the human vaginal microbiota in a variety of patient cohorts, the most common being swabs [152], but the use of cytobrushes [277], as well as cervicovaginal lavage [278], epithelial scrapes and biopsies [279] has also been reported. Heterogeneity of the vaginal microbiota at different locations throughout the vagina has been documented using the same sampling device[280], however a comparison of swabs and cytobrushes taken from the ectocervix has not been described.

Cytobrushes, unlike swabs, are made of polyethylene and lack any absorptive characteristics. Whilst the suitability of different swab types has not been reported in studies exploring the vaginal microbiota, experiments *in vitro* have shown that a significant difference exists in both absorbance and release of compounds and proteins from different swab types [281]. Cytobrushes have a greater exfoliative ability compared to swabs, possibly giving them the additional ability to disturb biofilms. We therefore hypothesised that cytobrushes would be associated with higher diversity due to these different properties.

In this pilot study, we compared the results obtained using swabs and cytobrushes, from a population of 30 women with high-grade SIL to determine whether these two techniques provide a comparable overview of the structure of the vaginal microbiota. We chose women with high-grade pre-invasive disease as opposed to low-grade or normal controls, since our previous study [229], which included women with various disease severity and healthy controls, indicated that women with HSIL have good representation of major vaginal CSTs.

This allowed us to compare the similarity of the two sample techniques in both low and relatively high-diversity vaginal communities.

Cytobrush sampling collected higher bacterial loads, as assessed using qPCR. Whilst the cervical microbiota has been demonstrated to be similar in composition to the vagina, it may have comparatively lower bacterial load [282], reinforcing the importance of collecting the greatest possible load. A small aliquot of the total 20ml LBC solution was used, and we were able to use qPCR to determine the volume to be used to give similar total bacterial load (Figure 4.1b, Table 4.2) to prevent biasing further sequencing experiments with a discrepancy in bacterial load between the two techniques. We also demonstrated the higher biomass collected by cytobrushes in a separate cohort of 20 further women in whom the mean weight of sample collected by swabs was 50 milligrams (mg), compared to 1560 mg by cytobrushes (unpublished data), providing further evidence that cytobrushes collect a higher biomass than swabs. As a result, the onefortieth of the original cytobrush sample is more than sufficient for 16S rRNA gene analysis, which mean additional investigations can be performed using the large volume remaining (e.g. cervical cytology, HPV genotyping, and general microbiology) that could otherwise not be performed if using a swab. However, metagenomic/whole genome shotgun sequencing analysis of cytobrush-collected samples may be hindered by a higher load of host DNA, however this was not assessed in the current study.

Overall our study demonstrated that swab and cytobrush samples provide comparable VM results at all taxonomic levels, as demonstrated by two-group/sample correlation coefficients, hierarchical clustering analysis and Bray-Curtis dissimilarity index. No significant differences in richness or diversity between the two sampling techniques were identified disproving our original hypothesis that cytobrush-collected samples would display higher microbial diversity. In spite of this, a greater number of unique taxa were observed in cytobrush samples and LefSe analysis identified *Proteobacteria, Betaproteobacteria, Burkholderiales, Burksholderiaceae* and *Comamondaceae* to be over-represented in the cytobrush-collected samples. However, levels of each were present at extremely low abundance and their presence has not previously been associated with the presence of HPV and cervical disease in studies using swabs for sampling.

Although overall correlation between swab and cytobrush data at an individual level was high, reduced correlation was observed in women with high-diversity CST IV. LEfSe analysis of this

smaller patient subset did not detect any differentially abundant taxa, but this may be due to a lack of statistical power. There was a discrepancy between the CST classification of sequencing data in 3/30 (10%) of women, all of whom had a swab sample which clustered with a *Lactobacillus* spp.-dominant CST, but with CST IV on their cytobrush sample. It is plausible that sampling with a cytobrush disrupts biofilms that are otherwise left intact when sampled with a rayon swab resulting in the isolation of taxa present in planktonic phase. Clearly further studies are required to confirm this however, our data indicates that swab-sampling techniques may be less suitable when studying diseases correlated with highly diverse communities. Cross-sectional data in high-grade pre-invasive cervical disease document high prevalence of dysbiosis [229] and therefore cytobrush-sampling techniques may reduce sample collection bias in these patients.

Although swabs are considered by some patients to be less invasive than a cytobrush, they are not used to collect samples for cytological screening due to their inability to exfoliate an adequate number of endocervical cells for cytological analysis [283]. Cytobrushes can not only be used for cervical cytology and HPV DNA testing, but we show that they provide a reliable and robust sampling tool for analysis of the vaginal microbiota. It should be noted however, that cytobrushes do not harbour absorbance qualities and thus dual sampling with a swab may provide useful material for analysis the proteomic and metabolic component of cervicovaginal mucosa.

One of the limitations of sequencing is that the results may be influenced by the choice of primer sets [284]. We have used primers for V1-V3 hypervariable regions of 16S rRNA genes, and acknowledge that these may not detect members of the *Bifidobacteriales* order, which includes *Gardnerella vaginalis* [284], a species frequently detected in the human vagina [285]. In order to determine whether primer choice influenced our results we performed *G. vaginalis* qPCR, and showed that where this species was detected, there was no significant difference between the two sampling techniques (Figure 4.7), and therefore do not consider this to be a significant limitation to our conclusions. Furthermore, the collection of both samples was performed during the same vaginal examination in order to ensure identical conditions and allow a direct comparison between the sampling techniques. The cytobrush, which has a greater exfoliative capacity compared to a swab was intentionally collected second to ensure that this does not disturb the biofilms prior to the swab collection. Given the wide surface of the cervix and the amount of discharge found in women, it is unlikely that the gentle tip of

the swab would be sufficient to disturb the microbiota in the cytobrush, hence the reason for such a study design.

This report is the first study to compare the VM in women sampled using swabs or cervical cytobrushes. A single clinician collected all samples, in an attempt to minimise the likelihood of intra-study variability in the sampling collection techniques. Our results indicate that resulting sequencing data derived from both sampling devices are comparable, yet cytobrushes permit the collection of a greater bacterial load. This may be in part due to their larger surface area, but these samples can also be used for additional cervical cytology and HPV DNA testing purposes. Looking beyond the current study, the results have implications in possible future attempts to synthesise the existing evidence and integrate existing multiple studies and datasets for the purpose of meta-analysis [286], as differential technique, device and site of sampling, whilst producing small variability may have a profound confounding effect on larger analyses. Further larger studies are required to confirm the findings of this study.

4.5 Conclusion

Analysis of our data shows that rayon swabs and polyethylene cervical cytobrushes produce comparable results when comparing the vaginal microbiota composition at species level, and did not show any significant difference in diversity or richness. However, cytobrushes were able to uncover CST IV VM's, not demonstrated by the corresponding swab sample in 10% of our sampled population, which may be due to the cytobrush having a greater exfoliative capacity, which could enable biofilm disruption. We have also shown that cytobrushes collect a higher bacterial load. These results should be taken into consideration when designing future prospective studies where multiple investigations on the sample3 are desired. Based on our findings we conclude that cervical cytobrushes are a valid sampling device for collection of samples for 16S rRNA gene analysis, which opens up the possibility of using historical biobank samples for the study of longitudinally collected patient samples.

The following chapter will describe a longitudinal observational cohort of women with untreated CIN2 obtained as part of a collaboration with Professor Anna-Barbara Moskicki, using biobanked samples collected using a cervical cytobrush.

CHAPTER 5. Untreated CIN2: the role of the vaginal microbiota in the natural history of disease outcomes

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Content from this chapter is currently under preparation as:

Mitra A, MacIntyre DA, Lee YS, Smith A, Marchesi JR, Bennett PR, Moscicki AB and Kyrgiou M. Untreated CIN2: the role of the vaginal microbiota in the natural history of disease outcomes (*Under preparation)*

5 Untreated CIN2: the role of the vaginal microbiota in the natural history

5.1 Background

CIN2 is regarded as a high-grade preinvasive lesion of the cervix, which is usually treated to reduce the risk of future progression to cervical cancer, although this may be controversial due to the high rates of regression cited by observational studies. A systematic review including 12 studies published between 1955 and 1990 observing the fate of CIN2 in 2247 women showed that 43% of CIN2 lesions regressed, 35% persisted and the remaining 22% progressed, with follow-up in the included studies ranging from four months to 18 years [42]. Notably this study was published before the widespread use of HPV detection methods. CIN2 it is often regarded as a heterogeneous disorder that can be caused by both low- and high-risk HPV (hrHPV) subtypes [287, 288], which will therefore have a substantial impact on the outcome of a particular lesion. Further studies have integrated HPV status and shown that regression is slower in women infected with hrHPV, compared to low-risk subtypes [289]. In a study of 100 women with CIN2/3, those infected with HPV16 were three times less likely to regress compared to women with a non-HPV16-associated lesion [290].

In view of the high rates of regression, particularly in younger women, it may be seen as too hasty to offer immediate treatment to women diagnosed with CIN2, which could unnecessarily put these often nulliparous women at an increased risk of future reproductive and obstetric complications to be discussed in Chapter seven. Clinicians are often understandably anxious about observing these women, due to the lack of objective tests that can be used to determine the fate of an individual CIN2 lesion, and in some patients, concerns regarding compliance with follow-up.

Analysis of the emerging evidence suggests that the vaginal microbiota (VM) plays a role in the persistence or regression of HPV infection, and in the natural history of subsequent disease [201, 202, 229, 269]. The current literature describes cross-sectional cohorts, which limits reported correlations between vaginal microbiota, HPV infection, cervical dysplasia and carcinogenesis to associations only, which lack any causal inference. The use of longitudinal cohort studies will enable temporal assessment and may detect causal associations between the vaginal microbiome, HPV infection and cervical disease. An understanding of the interplay between the vaginal microbiome and the fate of untreated CIN will not only improve our

understanding of the natural history of the disease, but could also be used in the future triage of such lesions.

5.2 Aims

- Compare the vaginal microbiota of women with CIN2 according to disease regression, progression or persistence using using Illumina MiSeq sequencing

5.3 Results

The study cohort were recruited from September 2002 to June 2006 at one of the 12 participating clinics within Kaiser Permanente Northern California and have previously been described by Moscicki et al. [291] and as outlined in section 2.3.5. Ninety-five women with CIN2 were recruited, however no baseline samples were available for bacterial sequencing in eight women. Eighty-seven women were therefore included in the final analysis, all of whom attended for at least two visits; a baseline visit during which CIN2 was diagnosed on a punch biopsy and at least one follow-up visit. The mean follow-up period was 27.4 months (Range 5.0 – 46.8 months) and the mean number of biopsies during follow-up was one (range 0 to 6). An exit biopsy was performed in all patients who attended their final visit and gave consent to a biopsy regardless of clinical need, which resulted in 58 exit biopsies being performed (67%). Patient characteristics at baseline visit are detailed in Table 5.1. The population described appeared to participate in relatively high-risk sexual behaviors evidenced by a high number of previous pregnancies (37/87, 42.5%), *C. trachomatis* infections (22/87, 25.3%), total number of lifetime sexual partners (mean = 8, range 1 – 35) and history of anal intercourse (13/87, 14.9%) compared to national data reported around the time of recruitment [292], as well as compared to a control population without cervical disease [293]. None of the study cohort were infected with HIV.

Table 5.1. Patient characteristics

	All, n=87
Age	
Mean (SD, range)	20.5 (16.0 – 26.5)
Ethnicity	
Caucasian	35/87 (40)
Black	21/87 (24)
Latin	19/87 (22)
Other ethnicity	11/87 (13)
Smoking	
Current smoker	23/87 (26)
Non-smoker	64/87 (74)
Contraception	
Nil	26/87 (30)
Condoms	6/87 (7)
Contraceptive injection	14/87 (16)
Other hormonal contraception	41/87 (47)

HPV status was known for 85 patients (97.7%), of whom six were HPV negative (6/85, 7.0%). Of the remaining 79 patients who were HPV positive, 73 were positive for at least one high-risk subtype (73/85, 85.8%). Thirty patients were HPV16 positive (30/85, 35.3%) and eight were infected with HPV18 (8/85, 9.4%). Thirty-three women were infected with a low risk HPV subtype (33/85, 38.8%), six of whom had only low-risk HPV infection (6/85, 7.0%). Twenty-eight women were infected with a single HPV subtypes (28/85, 32.9%), and forty-five were infected with multiple HPV subtypes (45/85, 52.9%).

5.3.1 Vaginal microbiota composition may predict outcome at 12, 24 and 36 months.

In total 397,782 reads were obtained from 87 samples with an average number of reads per sample of 4586 and the mean and median read lengths of 513 and 520 bp respectively. To avoid sequencing bias, operational taxonomic units (OTUs) were randomly sub-sampled to the lowest read count of 885, which retained 99.7% of OTU counts and still provided a minimum coverage of 98.7% for all samples. Following removal of singletons and rare OTUs, a total of 160 taxa were identified in the vaginal microbiota of the study cohort.

Figure 5.1 shows the distribution of CST's at the baseline visit, when all patients had a histologically confirmed diagnosis of CIN2.

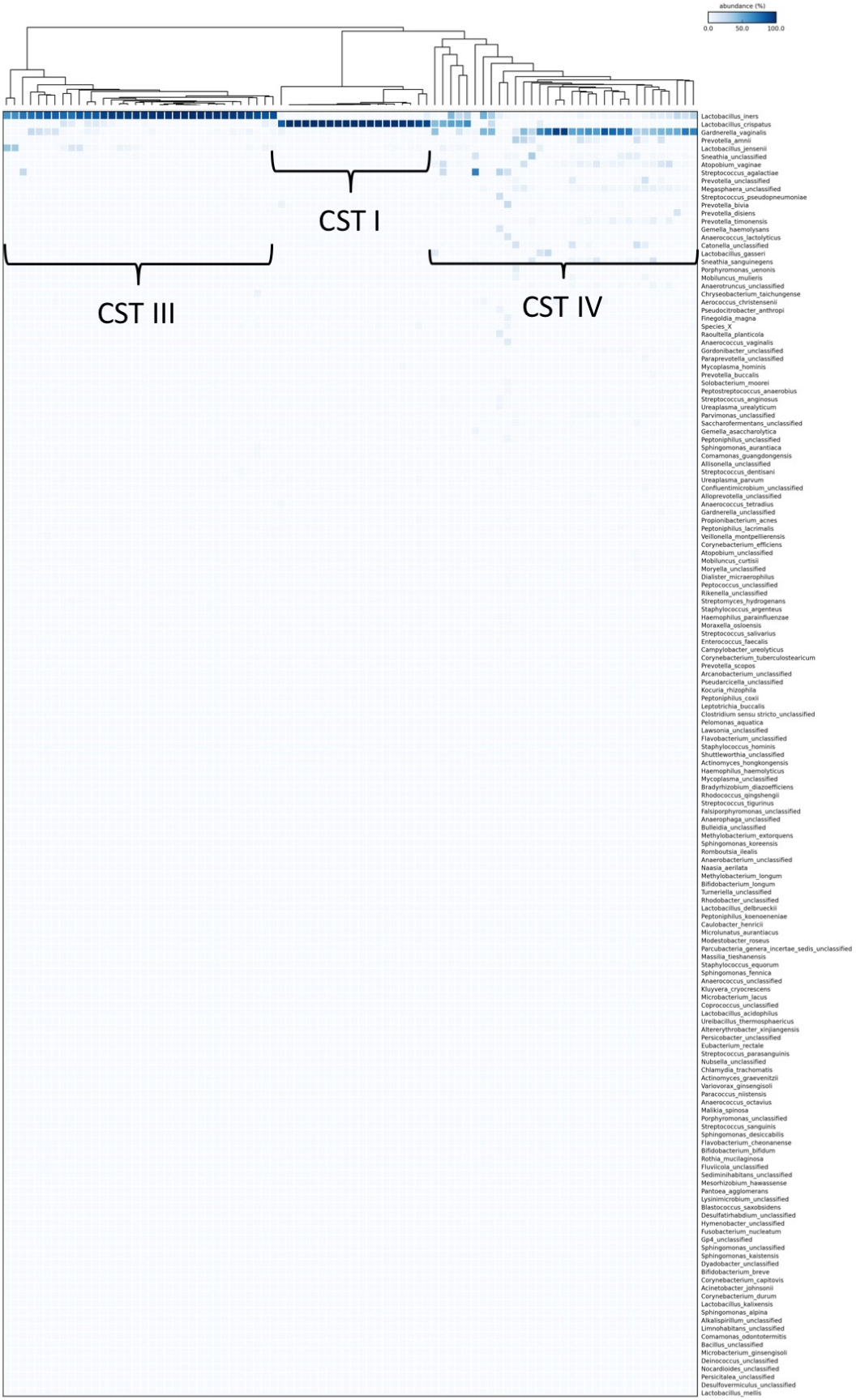


Figure 5.1. Heatmap of baseline CST's in women with CIN2

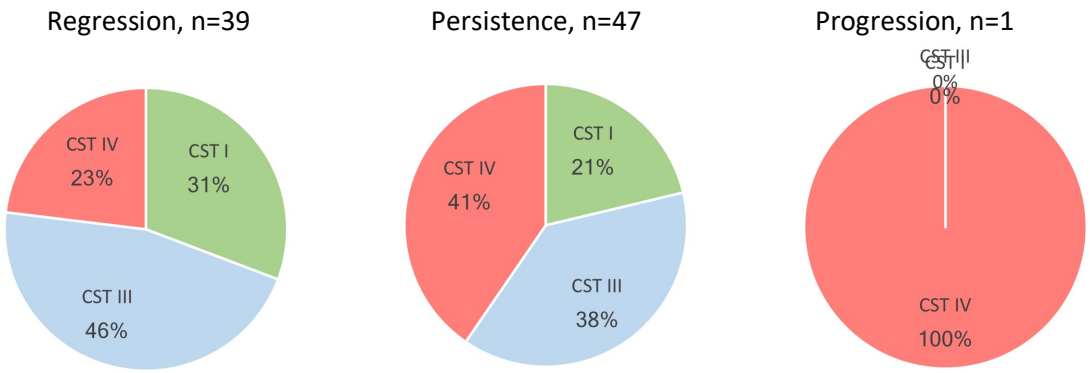
Hierarchical clustering analysis using ward clustering was used to classify samples into community state types (CSTs).

CST III was the most frequently observed (36/87, 41.4%), followed by CST IV (29/87, 33.3%), with CST I being the least common (22/87, 25.3%). CST II and CST V, dominated by *L. gasseri* and *L. jensenii* respectively, were not observed in this population. There was no difference when analyses were restricted to only HPV-infected, or hrHPV-infected women.

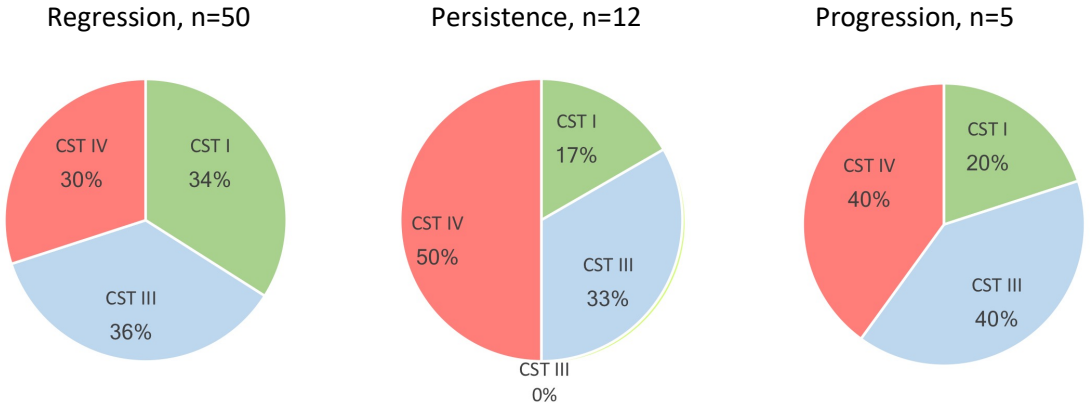
At 12 months, 39 patients had regressed (39/87, 44.8%), 47 had persistent disease (47/87, 54.0%) and one patient had progressed to CIN3 (1/87, 1.1%). The distribution of CST's is shown in Table 5.2. At 24 months, 20 patients were no longer under follow-up, and of the 67 remaining patients, 50 patients had regressed (50/67, 74.6%), 12 had persistent disease (12/67, 17.9%) and five patients had progressed to CIN3 (5/67, 7.5%). Of the 41 patients who were still under follow-up at 36 months, 36 patients had regressed by this timepoint (36/41, 77.0%), two patients had persistent disease. No further patients had progressed to CIN3, but of the five women known to have progressed by 24 months, three were still in follow-up and all continued to have CIN3 (3/41, 7.3%).

The distribution of baseline vaginal microbiota CST's according to outcomes at 12, 24 and 36 months are shown in Table 5.2 and Figure 5.3. At 12 months, CST III was seen most frequently in women who regressed and this was approaching significance (p=0.0536, one-way ANOVA), and women with CST IV were almost twice as likely to have persistent disease when compared to those who regressed (Regression with CST IV 9/39, 23.1% vs Persistence with CST IV 19/47, 40.4%, p= 0.6957, one-way ANOVA). At 24 months, women who regressed were significantly more likely to have a CST I microbiota, and less likely to have CST IV compared to those who persisted (p=0.0001, one-way ANOVA). At 36 months CST IV was also significantly associated with persistence compared to regression (p=0.0004, one-way ANOVA).

A – 12 months, n=87



B – 24 months, n=67



C – 36 months, n=41

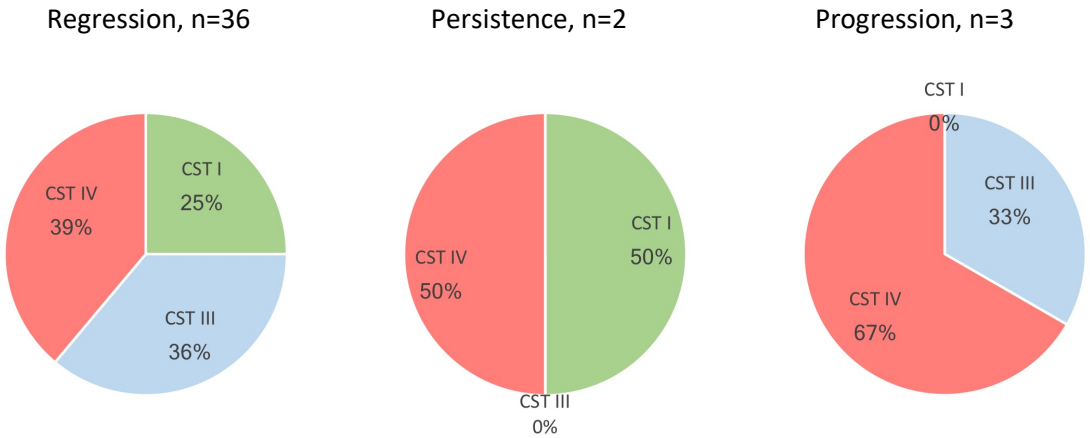


Figure 5.2. Outcomes according to baseline CST at 12 (A), 24 (B) and 36 (C) month follow-up

CST IV is associated with higher rates of persistence compared to regression at all time points.

Table 5.2. Outcomes according to baseline CST at 12, 24 and 36 month follow-up

Follow-up = 12 months, n = 87				
	Regression, n = 39	**Persistence, n = 47**	**Progression, n = 1**	**p value**
CST I (*L. crispatus*)	12/39 (30.8)	10/47 (21.3)	0/1 (0)	0.2934
CST III (*L. iners*)	18/39 (46.2)	18/47 (38.3)	0/1 (0)	**0.0306**
CST IV (*Lactobacillus* spp. deplete, high diversity)	9/39 (23.1)	19/47 (40.4)	1/1 (100)	0.1661
Follow-up = 24 months, n = 67				
	Regression, n = 50	**Persistence, n = 12**	**Progression, n = 5**	**p value**
CST I (*L. crispatus*)	17/50 (34.0)	2/12 (16.7)	1/5 (20.0)	0.1025
CST III (*L. iners*)	18/50 (36.0)	4/12 (33.3)	2/5 (40.0)	0.0603
CST IV (*Lactobacillus* spp. deplete, high diversity)	15/50 (30.0)	6/12 (50.0)	2/5 (40.0)	0.1286
Follow-up = 36 months, n = 41				
	Regression, n = 36	**Persistence, n = 2**	**Progression, n = 3**	**p value**
CST I (*L. crispatus*)	9/36 (25.0)	1/2 (50)	0/3 (0)	0.1708
CST III (*L. iners*)	13/36 (36.1)	0/2 (0)	1/3 (33.3)	**0.0431**
CST IV (*Lactobacillus* spp. deplete, high diversity)	14/36 (38.9)	1/2 (50)	2/3 (40.0)	**0.0226**

CST: Community state type, L.: Lactobacillus

CST did not vary significantly according to HPV status at baseline, although there was a trend for women with HPV18 to have a great proportion of CST IV (Table 5.3).

Table 5.3. CST according to HPV status and genotype

	HPV +ve, n=79	**hrHPV +ve, n=73**	**HPV16 +ve, n = 30**	**HPV18 +ve, n = 8**	**HPV -ve, n=6**
CST I (*L. crispatus*)	19/79 (24.1)	18/73 (24.7)	6/30 (20.0)	2/8 (25.0)	3/6 (50.0)
CST III (*L. iners*)	34/79 (43.0)	31/73 (42.5)	14/30 (46.7)	2/8 (25.0)	1/6 (16.7)
CST IV (*Lactobacillus* spp. deplete, high diversity)	26/79 (32.9)	24/73 (32.8)	10/30 (33.3)	4/8 (50.0)	2/6 (33.3)

CST: Community state type, hrHPV: high-risk Human Papilloma Virus, L.: Lactobacillus

5.3.2 CST IV is associated with slower clearance of CIN2.

Of the 87 women included in the cohort, 44.7% had regressed by 12 months (39/87), 66.7% (58/87) by 24 months, and 73.5% (64/87) by 36 months. At 12 months, 54.5% of women with CST I at baseline had regressed (12/22), 50.0% with CST III (18/36) and 31.0% with CST IV (9/29). At 24 months, of those with CST I at baseline 77.3% had regressed (17/22), 66.6% with CST III (24/36) and 58.6% with CST IV (17/29). At 36 months, 81.8% of women with CST I at baseline had regressed (18/22), 75.0% with CST III (27/36) and 65.5% with CST IV (19/29). Time to clearance of CIN according to vaginal microbiota composition is shown in Figure 5.3. Women with CST IV had a tendency to regress at a slower rate than women with a *Lactobacillus* spp. dominant microbiome and this was approaching significance (p=0.0964, Log-rank test).

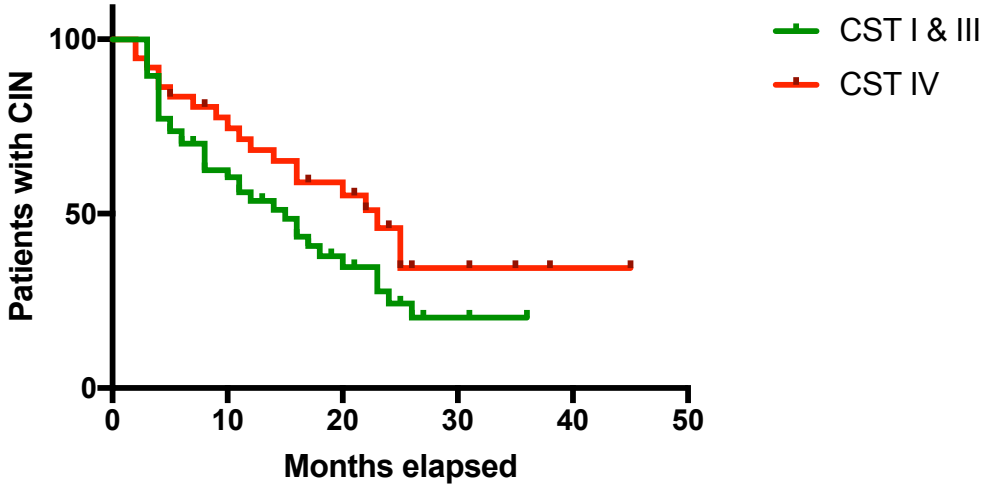


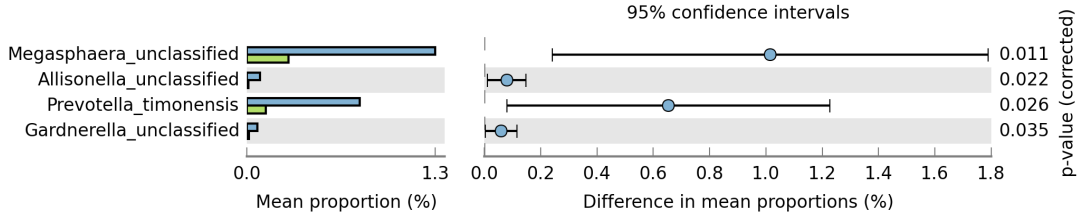
Figure 5.3. Time to clearance of CIN according to CST

Lactobacillus spp. dominant microbiome was associated with a faster rate of regression of CIN compared to a CST IV vaginal microbiome and this was approaching significance (p=0.0964, Log-rank test).

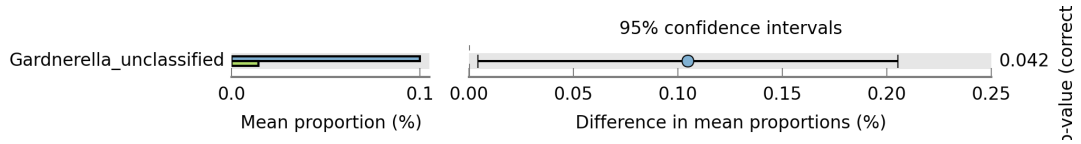
5.3.3 Specific species are associated with outcomes at 12, 24 and 36 month follow-up

Megasphaera spp. (p=0.011), *Allisonella* spp. (p=0.022), *Prevotella timonensis* (p=0.026) and *Gardnerella* spp. (p=0.035) were significantly more abundant in samples of women who had persistent disease at 12 months compared to those who regressed (Figure 5.4A). *Gardnerella* spp. was also significantly associated with persistence at 24 months (p=0.042) (Figure 5.4B). *Lactobacillus iners* was associated with regression by 36 months compared to persistence (p=0.00471(Figure 5.4C). It was not possible to identify any significant species associated with progression due to the small number of women who had progressed to CIN3 at each time point.

A – 12 months



B – 24 months



C – 36 months

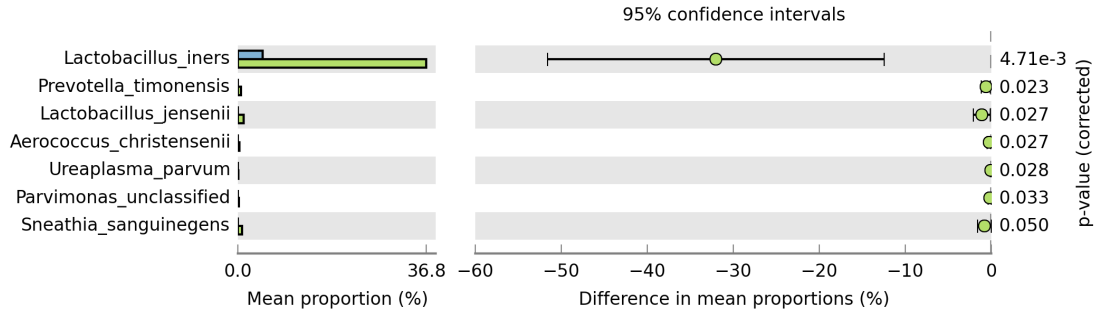


Figure 5.4. Significant species associated with persistence and regression at 12 (A), 24 (B) and 36 (C) month follow-up

Gardnerella spp. was associated with persistence at 12 and 24 months. *Lactobacillus iners* was associated with regression at 36 months. Corrected P-values were calculated using Storey's FDR approach.

Blue = persistence, Green = regression

5.4 Discussion

One of the current limitations of ongoing research into the 'oncobiome'; that is the microbiota associated with cancer development, is the lack of longitudinal studies to help differentiate causality from association [275]. This chapter represents a preliminary analysis of the impact of vaginal microbiota composition on the fate of CIN2 in a highly novel group of 87 young, ethnically-diverse American women who are known to have engaged in high-risk sexual behaviours. The data suggest that the composition of the vaginal microbiota at the time of CIN2 diagnosis may have an impact on the outcome of the disease and that CST IV may be associated with slower disease progression and a greater risk of eventual progression.

Vaginal *Lactobacillus* spp. prevent colonization of bacterial vaginosis-associated bacterial species through maintenance of a low pH [194-197], and bacteriocin production [294-296] An acidic environment can inhibit growth of several potentially pathogenic species, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Gardnerella vaginalis* [194-197], yet provides optimal support for cellular metabolic function of the cervix and the vagina [198]. This is important for maintenance of the cervical epithelial barrier function that inhibits entry of HPV to the basal keratinocytes [277]. When BV-associated strict anaerobes are able to colonise, they produce enzymes and metabolites, which may compromise this barrier, facilitating HPV entry. They also act on several cellular pathways that have been associated with increased levels of proinflammatory cervical cytokines [297-300] that enable a persistent, productive viral infection and subsequent disease development and progression [301-305] on a background of chronic inflammation, which is well-documented to promote neoplasia [306]. CST IV and CST III have both previously been associated with increased acquisition and persistence of HPV infection in a 16-week longitudinal study of sexually-active women who were not known to have cervical disease [202]. Although a control population permitting comparison of average VMB composition was not available, we observe comparatively high rates of CST III and CST IV, and lower rates of CST I previously seen in our cohort described in Chapter three. This may be explained by the relatively high-risk sexual behavioural characteristics of the women described in this chapter and as evidenced by higher rates of previous *Chlamydia trachomatis* infection, pregnancy and anal intercourse compared to the general population at the time of recruitment [292], as well as when compared to a control population without cervical disease [293]. Unprotected sexual intercourse is known to have an impact on the composition and stability of the vaginal microbiota, and was shown to increase the risk of *Gardnerella vaginalis* and *L. iners* colonisation in a longitudinal study of

52 sexually-active women [307], and to result in reduced abundance of *L. crispatus* [190]. This supports a mechanism which puts these women at higher risk of HPV acquisition, persistence and disease development. However, we did not observe any significant differences in VMB composition according to HPV status, although women with HPV 18 were more likely to have CST IV compared to those infected with other subtypes, yet this was not significant and may be a result of a relatively modest sample size.

It is intriguing that CST III was associated with a higher chance of regression at 12, 24 and 36 months, and that *L.iners* was a species found to be significantly associated with regression by 36 months. It has been suggested that *L.iners* does not posess many of the protective mechanisms of other *Lactobacillus* spp. and it also appears more capable of surviving and adapting to a wide range of pH and other metabolic stress-related conditions due to the constitutive and inducible expression of genes not seen in other lactobacilli [159, 160]. This may explainthe relative instability of this CST in comparison to other *Lactobacillus* spp.-dominant CSTs [158], allowing growth of strict anaerobes resulting in transition to CST IV, which as previously discussed is commonly found in association with dysplasia [229, 269, 279]. Furthermore *L.iners* exhibits quite a different metabolic profile depending on whether it grows in the absence or presence of strict anaerobes typically found in CST IV [160]. Classification of the vaginal microbiota structure into the five CST's originally described by Ravel *et al.* likely oversimplifies the comparison of the microbiota structure between women and therefore further investigation is required to determine whether subtle differences in composition and diversity of CST's dominated by *L.iners* yet with presence of other species, may in fact explain why some women with CST III regress quickly whilst others do not.

Our results indicate a relationship between CST IV-associated anaerobes and persistence and progression. This is consistent with a cross-sectional cohort of women with cervical disease previously described by Oh et al. [269] who included women with LSIL or HSIL on cytology vs normal controls (defined as normal or Atypical Squamous Cells of Undetermined significance (ASCUS) cytology). Their results suggested that microbiome patterns determined by paucity of *L. crispatus* and occupied predominantly by *A. vaginae* and secondarily *by G. vaginalis* and *L. iners* were associated by an almost 6-fold increase in the risk of cervical LSIL/HSIL disease (higher vs lower tertile, Odds Ratio (OR) 5.80, 95%CI 1.73–19.4), and thus the authors defined this as a 'risky microbial pattern' [269]. These clinical data, in addition to the *in vitro* studies mentioned above are clearly suggestive that *Lactobacillus* spp. play a protective role, and

indicate that strict anaerobes have an inflammatory impact on the cervicovaginal environment thus enabling viral entry and facilitating persistence of HPV which is necessary for subsequent high-grade disease, its persistence and progression.

Regression rates in this cohort were high, with 44.7% regressing by 12 months, 66.7% by 24 and 73.5% by 36-month follow-up. This is much higher than many other described cohorts [42], which is likely due to the young age of the included patients, with previously published cohorts of young women also showing similarly higher rates of regression compared to the general population [288]. This supports the principle for screening from an older age, which is often debated in the lay media, because it is clear that screening from a younger age would detect high-grade disease with a very high regressive potential, thus resulting in overtreatment and an excess risk of the reproductive and obstetric outcomes, such as second trimester miscarriage and preterm birth as explored further in Chapter seven. Opinions regarding treatment of CIN2, and safe duration of watchful waiting are often divided, with many people now suggesting that CIN2 may not have such an aggressive potential as CIN3 [308]. Furthermore CIN2 may in fact be a heterogeneous disorder that is caused by both low- and high-risk HPV (hrHPV) subtypes [287, 288], as evidenced by our cohort in whom 7% were HPV negative at the time of CIN2 diagnosis.Our provisional findings suggest the interaction between the vaginal microbiota and natural history of CIN warrants further investigation, because it may be possible in the future to use VMB composition as one of the ways in which to identify women most at risk of persistence and progression, and subsequently most likely to benefit from treatment, consequently preventing unnecessary treatments that may put women at risk of adverse pregnancy outcomes. Whilst many of our findings are suggestive of an association but lack statistical significance, this may be due to the modest sample size, exacerbated by the high rates of regression, even in the presence of CST IV.

There are several potential confounders and limitations of the data presented in this chapter. Firstly, the act of taking a biopsy has been suggested to influence the natural history of CIN. There is some evidence to indicate that taking a biopsy may cause acute inflammation, which has been suggested by some to increase the chance of clearance [45], however this has been debated by others [46]. All patients had a biopsy at the initial visit because a histological diagnosis of CIN2 was a prerequisite for inclusion in the study cohort. The number of subsequent biopsies carried out during follow-up ranged from zero to six, and this therefore may represent a confounding factor not only because the biopsy may alter the natural history,

but it also may uncover a higher number of cases of high-grade disease that could be missed by the relatively low sensitivity of cervical cytology [309, 310].

A further confounder to consider is that it is difficult to ascertain how long a lesion has been present. HPV16 and to a lesser extent the phylogenetically-related subtypes HPV31, 33, 35, 52 and 58 are consistently associated with higher rates of persistence and progression [311], with HPV16 and 18, followed by the aforementioned high-risk non-16/18 subtypes, most commonly detected in cervical cancer cases [312]. This may be difficult to control for due to the logistics of how and when to recruit women. Finally, we do not have a control cohort of disease-free women for comparison. It is clear that these women exhibit certain behavioural characteristics which could both alter their VMB structure and also disease outcomes.

5.5 Conclusion

Cross-sectional studies exploring the associations between the VM, HPV infection and cervical pre-invasive and invasive disease have shown that a high-diversity VM, and to a lesser extent *L. iners*-dominant VM's correlate with increasing cervical disease severity [229, 269, 270], and in acquisition and persistence of its causative agent HPV [202]. This chapter describes a novel longitudinal cohort of 87 young women with CIN2. Whilst there were high rates of regression, the preliminary analysis of these data suggest that absence of *Lactobacillus* spp. and presence of a diverse population of strict anaerobes may be associated with slower disease regression. There are several plausible mechanisms for how this may arise, largely related to the development of a proinflammatory environment, which will be further explored in Chapter six. The findings discussed here may be useful for future triage of patients in order to prevent over-treatment of lesions destined to regress, which may put them at unnecessary risk of the adverse pregnancy outcomes to be discussed in Chapter seven.

CHAPTER 6. Antimicrobial peptides and cervical inflammatory cytokines are altered in CIN

Content from this chapter is currently under preparation as:

Mitra A, MacIntyre DA, Whelan E, Lee YS, Smith A, Marchesi JR, Lyons D, Bennett PR and Kyrgiou M. Cervical intraepithelial neoplasia is associated with an altered vaginal microbiome and innate immune disruption that is not altered following excision of disease. (*Under preparation)*

6 Antimicrobial peptides and cervical inflammatory cytokines are altered in cervical intraepithelial neoplasia

6.1 Background

Antimicrobial peptides (AMPs) are small, cationic peptides that play a role in innate immune protection at various mucosal surfaces, including the lung, gastrointestinal tract as well as the female reproductive tract. They are sometimes referred to as 'endogenous antibiotics' because their cationic nature enables them to selectively attract and destroy the anionic phospholipid and lipopolysaccharide (LPS)-containing microbial membranes [206]. They have also been shown to demonstrate antiviral activity against non-enveloped viruses such as HPV through their ability to inhibit various points in the viral entry process and replication within human cells [207]. AMPs are also involved in tissue homeostasis, wound healing and scarring and subsequently their expression is both constitutive, to provide prophylactic background protection, and inducible at times of infection, inflammation or tissue injury [208].

Human beta-defensin-1 (hBD-1) is both constitutively and inducibly expressed throughout the female reproductive tract [211], and displays broad spectrum antimicrobial activity, particularly against Gram-negative species including *Ureaplasma* spp. [215] and *Escherichia coli* [216] as well as the yeast, *Candida albicans* [217]. Polymorphisms in the DEFB1 gene that encodes hBD-1 have been associated with increased susceptibility to HPV infection [213] and upregulated hBD-1 expression has been observed in low-risk HPV-mediated genital warts [214].

Secretory leucocyte protease inhibitor (SLPI) is also expressed in both a constitutive and inducible manner [220] by squamous and glandular cervical epithelium [219], and by immune cells including neutrophils and macrophages [220, 221]. It is both anti-inflammatory and antimicrobial and has been shown to possess potent anti-HIV [222] and anti-HSV activity [223] and up-regulation is seen in cervical cancer [225]. Interestingly bacterial vaginosis has been associated with lower levels of SLPI and we have recently shown that an increasing prevalence of a BV-like, high-diversity microbiome with *Lactobacillus* spp. depletion is associated with increased CIN severity [229]. It is possible that such microbiome structures may interact with the innate immune system via AMP modulation to facilitate HPV persistence and disease development and progression.

The expression of antimicrobial peptides has not been examined in women with cervical disease. Increased expression may represent a point at which the innate immune system is disturbed facilitating a chronic HPV infection and subsequent carcinogenesis.

Smoking is a well-defined risk factor for chronic HPV infection, CIN and development of cervical cancer based on numerous epidemiological studies [51, 54]. There is also a wealth of *in vitro* data supporting a mechanistic link between smoking and HPV-related cervical disease. Nicotine and its carcinogenic metabolites can be detected in cervical mucous [56] and smoking appears to have an immunosuppressive effect on the cervical environment with a decrease in Langerhan's cells observed in the cervical epithelium of smokers [57]. The impact of smoking on cervical antimicrobial peptides has not previously been described, and may contribute to the immunosuppressive effect, thus facilitating microbial dysbiosis, as well as HPV persistence.

6.2 Aims

- Assess the expression of hBD1 and SLPI in women with CIN and compare to healthy controls.
- Correlate AMP levels with the vaginal microbiome

6.3 Results

A total of 232 women recruited at Imperial College London according to the criteria in section 2.3.1 were included in the analysis and classified into three groups; 34 normal, 81 LSIL, and 117 HSIL. Patient metadata were analysed as shown in Table 6.1.

Table 6.1. Characteristics of included patients

Characteristics	Normal, n=34	LSIL, n=81	HSIL, n=117	Total, n=232	*P* value
Age					**0.26**
Mean (SD, range)	30 (4.415, 20-43)	31 (5.348, 22-45)	31 (4.662, 25-43)	31 (4.889, 20-45)	
Ethnicity					**0.12**
Caucasian	26/34 (77)	71/81 (88)	98/117 (84)	195/232 (84)	
Asian	3/34 (1)	5/81 (6)	13/117 (11)	19/232 (9)	
Black	5/34 (14)	5/81 (6)	6/117 (4)	18/232 (7)	
Parity					**0.51**
Nulliparous	27/34 (81)	59/81 (73)	84/117 (72)	170/232 (76)	
Parous	7/34 (19)	22/81 (27)	33/117 (28)	62/232 (24)	
Smoking					**0.52**
Current smoker	5/34 (15)	20/81 (25)	29/117 (25)	54/232 (23)	
Non-smoker	29/34 (85)	61/81 (75)	88/117 (75)	178/232 (77)	
Menstrual cycle					**0.35**
Luteal	12/34 (35)	42/81 (52)	58/117 (50)	112/232 (54)	
Follicular	18/34 (55)	35/81 (43)	48/117 (41)	101/232 (39)	
Unknown	4/34 (10)	4/81 (5)	11/117 (9)	19/232 (7)	
Contraception					**0.96**
Nil	16/34 (47)	29/81 (36)	36/117 (32)	81/232 (35)	
Condoms	5/34 (15)	16/81 (19)	21/117 (19)	42/232 (19)	
COCP	9/34 (26)	22/81 (26)	39/117 (34)	70/232 (30)	
POP	1/34 (3)	4/81 (5)	6/117 (4)	11/232 (4)	
Copper IUD	0/34 (0)	3/81 (4)	4/117 (3)	7/232 (3)	
Mirena IUS	2/34 (6)	4/81 (5)	6/117 (4)	12/232 (5)	
Vaginal Ring	0/34 (0)	0/81 (0)	1/117 (1)	1/232 (1)	
Contraceptive implant	1/34 (3)	2/81 (3)	4/117 (3)	7/232 (3)	
Contraceptive injection	0/34 (0)	1/81 (2)	0/117 (0)	1/232 (1)	
Time since intercourse					**0.74**
>48 hours	30/34 (87)	69/81 (85)	98/117 (84)	197/232 (85)	
<48 hours	4/34 (13)	12/81 (15)	19/117 (16)	35/232 (15)	

CIN: Cervical intraepithelial neoplasia; COCP: Combined oral contraceptive pill; IUD: Intrauterine device; IUS: Intrauterine system; POP: Progesterone-only pill

There was equal distribution in the samples collected at the follicular or luteal phase of the cycle and in the rate of women that had intercourse within 48 hours from sample collection. For the untreated and pre-treatment analysis cohort histology was available in all HSIL cases (100%), and for 72% (58/81) and 9% (3/34) of LSIL and normal cases, respectively. Where histology was not available cytology was used for patient classification and was available in 100% of cases.

6.3.1 hBD-1 and SLPI levels are increased in high-grade CIN compared to healthy controls

Total levels of hBD1 and SLPI were measured from high vaginal swabs and normalised to total protein levels. There was no significant difference in total protein concentration according to disease severity (p=0.7513, Kruskal-Wallis test) (Table 6.2, Figure 6.1a). Levels of both hBD1 and SLPI increased in women with HSIL who displayed significantly greater levels of both peptides, compared to healthy controls (p=0.0129 and p=0.0308 respectively, Kruskal-Wallis test) (Table 6.2, Figure 6.1b & c).

HPV status was known for 109 women and did not have a significant impact on hBD-1 or SLPI levels across the cohort (p=0.5888, p=0.3896, unpaired t-test). Similarly, no differences were detected between sub-group analyses of women classified as either normal/LSIL HPV negative, or normal/LSIL HPV positive (p=0.6704, p=0.1879, unpaired t-test) (Table 6.1, Figure 6.2, data shown only for hBD-1).

Given that these peptides are produced by the squamous and columnar cervical epithelium an analysis was performed to determine whether levels correlate with cervical length and volume. However, no correlation between levels of hBD1 or SLPI and either measurement, as determined by transvaginal ultrasound scanning was observed.

Table 6.2. Total protein, hBD-1:total protein and SLPI:total protein according to disease severity

	Total protein (µg/ml) (median ± IQR)	**hBD-1:total protein (pg/µg protein)** (median ± IQR)	**SLPI:total protein (pg/µg protein)** (median ± IQR)
Normal, n = 34	619 ± 409	2096 ± 1164	90486 ± 41324
LSIL, n = 81	552 ± 314	3642 ± 1715	140764 ± 71098
HSIL, n = 117	546 ± 345	4002 ± 2423	164094 ± 60236
Any disease state, HPV negative, n = 23	602 ± 389	4034 ± 2036	117386 ± 52243
Any disease state, HPV positive, n = 86	533 ± 311	3783 ± 2038	143125 ± 61573
N/LSIL, HPV negative, n = 19	588 ± 377	2740 ± 1677	59020 ± 43070
N/LSIL, HPV positive, n = 29	578 ± 359	2802 ± 1482	126744 ± 65386

IQR; interquartile range

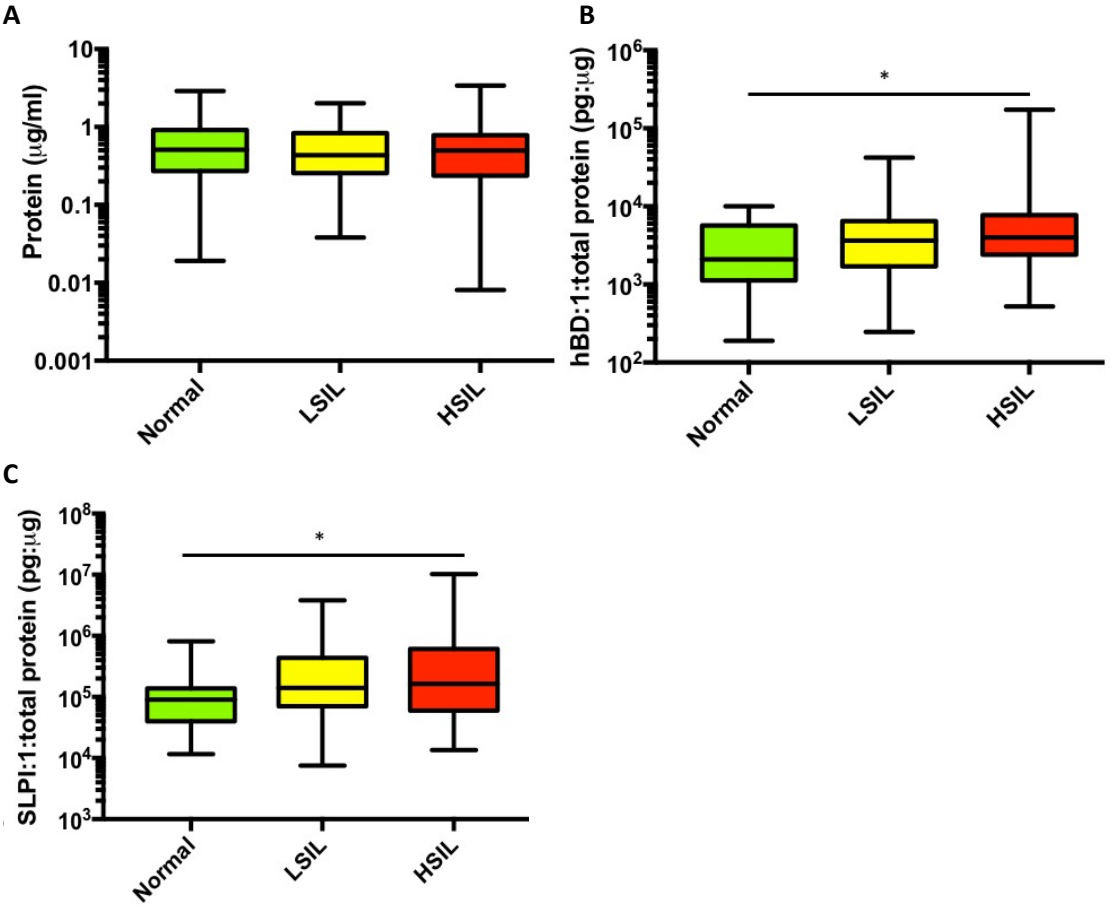


Figure 6.1. Total protein levels (A), hBD-1:total protein (B) and SLPI:total protein (C) according to disease severity

There was no significant difference in total protein levels according to disease severity. Levels of hBD-1 and SLPI were both significantly higher in women with HSIL compared to normal controls. Kruskall-Wallis test, * $p<0.05$.

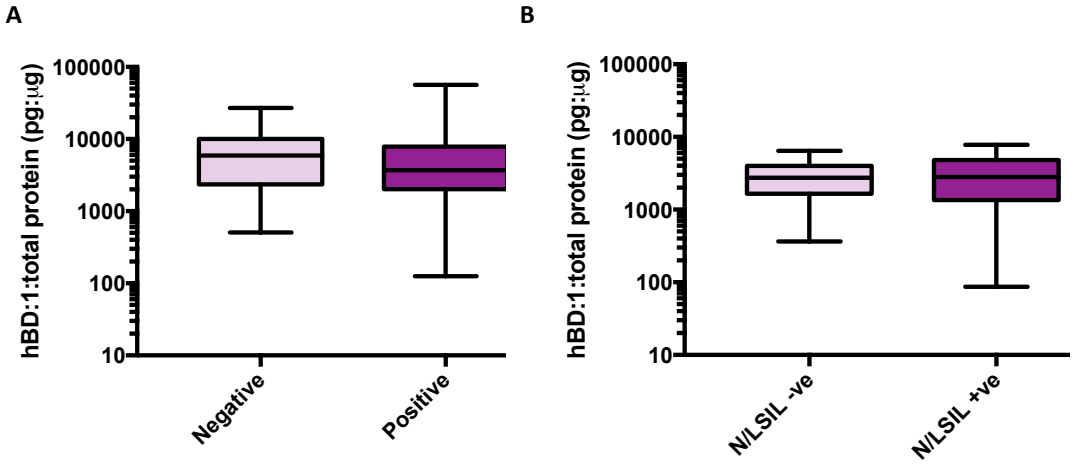


Figure 6.2. hBD-1 levels according to HPV status (A) and disease and HPV status (B)

There was no significant difference hBD-1 according to HPV status, and in a subgroup analysis of women who were normal or had LSIL according to HPV status. Kruskall-Wallis test.

6.3.2 The influence of the vaginal microbiota on AMP levels

An analysis was performed comparing hBD1 and SLPI levels across the three most common CST's; CST I, III and IV, according to disease status. There was a trend for levels of both peptides to be higher in CST III and IV, compared to women with CST I. Levels of hBD1 were significantly higher in women with HSIL and CST IV compared to normal controls with the same CST (p=0.0101, Kruskal-Wallis test) (Figure 6.4).

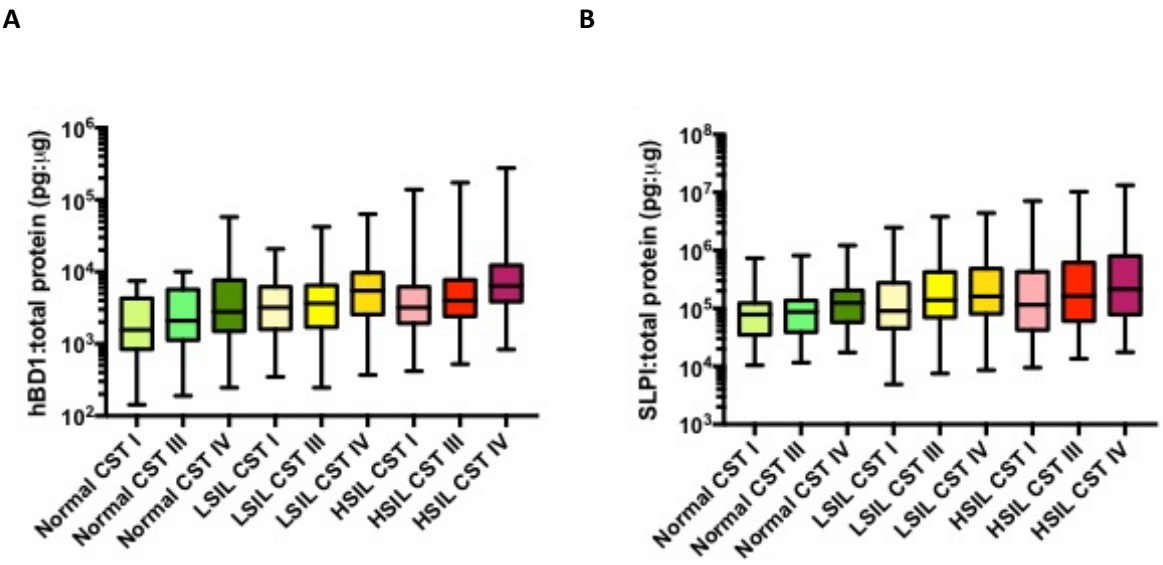


Figure 6.3. hBD-1:total protein (A) and SLPI:total protein (B) levels

There was a trend for levels of both peptides to be higher in CST III and IV, compared to women with CST I in each disease state. Kruskall-Wallis test.

Table 6.3. hBD-1:total protein and SLPI:total protein according to disease severity and microbiome status

	hBD-1:total protein (pg/μg protein) (median ± IQR)	**SLPI:total protein (pg/μg protein)** (median ± IQR)
Normal CST I, n = 17	1572 ± 843	78006 ± 34844
Normal CST III, n = 12	2096 ± 1123	86673 ± 38716
Normal CST IV, n = 5	2791 ± 1496	124291 ± 57876
LSIL CST I, n = 41	3192 ± 1587	90788 ± 45505
LSIL CST III, n = 21	3642 ± 1711	139673 ± 70008
LSIL CST IV, n = 19	5464 ± 2567	160624 ± 80510
HSIL CST I, n = 48	3202 ± 1925	114933 ± 42212
HSIL CST III, n = 34	4002 ± 2407	164190 ± 60303
HSIL CST IV, n = 32	6404 ± 3851	213447 ± 78393

6.3.3 Proinflammatory cytokine levels are elevated in high-grade disease

Levels of pro- and anti-inflammatory cytokines were examined as potential modulators of AMP expression. In women with HSIL, levels of interleukin (IL)-1β and IL-8 were significantly greater compared to healthy controls (p=0.0003 and p=0.0070 respectively, Kruskall-Wallis test), whereas interferon (IFN)-γ levels were significantly lower in women with HSIL compared to both LSIL and normal controls (0<0.0001 and p=0.0103 respectively, Kruskall-Wallis test) (Table 6.4, Figure 6.5). Tumour necrosis factor (TNF)-α was lower in LSIL compared to HSIL (p=0.0008, Kruskall-Wallis test) (Table 6.4, Figure 6.4).

There was no significant difference in IL-1α, IL-2, IL-4, IL-6 or IL-10 levels according to disease severity. No association between any single cytokine and hBD-1 or SLPI levels was indentified although the trends in expression level were similar for IL-1β and IL-8.

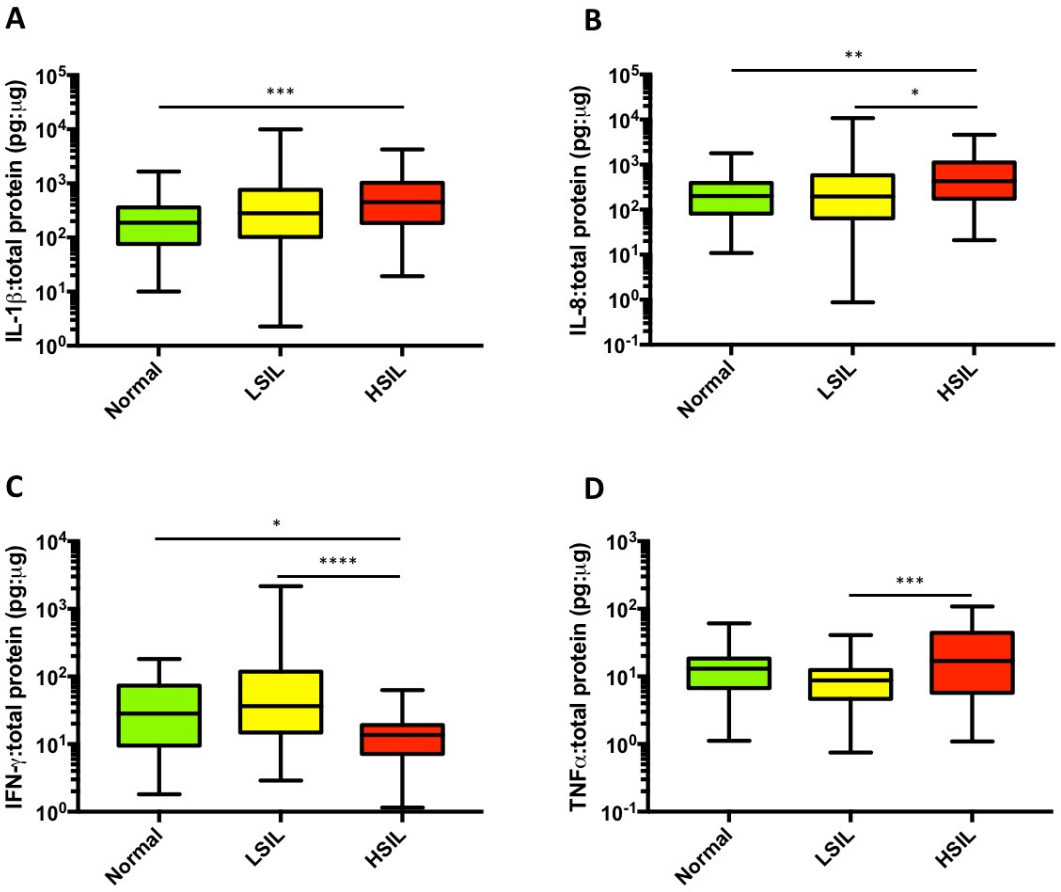


Figure 6.4. Levels of IL-1β (A), IL-8 (B), IFN-γ (C) and TNF-α (D) according to disease severity.

Interleukin (IL)-1β and IL-8 levels were significantly greater in HSIL compared to healthy controls (A&B). Interferon (IFN)-γ levels were significantly lower in women with HSIL compared to both LSIL and normal controls (C). Tumour necrosis factor (TNF)-α was lower in LSIL compared to HSIL (D).
Kruskall-Wallis test, * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table 6.4. Cytokine levels according to disease state and vaginal microbiome composition

	IL-1β:total protein (pg/μg protein) (median ± IQR)	**IL-8:total protein (pg/μg protein)** (median ± IQR)	**Interferon-γ pg/μg protein)** (median ± IQR)	**TNF-α:total protein (pg/μg protein)** (median ± IQR)
Normal, n = 34	187.9 ± 284.1	199.9 ± 309.5	28.2 ± 74.5	13.1 ± 11.4
LSIL, n = 81	281.1 ± 658.1	196.7 ± 511.8	36.4 ± 114.1	8.8 ± 7.6
HSIL, n = 117	447.6 ± 835.4	431.4 ± 933.5	13.4 ± 12.1	19.9 ± 38.4
Normal CST I, n = 16	114.5 ± 177.3	146.7 ± 232.2	31.0 ± 65.3	13.1 ±15.7
Normal CST III, n = 12	222.5 ± 345.2	215.2 ± 341.7	32.5 ± 73.6	6.7 ± 9.8
Normal CST IV, n = 5	785.4 ± 1236.1	559.5 ±647.1	37.8 ± 77.5	22.1 ± 16.7
LSIL CST I, n = 39	236.1 ± 581.0	170.8 ± 436.3	32.8 ± 93.7	13.5 ± 26.3
LSIL CST III, n = 21	153.6 ± 378.8	238.8 ± 610.6	38.3 ± 108.4	13.9 ± 54.6
LSIL CST IV, n = 19	370.4 ± 584.6	223.2 ± 448.5	15.9 ± 20.3	9.9 ± 12.0
HSIL CST I, n = 47	276.3 ± 598.0	355.7 ± 834.0	15.3 ± 14.3	9.1 ± 11.1
HSIL CST III, n = 33	489 ± 1058.0	449.7 ± 1054.4	11.9 ± 11.1	9.1 ±23.3
HSIL CST IV, n = 32	734 ± 1589.3	581.7± 1364.4	13.1 ± 25.2	25.2± 44.8

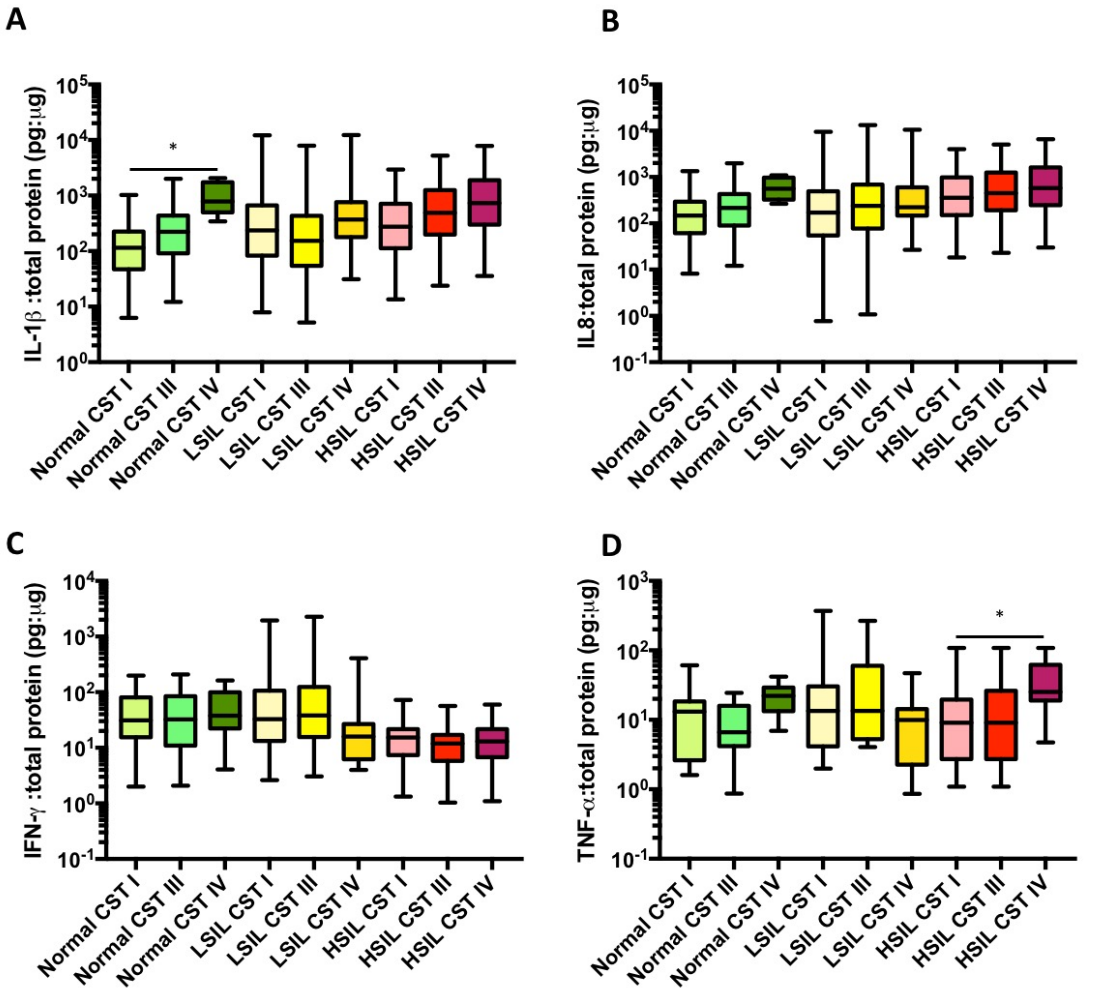


Figure 6.5. Levels of IL-1β (A), IL-8 (B), IFN-γ (C) and TNF-(D) according to microbiome structure

IL-1β was significantly higher in healthy controls with CST IV compared to those with CST I (A). IL-8 and IFN-γ were not significantly different according to CST (B&C). There was no significant difference in TNF-α was higher in women with HSIL who had a CST IV vaginal microbiome structure, and approached significance between those with CST III and IV (D). Kruskall-Wallis test, *p<0.05

When cytokine levels were examined in relation to vaginal microbiome structure, IL-1β was found to be significantly higher in healthy controls with CST IV compared to those with CST I (p=0.0178, Kruskal-Wallis test). A similar pattern was seen in women with HSIL but did not reach statistical significance (p= 0.0725, Kruskal-Wallis test). TNF-α was higher in women with HSIL who had a CST IV vaginal microbiome structure, and approached significance between those with CST III and IV (p=0.0200 and p=0.0693, Kruskal-Wallis test).

6.3.4 The influence of smoking on AMP levels

The impact of cigarette smoking was assessed within each disease state. There was a total of 54 smokers (Normal 5/34 (14.7%), LSIL 20/81 (24.7%), HSIL 29/117 (24.8%)). Levels of hBD1 was significantly lower in smokers classed as normal or LSIL (p=0.0005 & p=0.0006 respectively, unpaired t-test). There was a trend for hBD1 to be lower in smokers with HSIL which was approaching statistical significance (p= 0.0703, unpaired t-test). There did not appear to be a clear correlation between SLPI levels and smoking status (Table 6.3)

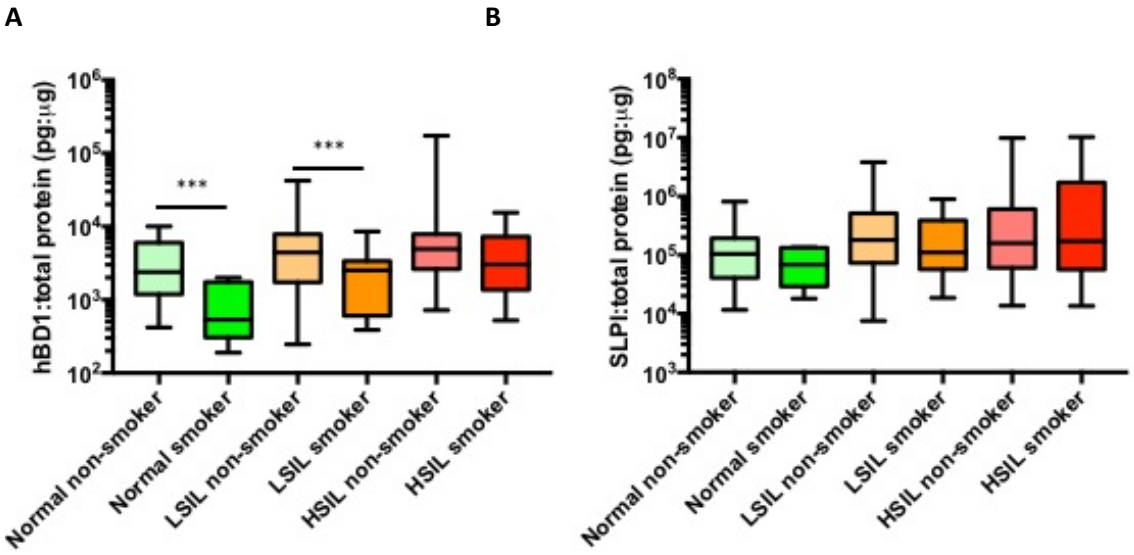


Figure 6.6. The impact of smoking on hBD1 (A) and SLPI (B) levels

hBD1 was significantly lower in normal or LSIL patients who smoked (unpaired t-test). There was a trend for hBD1 to be lower in smokers with HSIL (unpaired t-test) (A). There was no clear correlation between SLPI levels and smoking status (B). Unpaired t- test, ***p<0.001.

Table 6.5. hBD-1 and SLPI levels according to disease severity and smoking status

	hBD-1:total protein (pg/ µg protein) (median ± IQR)	**SLPI:total protein (pg/ µg protein)** (median ± IQR)
Normal, non-smoker, n= 5	2396 ± 1193	103936 ± 40598
Normal, smoker, n = 19	5369 ± 304	68574 ± 29018
LSIL, non-smoker, n= 20	4406 ± 1735	180951 ± 73179
LSIL, smoker, n = 61	2525 ± 606	110675 ± 57048
HSIL, non-smoker, n= 29	4915 ± 2611	159355 ± 60236
HSIL, smoker, n = 88	3036 ± 1360	171763 ± 56676

6.4 Discussion

AMPs play a key role in the innate immune system of the cervix, and are inducible in the presence of infection and inflammation, which is important for protection against viral and bacterial pathogens. HPV is necessary for cervical carcinogenesis, but the data presented in Chapters three and five suggest bacteria also play a role in influencing disease outcomes. The data presented in this chapter show that levels of hBD1 and SLPI both increase with increasing disease severity in our study population. This observation suggests that cervical tissue retains the ability to secrete these peptides and afford protection against pathogens with increasing disease severity. Just as HPV is known to evade immune recognition through increased expression of anti-inflammatory cytokines [24], it may be possible for the virus to induce expression of these AMPs as a further layer of immune evasion, ensuring survival and replication of the virus within the cervical epithelium, resulting in on-going infection and development of clinically significant lesions. However, there was no significant difference in AMP levels in the context of HPV status in a sub-group analysis of women who had LSIL or were healthy controls, which suggests other mechanisms besides the viral infection may also be associated with their increased expression in higher disease states.

A further factor to consider is the activity of the proteins present. Post-translation regulation of SLPI activity in particular can be modulated through cleavage by other extracellular proteases including matrix metalloproteinases, cathepsins and neutrophil elastase, which has been demonstrated in the respiratory epithelium to reduce SLPI activity and furthermore the cleaved protein has been shown to exhibit proinflammatory properties [313], which could facilitate HPV persistence and carcinogenesis. Polymorphisms in the DEFB1 gene encoding hBD-1 have been associated with increased susceptibility to HPV infection [213] and therefore higher protein levels may not directly correlate with optimal function. A limitation the ELISA technique used to quantify levels is that it does not give any information about protein activity levels. Future work could focus on evaluating the factors regulating both AMP expression and activity to better understand whether these peptides play a role in determining cervical disease outcome.

Carcinogenesis involves modification of the tissue environment to facilitate tumour cell growth, invasion, angiogenesis and metastasis, which involves extensive tissue remodelling [314]. Cancer cells, and their surrounding stroma produce proteases to assist in this transformation, which subsequently results in production of signalling molecules such as

cytokines and growth factors to support this process. One would therefore expect protease inhibitors to be tumour suppressors, yet they are often overexpressed by cancer tissues [315]. Specifically, SLPI overexpression has been documented in cervical cancer, as well as breast, endometrial, lung and ovarian cancers [316], and has been shown to be associated with poor prognosis in colorectal cancer [317]. CIN can be regarded as a novel entity in the female reproductive tract, because along with only a small handful of other disease in the body it represents an identifiable precancerous state that can be relatively easily identified by screening and thus studied to understand the changes that take place prior to development of overt invasive disease. Both AMPs studied were increased with increasing disease severity however we did observe a large range in levels. It is well known that a large number of cases of high-grade disease will ultimately regress and it is estimated that if untreated, 12% of cases of CIN3 will eventually progress to cancer [42]. It is therefore tempting to speculate that higher levels may correlate with lesions that have a greater progressive potential, but given that the majority of these premalignant lesions are removed to prevent progression to invasive disease, such a theory will be difficult to prove. The role of hBD1 in cancer development is less well understood, and levels have been shown to be elevated in lung and pancreatic cancer tissue, but decreased in renal and prostate cancers [316], and may be a predictor of survival in oral squamous cell carcinoma [318]. Human beta defensin-2, which has a similar structure to hBD1, is known to be overexpressed in high-grade CIN and early squamous cell carcinoma of the cervix with relatively lower levels of expression in normal squamous cells [319]. The same study also used HeLa cells, an HPV18 positive adenocarcinoma cell line, to demonstrate that hBD2 was able to drive cell proliferation in a dose-dependent manner [319]. However far fewer molecular mechanisms have been suggested for how defensins either facilitate or play a co-factor role in carcinogenesis compared to SLPI, and therefore its role in the cervix may be more closely related to its relationship with the viral and bacterial environment.

Bacterial elements including LPS are able to stimulate AMP expression, and in light of the data presented in Chapters three and five suggesting a difference in vaginal microbiome composition according to disease state, we examined the impact of this on AMP expression. CST II and V were infrequently observed in our patient cohort (Chapter three) along with in other published studies [153, 320], and analysis was thus restricted to CSTs I, III and IV. A trend for increased levels of both hBD-1 and SLPI in women with CST IV which suggests the vaginal microbiome may partly influence their production, but the cervical disease state appears more influential. It is interesting to note that CST III, which is dominated by *Lactobacillus iners*,

is often regarded as an 'unhealthy' *Lactobacillus* species [159], and its presence in this study was generally associated with higher AMP and proinflammatory cytokine levels. It is also interesting to note that particular strains of *L. iners* have been shown to be more susceptible to AMP induced-death compared to *L.crispatus* and *L.jensenii* in *in vitro* studies using LL-37, an intrinsic AMP belonging to the cathelicidin family [321]. Not only does this support the concept of *Lactobacillus* spp. having a protective niche in the human vagina, it also suggests that certain strains of *L.iners* are better tolerated in the vagina than others.

Both CIN and bacterial vaginosis, which can be likened to CST IV, have been associated with increased levels of proinflammatory cervical cytokines [297-300]. Bacteria can stimulate monocytes, macrophages and dendritic cells to produce hBD1 via lipopolysaccharide (LPS) and interferon (IFN)-γ [322], which is a proinflammatory cytokine that is critical to the antiviral response. In our cohort, there was no correlation between hBD1 and IFN-γ levels. Instead, IFN-γ levels were lower in women with higher grade disease, which is in keeping with previously observations in both high-grade CIN [297] and cervical cancer [323]. High-risk HPV is able to down-regulate this interferon response via the E6 and E7 oncoproteins [31, 32], which suggests there are other factors inducing hBD1 levels in these patients. Epithelial cells are known to produce defensins in response to other proinflammatory cytokines, such as IL-1β, and TNF-α [324], both of which were increased according to disease severity, but also with presence of a CST IV, high-diversity, *Lactobacillus* spp. deplete vaginal microbiome, which is seen at a higher frequency in women with high grade disease as discussed in Chapter three. Bacterial vaginosis has previously been correlated with higher levels of IL-1β [303], IL-8 [325] and TNF-α [326] and also suggested as one of the mechanisms contributing to human immunodeficiency (HIV) transmission [326], and may provide an putative link between BV and HPV-related disease that warrants further investigation. One previous study has aimed to specifically correlate the vaginal microbiome composition with cytokine production in women with CIN, and found IL-10 mRNA levels to be higher in women with CIN compared to healthy controls, and noted that IL-4 and TGF-β 1 mRNA levels were higher in microbiomes dominated with *Fusobacterium* spp. although only in two samples. Furthermore, there are some clear methodological flaws in the study, largely based around sample collection techniques which varied between swabs, cytobrushes and biopsies according to different patient groups, which as shown in Chapter four could be a confounding factor.

There is a general lack of consensus regarding how the cytokine profile changes in both cervical disease and in the context of vaginal microbial community composition. One consideration is the heterogeneity surrounding diagnosis of bacterial vaginosis, with use of microscopy techniques being potentially subjective compared to the molecular techniques used to classify microbiome composition in the study described in this chapter. It is pertinent to note however, that it can be difficult to compare levels of cytokines to other studies, due to differences in sample collection (eg, vaginal swabs compared to cervicovaginal lavage samples), processing and also normalisation. In this chapter, all samples were collected by a single clinician using a vaginal swab and the levels of all proteins and cytokines obtained have been normalised to the total protein content of the sample. Differences behavioural and environmental characteristics within patient populations may also be a factor such as phase of the menstrual cycle, contraceptive use, and sexual practice [327]. Analyses were performed to examine these factors, and we did not find any significant differences (data not shown). There is compelling evidence to suggest that the contraceptive injection (medroxyprogesterone acetate) elicits a proinflammatory response in the cervix [328]. However, restricting analyses to types of contraception in our cohort resulted in a very small sample size, which did not yield any significant results.

Another possible reason for the discrepancy between levels in our cohort, and others may be the difference in the stage of the disease process, irrespective of the histological or cytological diagnosis. Whilst the mechanisms of CIN regression are unclear, there appears to be a shift in the balance between the Th1:Th2 response with [24]. Notably the levels detected were the most variable in the LSIL group. There are two plausible explanations for this. Firstly, one would expect women with low-grade disease to be the most heterogeneous in terms of natural history of the disease, including women with acute HPV infections, chronic HPV infections, those who have cleared an HPV infection and are in the process of regressing back to normal, those progressing to high-grade disease, or those with a stable low-grade lesion that may have been present for a long time. All of these different states are likely to be characterised by subtly different cytokine profiles. Secondly, 81% of women with LSIL were classified according to cytology, compared to 100% in the HSIL group, with histology, which is more accurate being used in the remainder of cases. This is due to histology being taken only due to clinical need, but may result in some inaccuracy in the classification of disease status, which overlaps with the first point.

Smoking is known to be associated with an increased risk of chronic HPV infections and

cervical cancer studies [51, 54] and has been shown to induce a degree of immunosuppression in various tissues within the body, including the respiratory mucosa, where AMPs have also been well studied. We have shown a novel interaction between smoking and the innate immune system of the cervix, with hBD1 levels being significantly reduced in smokers. The same pattern has been observed in the oral mucosa of smokers [329]. SLPI upregulation may be seen in the sputum and nasal passages of smokers [330], as well as in smoking-related diseases such as chronic obstructive pulmonary disease (COPD) [331] and lung cancer [332]. However, we did not observe any pattern with SLPI levels and smoking in the cervix in our patient cohort.

6.5 Conclusion

The results of this chapter suggest an interaction between the AMPs, cytokines and vaginal microbiome, which are pivotal to the effective workings of the innate immune response and are likely to modulate the fate of a cervical lesion at some point in its natural history. Whilst these factors have previously been examined in isolation, this is the first study that has attempted to integrate these factors and begin to understand their interplay. Further investigation is required to understand how other parts of the innate immune system, including the immune cells, are affected in HPV infection and cervical dysplasia and how the findings in this chapter may interlink.

CHAPTER 7. Fertility Early and Late Pregnancy Outcomes after Treatment for Cervical Intraepithelial Neoplasia: Systematic Review and Meta-Analysis

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Kyrgiou M, Athanasiou A, Paraskevaidi M, Arbyn M, **Mitra A**, Martin-Hirsch P, Bennett P, Paraskevaidis E. The risk of preterm birth after treatment for cervical pre-invasive and early invasive disease increases with increasing cone length: a systematic review and meta-analysis. *British Medical Journal 2016 Jul 28;354:i3633.*

Kyrgiou M, **Mitra A**, Paraskevaidis E. Fertility and early pregnancy outcomes after treatment for cervical intra-epithelial neoplasia: a systematic review and meta-analysis. *JAMA Oncol. 2016 Jun 23. doi: 10.1001/jamaoncol.2016.1839.*

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Kyrgiou M, **Mitra A**, Arbyn M, Stasinou SM, Martin-Hirsch P, Bennett P, Paraskevaidis E. Fertility and early pregnancy outcomes after treatment for cervical intra-epithelial neoplasia: a systematic review and meta-analysis. *British Medical Journal, 2014;349:g6192*

7 Fertility, Early and Late Pregnancy Outcomes after Treatment for Cervical Intraepithelial Neoplasia: Systematic Review and Meta-Analysis

7.1 Background

Cervical cancer is the 12th most common cancer in women in the UK, however the incidence has fallen 44% since the 1970's through the introduction of an organised screening programme. Current clinical practice in the UK involves excisional treatment to remove the dysplastic cells detected through screening. Meta-analyses [84, 93] and large retrospective linkage studies [333, 334] suggest that these excisional methods of treatment increase the risk of preterm birth, low-birth weight, premature rupture of the membranes and perinatal mortality in subsequent pregnancies; CIN itself and other confounders may contribute to that risk [85, 96, 113]. During the decade following publication of the first meta-analysis describing obstetric risks associated with treatment [84], more than 50 observational studies have been published confirming [335, 336] or disputing these associations [96, 337]; some of these reporting data from large population-based datasets and further systematic reviews and meta-analyses have reached contradictory conclusions [84, 86, 93, 95, 338-341] and initiated debates and confusion amongst the clinical and academic community [93, 338-341]. Whether these discrepancies were due to differences in quality of the studies or heterogeneity in the explored comparisons [95], there is a clear need to perform a comprehensive high-quality synthesis of the existing evidence as to appropriately inform women and clinicians.

Furthermore, the mean age of women undergoing treatment for preinvasive cervical disease is similar to the age of women having their first child and its effect on the ability to conceive and the early pregnancy outcomes has been relatively under-reported [342, 343], yet is often the most common concern voiced by patients in the colposcopy, general gynaecology and obstetric clinics. Second-trimester miscarriage in particular is of relatively low incidence, and therefore large numbers of women are required to give an accurate overview of the risk to treated women, which also prompted the need for a systematic review and meta-analysis.

7.2 Aims

To perform a systematic review and meta-analysis to determine the impact of treatment for CIN on

- Fertility
- Early pregnancy outcomes (<24 weeks gestation)

- Obstetrics outcomes (>24 weeks gestation)
    - According to length and volume of cone excised
    - According to control group e.g. internal, external

7.3 Results

7.3.1 Impact of treatment on fertility and early pregnancy outcomes

One-hundred and sixty-seven studies were identified; 15 of which met the inclusion criteria [126-129, 333, 344-353] and are show in the PRISMA flowchart (Figure 7.1), along with reasons for exclusion of the remaining studies. No unpublished studies could be identified.

There were no randomised controlled studies found. One prospective cohort study was identified [347], and the remaining 14 were retrospective cohorts. Five studies included multiple treatment techniques, six studied the impact of LLETZ, three of CKC, and one of LC. Five studies adjusted the untreated population for known risk factors for adverse reproductive outcomes, one included women with CIN but no treatment, one did not match for any confounders, four used internal controls (the outcomes of the same women before and after treatment, whilst four performed a logistic regression to control for known confounders [126, 127, 333, 350]. Sources of data included national registries, hospital records, and patient questionnaires. The number of participants in the treated and untreated group ranged from 21 to 15108 and 20 to 2164006, respectively. Further characteristics of the included studies and the outcomes examined are shown in Appendix 3.

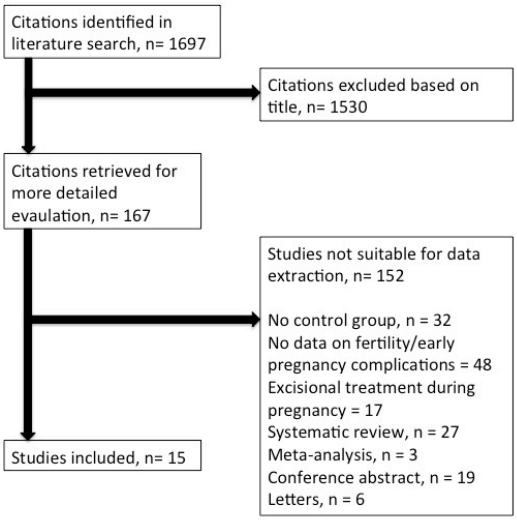


Figure 7.1. PRISMA flowchart for fertility and early pregnancy outcomes

7.3.1.1 Fertility outcomes

The meta-analysis concluded that treatment for CIN does not appear to impair the chance of successful conception. Overall pregnancy rates was higher for treated versus untreated women (43% vs. 38%, 4 studies, 38050 women, RR=1.29, 95%CI 1.02-1.64), although the interstudy heterogeneity was high (I^2 88%) (Figure 7.2). The pregnancy rate in women with an intention to conceive was assessed in two small studies [129, 353] and was not significantly

different in treated and untreated women (RR=0.93, 95% CI 0.80-1.08). The time needed to conceive was described in three small studies [127-129] and the pooled meta-analysis suggested that treatment did not increase the proportion of women requiring >12 months to conceive (14.7% vs 9.2%, 1348 women, RR=1.45, 95%CI 0.89-2.37, p=0.14) (Figure 7.3). All other intervals to conception that were assessed were also not significantly affected p>0.05.

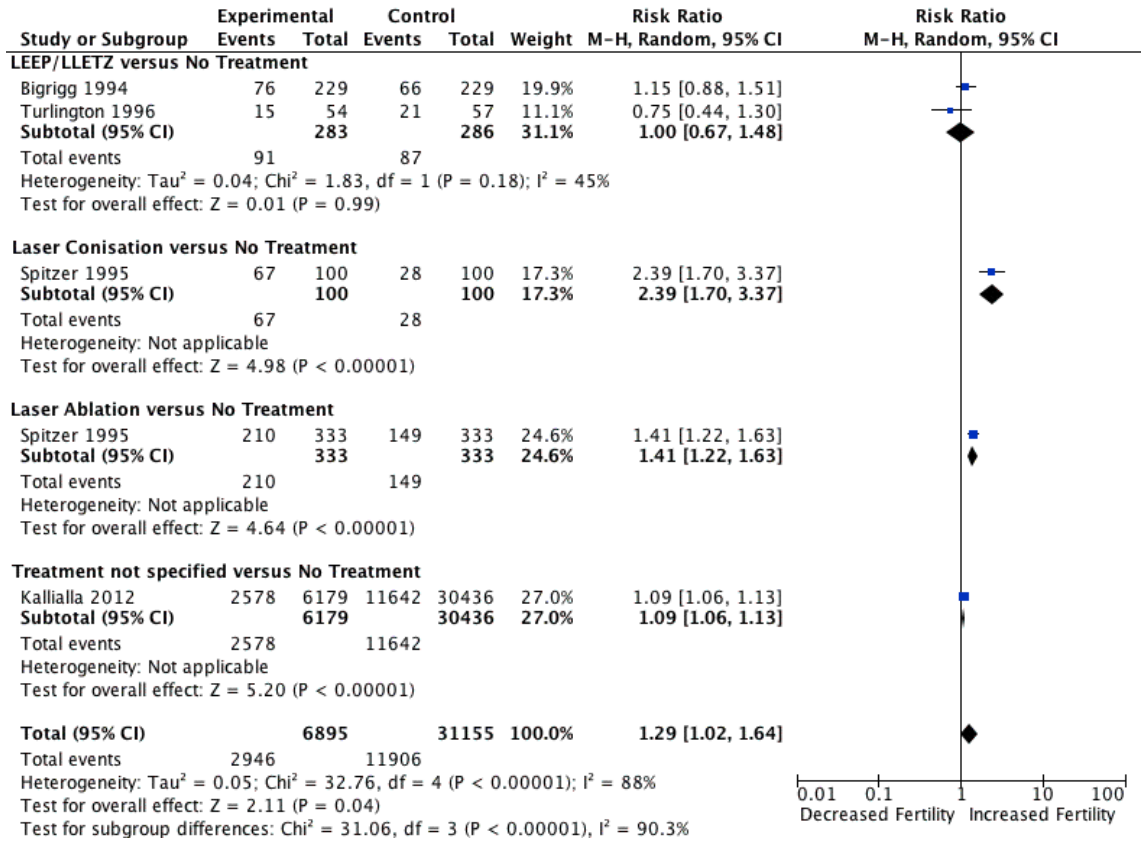


Figure 7.2. Meta-analysis of pregnancy rates in treated vs. untreated women

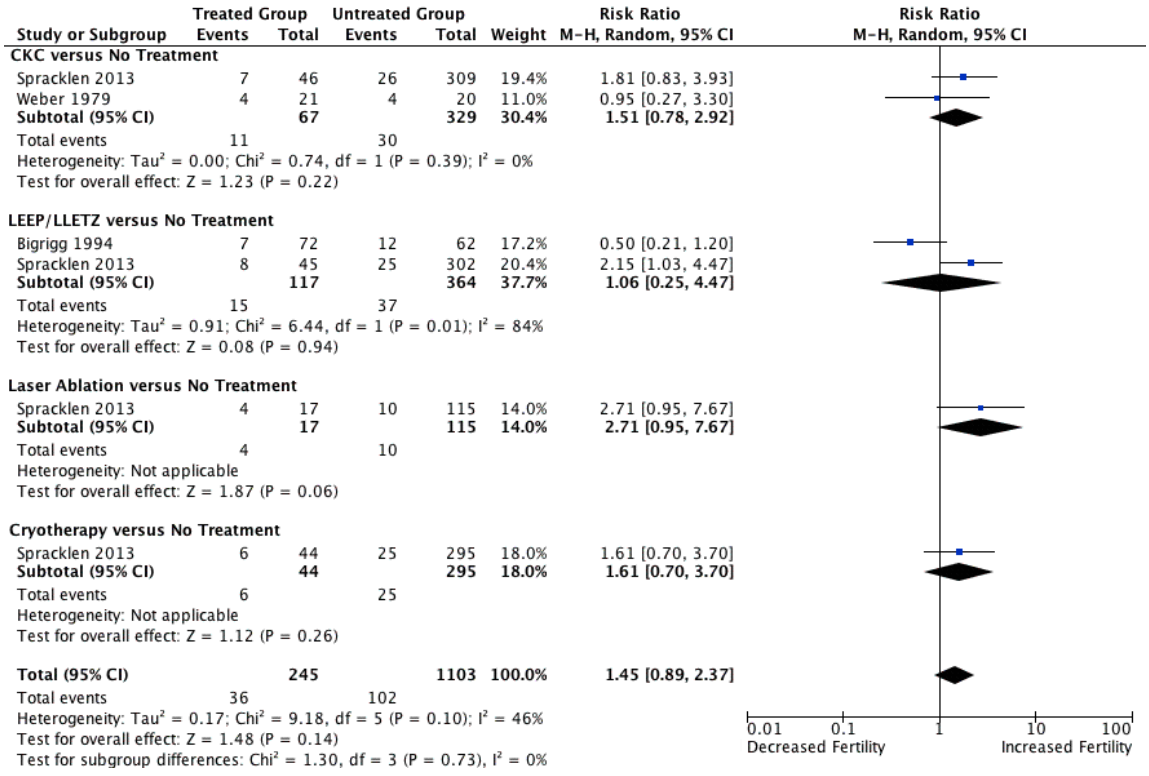


Figure 7.3. Meta-analysis of rate of women requiring >12 months to conceive in treated vs. untreated women

7.3.1.2 Early pregnancy outcomes (<24weeks of gestation)

Early pregnancy outcomes were assessed in 14 studies. There was no significant difference in the total miscarriage rate (at any gestation) between treated and untreated women (4.6% vs. 2.8%, 10 studies, 39504 women, RR=1.04, 95%CI 0.90-1.21).

Four studies reported on 1st trimester miscarriage rates, separately [129, 345, 348, 349]; and the pooled meta-analysis did not indicate a significant difference between treated and untreated groups (9.8% vs. 8.4%, 1103 women, RR=1.16, 95%CI 0.80-1.69) (Figure 7.4).

Eight studies reported on 2nd trimester miscarriage rates [129, 333, 344-346, 348-350]. In the pooled meta-analysis, we found that cervical treatment significantly increased the risk of 2nd trimester miscarriage. The rate was significantly increased in treated (1.6%) versus untreated women (0.4%) (2182268 women, RR=2.60, 95%CI 1.45-4.67) (Figure 7.5). There was no significant inter-study heterogeneity.

The rate of ectopic pregnancy was also higher for treated (1.6%) women (0.8%) (6 studies, 38193 women, RR=1.89, 95%CI 1.50-2.39) (Figure 7.6), while he molar pregnancy rate did not differ (p>0.05). The termination of pregnancy rate was higher in women with a history of treatment (12.2%) versus untreated controls (7.4%) (7 studies, 36809 women, RR=1.71, 95%CI 1.31-2.22) (Figure 7.7).

The risk of publication bias could not be assessed formally given the low number of studies included in each of the meta-analyses.

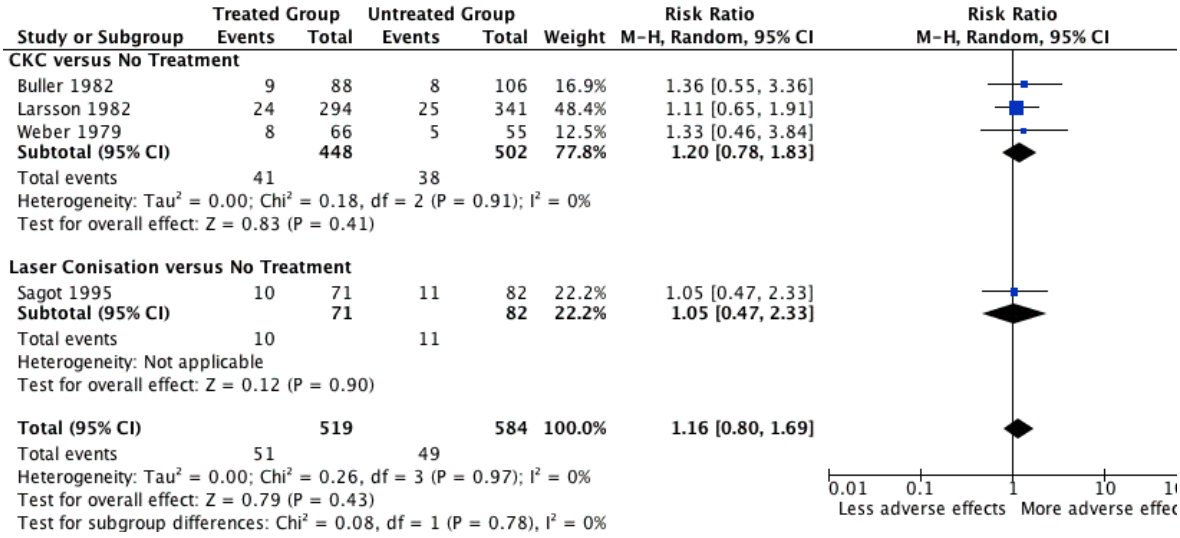


Figure 7.4. Meta-analysis of 1st trimester miscarriage rates in treated vs. untreated women

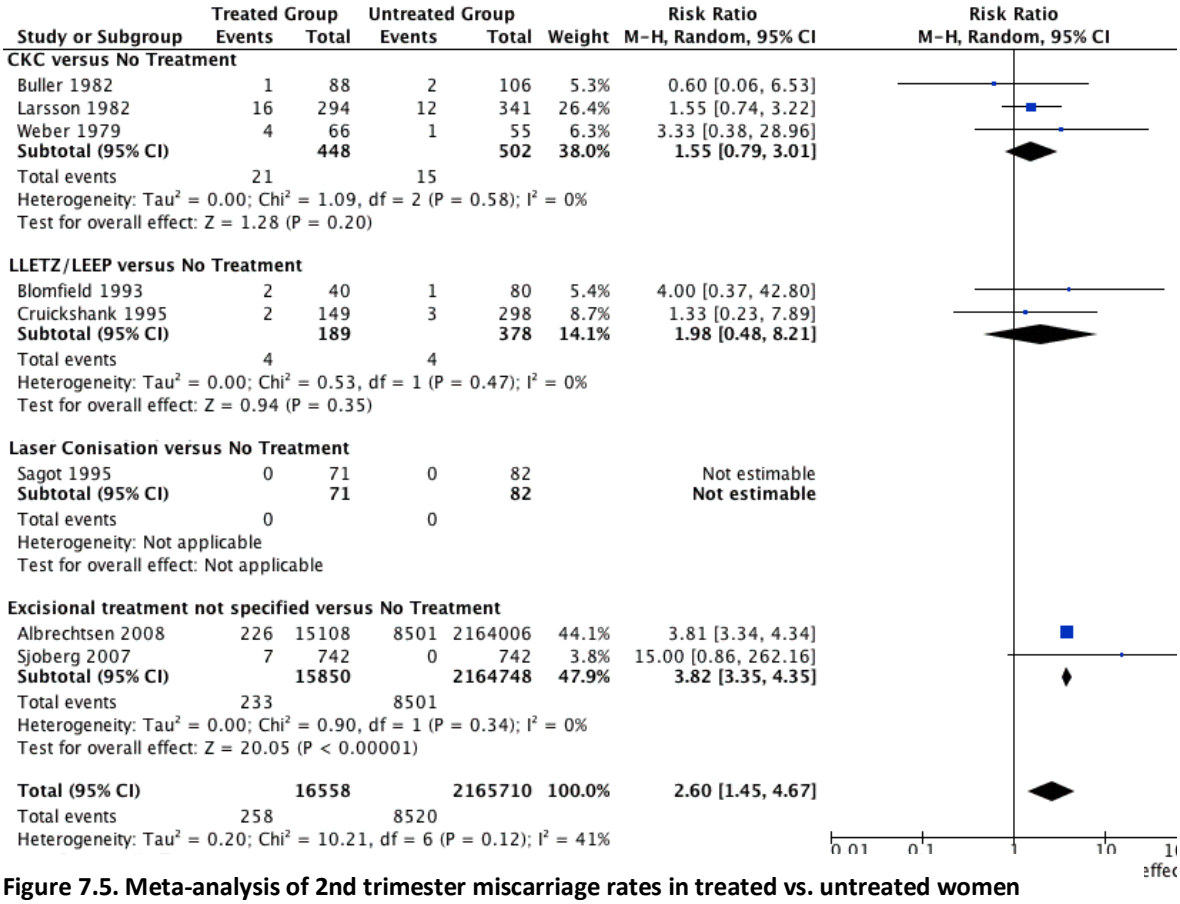


Figure 7.5. Meta-analysis of 2nd trimester miscarriage rates in treated vs. untreated women

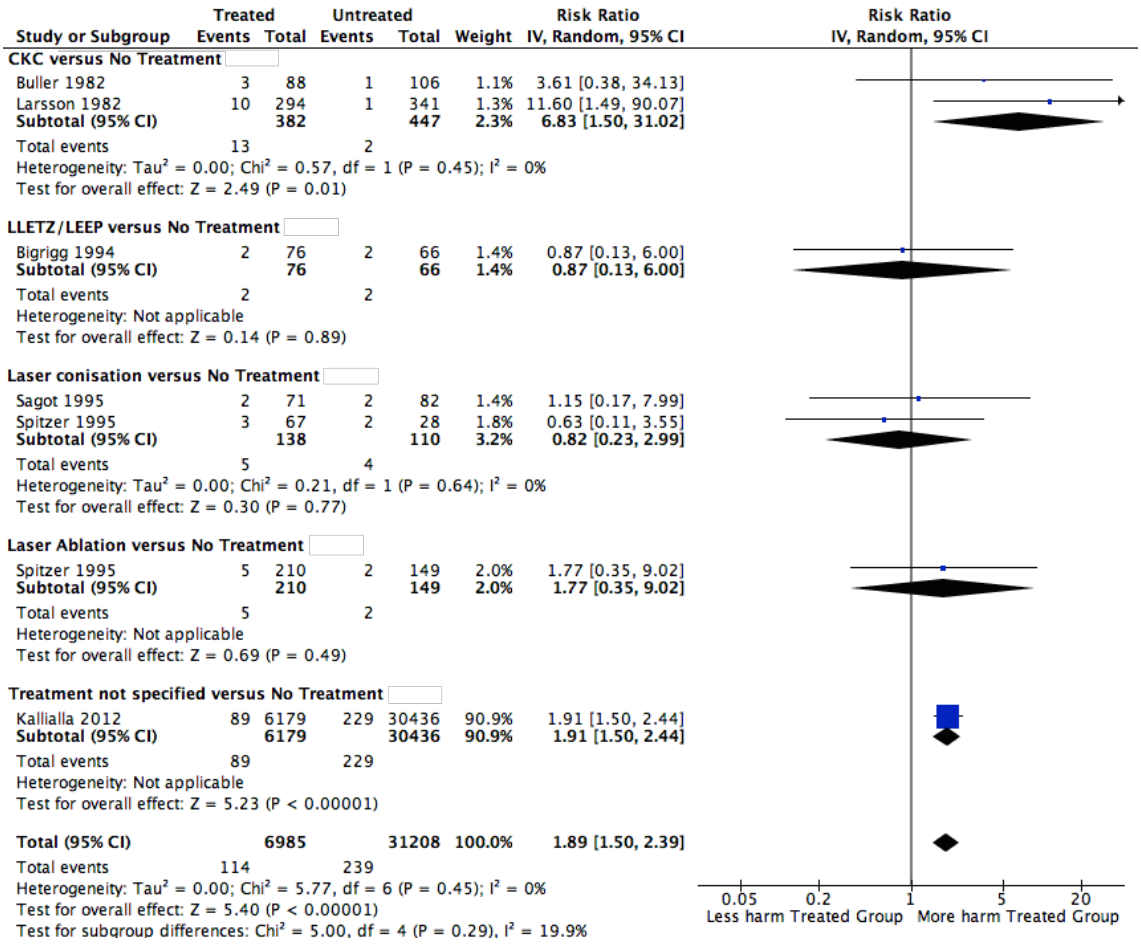


Figure 7.6. Meta-analysis of ectopic pregnancy rates in treated vs. untreated women

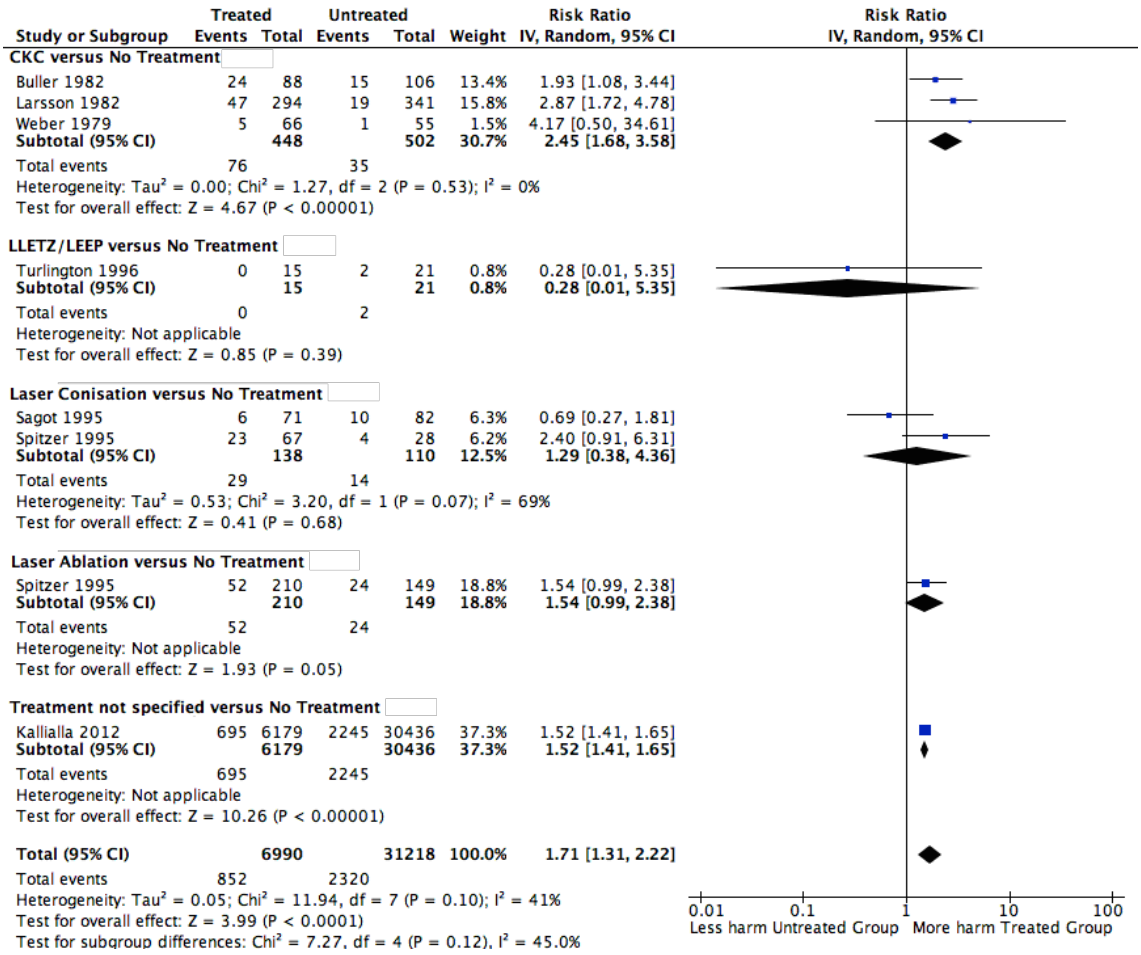


Figure 7.7. Meta-analysis of termination of pregnancy rates in treated vs. untreated women

7.3.2 Obstetric outcomes (>24 weeks gestation)

Three-hundred and eighty-one potentially eligible studies were identified; 69 of which [96, 110, 113, 129, 333, 335-337, 344-402] fulfilled the inclusion criteria of this review. Figure 7.8 demonstrates the reason for exclusion of the remaining 312 publications. No unpublished studies were identified. The detailed characteristics of the included studies and the Newcastle-Ottawa score of quality assessment for observational studies are shown in Appendix x. The majority of the studies were retrospective with only five prospective reports. All were cohort studies, apart from one case-control study by Castanon 2014 [397]. There were no randomised controlled studies. Fourteen studies examined the impact of CKC, 11 of LC, one of NETZ, 35 of LLETZ [110, 113, 334, 336, 344, 346, 347, 352, 353, 362, 363, 366, 373-377, 379-382, 385-387, 389-391, 393-395, 398, 399, 401, 402], eight of LA, one of RD, two of CT, 13 of Excision NOS, five of Ablation NOS, and three of Treatment NOS. There were five types of untreated comparison groups; three types of external comparator (1. External population adjusted for known risk factors related to adverse obstetric sequelae, 2. women attending colposcopy with or without CIN and/or biopsy but no treatment and 3. women with untreated high-grade disease) and two types of internal matching (1. comparing pre-treatment pregnancies of the treated population or 2. self-matching for women that delivered both before and after treatment.) All studies that used an external comparison group either matched for known risk factors or performed a regression analysis to control for known confounders. Four studies did not include any control for confounders.

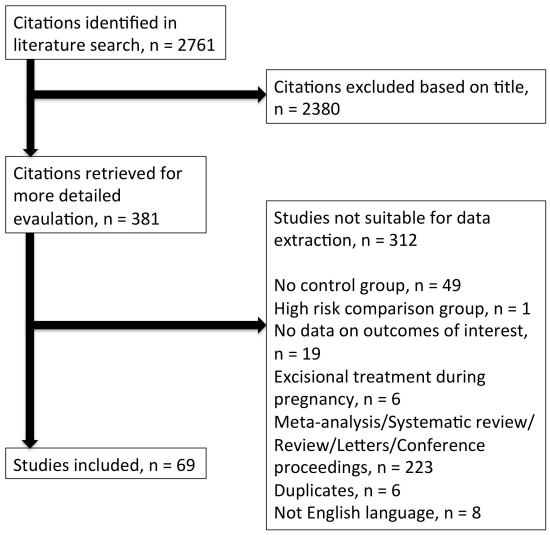


Figure 7.8. PRISMA flowchart for obstetric outcomes

The results relating to impact of treatment on preterm birth are presented in the following section. Other maternal and neonatal outcomes can be found in the published manuscripts [94, 403].

7.3.2.1 Preterm birth (<37 weeks of gestation)

The risk preterm birth was significantly increased after cervical treatment compared to untreated controls (Figure 7.9), including prematurity overall at less than 37 weeks of gestation (57 studies, 5198352 women, 10.8 v 5.5%, RR=1.71 [1.52, 1.92]), for severe prematurity less than 34/32 weeks of gestation (24 studies, 3794833 women, 3.5 v 1.4%, RR=2.45 [1.96, 3.06]) and extreme prematurity less than 30/28 weeks of gestation (eight studies, 3906697 women, 1.0 v 0.3%, RR=2.64 [1.81, 3.86]). The risk of preterm birth under 37 weeks was greatest for excisional compared to ablative techniques. Highest risk was following CKC (RR=2.11 [1.24, 3.57]), excision NOS (RR=2.13 [1.66, 2.74]) and LLETZ (RR=1.56 [1.36, 1.79]), compared to ablation NOS (RR=1.46 [1.27, 1.66]). Similar trends were noted for severe and extreme prematurity. Following multiple treatments the risk of prematurity was substantially higher than after a single treatment (repeat treatment: 11 studies, 1317284 women, 13.2 v 4.1%, RR=3.78 [2.65, 5.39]; single treatment: 17 studies, 1367023 women, 7.5 v 4.2%, RR=1.75 [1.49, 2.06]).

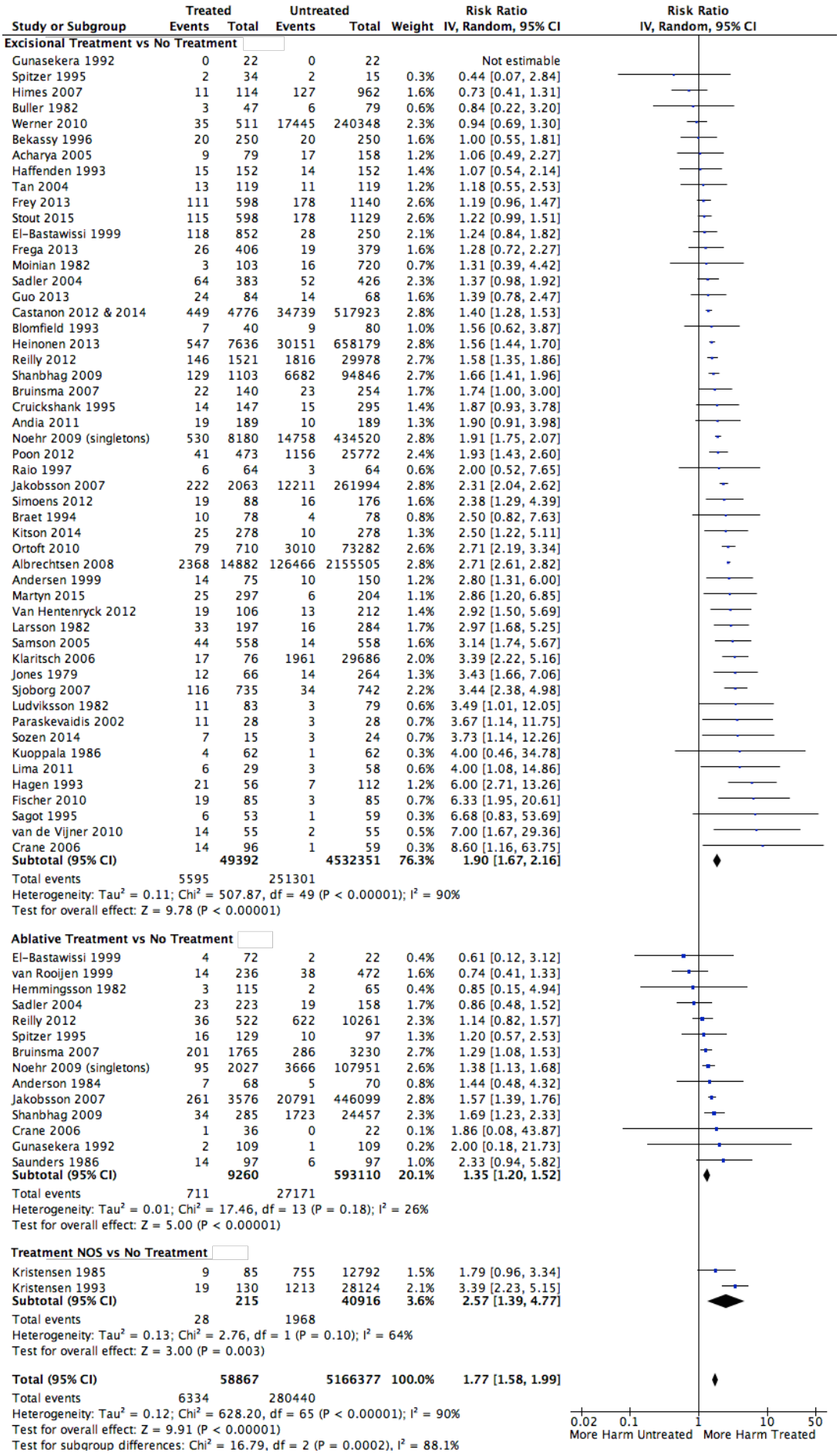


Figure 7.9. Meta-analysis of preterm birth rates in treated vs. untreated women

7.3.2.2 Preterm birth and cone depth and volume

The risk of preterm birth at less than 37 weeks of gestation according to the cone dimensions demonstrated that the risk increases progressively with increasing cone depth (Figure 7.10) and volume. The risk for treated versus untreated women was significantly higher for women with even relatively shallow cone depth of less than 10/12mm (eight studies, 550929 women, 7.1 v 3.4%, RR=1.54 [1.09, 2.18]. The magnitude of effect increased with cone depth (≥10/12mm: eight studies, 552711 women, 9.8 v 3.4%, RR=1.93 [1.62, 2.31]; ≥ 15/17mm: four studies, 544248 women, 10.1 v 3.4%, RR=2.77 [1.95, 3.93]; ≥ 20mm: three studies, 543750 women, 10.2 v 3.4%, RR=4.91 [2.06, 11.68]). The trend was similar with increasing cone volume, although was reported by one study only (<6mm, 550 women, 8.1 v 3.6%, RR=2.25 [1.09, 4.66]; >6cc: 284 women, 50.0 v 3.6%, RR=13.9 [5.09, 37.98]).

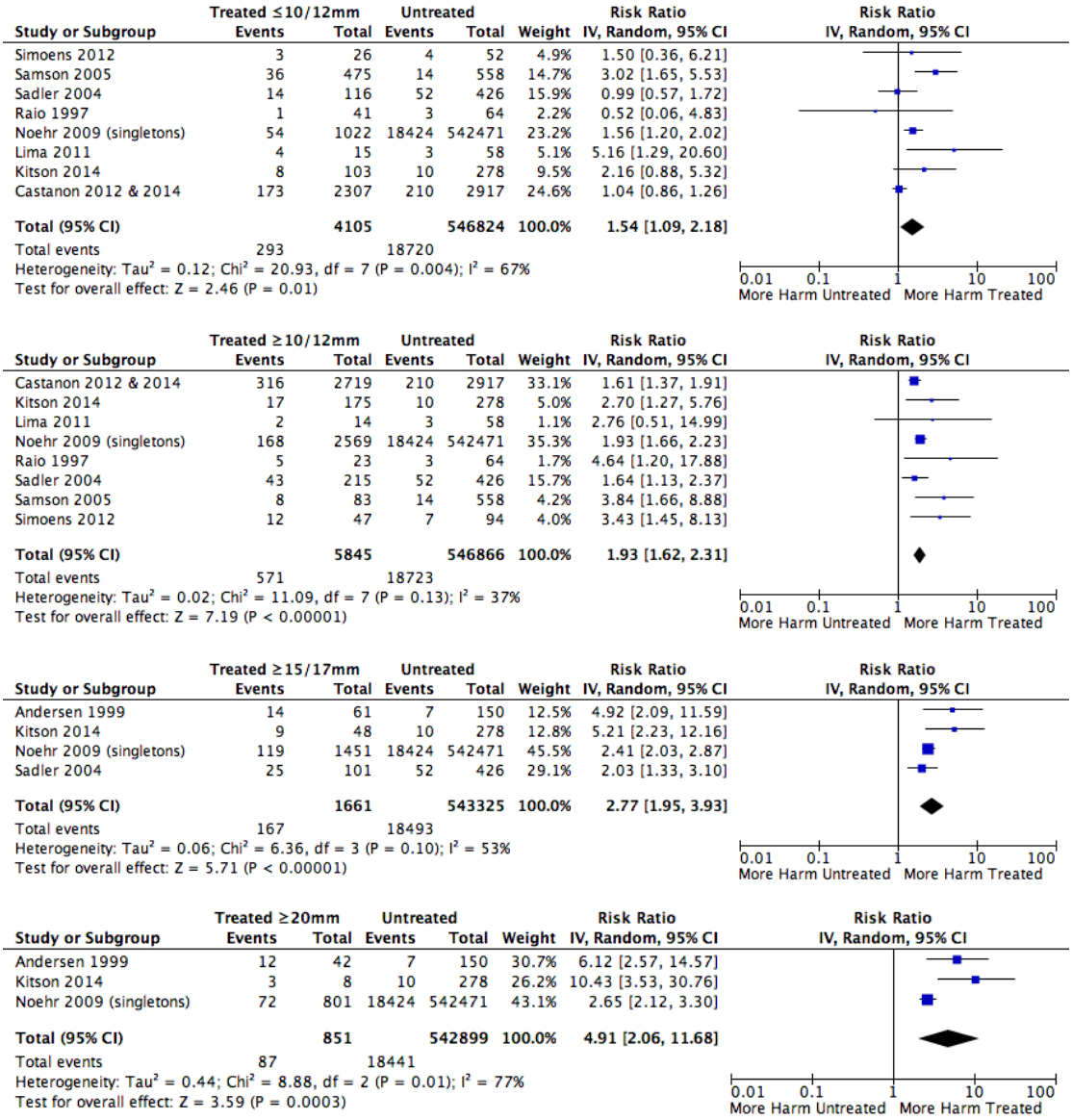


Figure 7.10. Meta-analysis of preterm birth rates according to cone depth

7.3.2.3 Choice of comparison group & impact magnitude of obstetric risk

The impact that the choice of comparison group may have on the magnitude of effect was assessed by a subgroup analysis that classified different studies according to the comparator used. The results suggested that treatment significantly increased the risk of preterm birth at less than 37 weeks of gestation irrespective of the comparison group used. The magnitude of effect was highest when an external comparison group was used (44 studies, 5177986 women, 10.6 v 5.4%, RR=1.96 [1.74, 2.21]), followed by internal comparators (self-matching: seven studies, 3132 women, 10.9 v 7.1%, RR=1.55 [1.2, 1.99]; pre-treatment pregnancies: 13 studies, 83086 women, 14.1 v 6.4%, RR=1.41 [0.98, 2.02]) and lastly women with disease but no treatment (11 studies, 70061 women, 8.7 v 6.0%, RR=1.25 [1.11, 1.4]). When women with disease but no treatment and the pregnancies of the same women before treatment were compared to the general population, the risk of preterm birth was significantly increased (16 studies, 4342190 women, 5.9 v 5.6%, RR=1.27 [1.16, 1.39]).

7.4 Discussion

Cervical treatment has been associated with an increased risk of adverse obstetric sequalae and preterm birth in subsequent pregnancies [84, 85, 93, 335]. More recent data suggests cervical intra-epithelial neoplasia itself may partly contribute to that risk [96], while there seems to be a 'dose-effect' relation documented for treatment; the larger the excision or proportion of cervix removed, the larger the treatment effect [85, 106, 108, 115, 382]. Cervical treatment may impair future fertility for women as a result of cervical stenosis and the reduction of cervical glands and mucus [123, 343]. A large number of contradictory studies, systematic reviews and meta-analyses exist on the topic of reproductive complications in this group of treated women. A comprehensive review was performed in order to clarify the risk to allow clinicians to better inform and triage their patients according to need for future preventative interventions.

The results of the systematic review and meta-analysis to assess fertility and early pregnancy outcomes did not find any evidence to support the hypothesis that treatment has a negative impact on fertility outcomes and the ability to conceive. Whilst there are several plausible mechanisms including cervical stenosis and removal of mucus-secreting glands, which could cause physical barriers to conception, there was no difference in pregnancy rates in women with an intention to conceive. The overall pregnancy rate was in fact higher in treated women (Figure 7.2), which may be explained by sexual and behavioural characteristics in these women increasing the likelihood of pregnancy and HPV infections. UK guidelines state women may be investigated for fertility problems if they do not become pregnant within 12 months of regular, unprotected intercourse, and this time point was therefore chosen as a further measure of fertility outcomes. Treated women did not take significantly longer to conceive overall, however there was a slight trend towards longer time to conception in this group (Figure 7.3). A likely explanation for this may be clinicians' recommendation to avoid conception during the early post-operative period and until the first follow-up assessment at 6 months confirms the absence of residual disease, which could necessitate further treatment. This data may also be less reliable due to the retrospective nature of the studies, and the uncertainty with which many patients may answer this question.

The rate of all miscarriages (first and second trimester) and those occurring specifically in the first trimester were not different between treated and untreated women (Figure 7.4). However, this meta-analysis is the first to suggest treatment for CIN significantly increases the

risk of second trimester miscarriage (Figure 7.5). Previous smaller cohorts have often failed to demonstrate a significant effect of treatment, which is likely due to the low incidence of 2nd trimester miscarriages. Although the results of this analysis are dominated by one large study [333], this is not an outlier and exclusion of this study from the analysis yielded similar results. Around 20% of pregnancies end in miscarriage, and the vast majority of these (80%) occur in the first trimester (≤12 weeks) [131]. Between 50 and 85% are caused by fetal chromosomal abnormalities [132], with inherited thrombophilia, uterine malformation, uncontrolled diabetes and thyroid disease cited as less common causes [131], all of which are unlikely to be mechanistically linked to treatment of CIN. Second trimester miscarriage (12-24 weeks gestation) and may be associated with cervical incompetence and infection [135, 136], in addition to the causes of early miscarriage. It is plausible that this phenomenon, relating to second-trimester miscarriage, is a continuum of the factors causing PTB in this patient group. An analysis to stratify risk according the depth of the cone was not possible, due to lack of reported data, although is likely that the risk increases with higher proportions of the cervix removed and future studies should aim to address this.

The higher number of ectopic pregnancies (Figure 7.6) and terminations (Figure 7.7) in the treated population may be a reflection of the behavioural characteristics of women with cervical precancer, known to be at higher risk of sexually transmitted disease and unplanned pregnancies.

Excisional treatment has been demonstrated to cause preterm birth by numerous studies and meta-analyses, but a rapidly evolving evidence base and inconsistencies in the published literature [96, 337, 339, 340, 381, 404] a high-quality synthesis of the evidence should be available for effective patient counseling at colposcopy and antenatal clinics. The current meta-analysis confirms that all types of local cervical treatment, both excisional and ablative, increases the risk of preterm birth and other adverse fetal and maternal outcomes. The magnitude of this effect of treatment was higher for more radical techniques (ie. CKC followed by LLETZ and LA) and for excision rather than ablation. Multiple conisations increased four-fold the risk of preterm birth as compared to untreated controls overall. These findings may be explained by the subgroup analyses, which clearly demonstrated that the risk of preterm birth directly correlates to the cone dimensions (depth/volume) and progressively increases with increasing cone depth (‘dose-effect’). Although the risk was increased even for excisions measuring less than 10mm in depth, this was almost two-fold higher for excisions of more

than 10mm, three-fold higher for more than 15/17mm and almost five-fold higher for excisions exceeding 20mm in depth. This information should help clinicians to inform their patients of their personal risk, and to stratify women according to those in greatest need of increased antenatal surveillance, with a view to preventing unnecessary procedures and interventions that themselves could increase the risk of a poor pregnancy outcome [156].

It has previously been suggested that the impact of treatment on the risk of preterm birth may not be a consequence of treatment but rather a product of other confounders present in women with cervical disease [85, 96, 337]. Subgroup analyses to stratify the risk to the comparator used, clearly demonstrates that although the risk of preterm birth is significantly increased after treatment irrespective of the comparison group used, the choice of comparator may over-inflate or under-estimate the effect from treatment. The magnitude of effect was highest when external controls were used, followed by internal control, followed by women that had disease but were not treated. The analyses in women with HSIL but no treatment only included three studies and 3764 participants; we were unable to draw any firm conclusions from this comparison.

There are several limitations of the results presented. Firstly, there was a high degree of heterogeneity with regards to study design including populations, matching for confounders, length of follow-up and outcome verification. Secondly, the majority of included studies describe retrospective cohorts that are prone to possible bias. The collected data are derived from clinic datasets, telephone contacts and mailed questionnaires and may represent incomplete and selected data. Finally, with respect to the fertility outcomes the number of studies and the study size was often small for many of the reported outcomes. Furthermore, studies that assess fertility outcomes are difficult due to the multifactorial nature of the condition (male factor, life-style etc). It is often difficult to design such a study and ensure all confounding variables have been eliminates.

7.5 Conclusion

Despite the limitations, this meta-analysis provides reassuring evidence that fertility is not affected after CIN treatment, although the risk of second trimester miscarriage is significantly increased. These results also confirm that although women with CIN have a higher baseline risk of prematurity as compared to the general population, cervical treatment and particularly long cones further increase such risk. The exact mechanisms remain unknown, and the

prospective cohort study conducted and reported in Chapters eight, nine and ten will explore possible mechanisms relating to changes in the cervical structure, immune system and microbiome. Caution should prevail when considering treatment in young women desirous of future pregnancies. If a decision is made to treat, it is important to remove as little tissue as needed, especially in nulliparous women with a small cervix without compromising the eradication of the disease. Every effort should be made to optimise both reproductive but also oncological outcomes for patients requiring treatment [106, 405].

CHAPTER 8. Ultrasound assessment of cervical length and volume before and after cervical treatment

8 Ultrasound assessment of cervical length and volume before and after excisional treatment

8.1 Background

Excisional treatment of CIN is effective for preventing future invasive cervical cancer. Its main benefit over ablative techniques is the ability to obtain an adequately-sized tissue biopsy for in-depth histological diagnosis [406]. There exists some discrepancy in the literature concerning whether excisional treatment for CIN leads to adverse obstetric outcomes [84, 396], which as shown in Chapter seven may also be attributed to several factors including the choice of control group [95], technique used [84, 111] and length or volume of cervical tissue excised [107, 108, 110]. Tissue shrinkage following formalin fixation may also underestimate the true size of the cervical tissue excised [407]. Furthermore, there may be reporting bias with regards to the terminology used to refer to the size of a cervical biopsy. The terms 'cone length' and 'cone depth' have previously been used interchangeably and may in fact refer to two subtly different measurements. The consensus within the academic colposcopy community is currently to refer to the cone length as the slant height or hypotenuse, and the depth as the height from the external os on the base of the cone, directly upwards into the cervical canal, as represented in Figure 8.1.

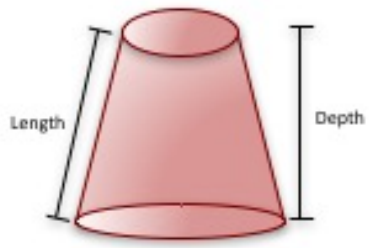


Figure 8.1. Length and depth of cervical cone biopsy

According to traditional anatomy texts the female uterine cervix is between 3–4cm in length and 2.5cm in diameter. However, there is likely to be wide variation in the general population, and therefore removal of for example, a cone of 15mm in length will represent a different proportion of excision across the population. It has been suggested that the proportion of excision may be more important for predicting pregnancy outcomes than the absolute length or volume of tissue removed [109]. This chapter will test the following hypotheses; firstly, that there is wide variation in cervical length and volume in the general population, and therefore it is plausible that proportion of excision is more clinically relevant for determining risk, compared to absolute values relating to length or volume of cervical tissue excised. Secondly, cervical deficit (length and volume) at six months post-treatment is correlated with proportion of excision.

8.2 Aims

1. Establish the variation in cervical length and volume in our population
2. Correlate proportion of cervical length and volume excision with regeneration at six months.

8.3 Results

Complete ultrasound data including cervical length, 2D and 3D volume was available for 288 women, recruited at Imperial College London according to the criteria in section 2.3.1. Of these, 13 gave a history of previous treatment and were excluded from subsequent analysis in this section giving a total of 275 women including in the final analysed cohort. A summary of patient characteristics is presented in Table 8.1.

Table 8.1. Baseline characteristics of untreated patients

Age	30.8 years (range 20 – 45 years, SD 4.9 years)
Disease status	
Normal	47 (17.1%)
LSIL	90 (32.7%)
HSIL	132 (48.0%)
Cancer	6 (2.2%)
Cervical length	34.2mm (range 23.6 – 51.8 mm, SD 4.6mm)
Cervical volume (2D)	14.01cm^3 (range 7.4 – 46.1cm^3, SD 5.6cm^3)
Cervical volume (3D)	19.92cm^3 (range 7.2 – 53.1cm^3, SD 8.8cm^3)

8.3.1 Cervical length and volume do not differ according to disease status

The mean cervical length in the final cohort of 275 untreated women was 34.2mm (range 23.6 – 51.8 mm, SD 4.6mm). The mean cervical 2D volume was 14.01cm^3 (range 7.4 – 46.1cm^3, SD 5.6cm^3), and mean 3D volume was significantly greater at 19.92cm^3 (range 7.2 – 53.1cm^3, SD 8.8cm^3)($p<0.001$, paired t-test)) (Table 8.1, Figure 8.2), however there was a positive correlation between 2D and 3D volume (Pearson correlation coefficient = 0.7153) (Figure 8.3).

There was no difference in cervical length ($p=0.99$), 2D volume ($p=0.48$) or 3D volume ($p=0.92$) according to disease severity as baseline (Figure 8.4). Figure 8.5 demonstrates the variation in cervical length and volume that exists in the population.

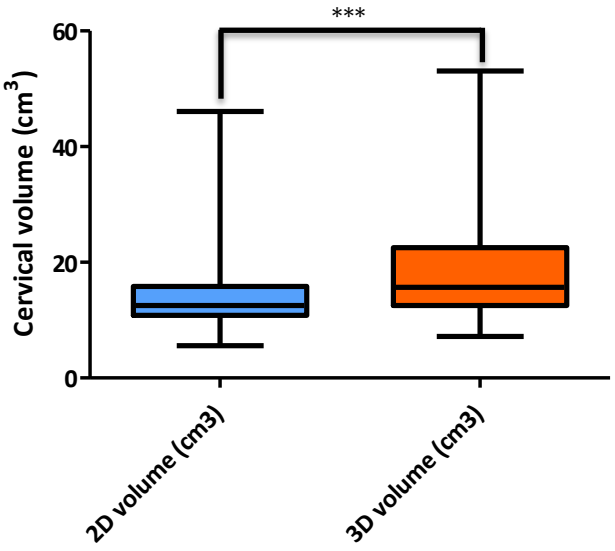


Figure 8.2. 3D cervical volume is significantly greater than 2D volume.

Paired t-test, ***p<0.001.

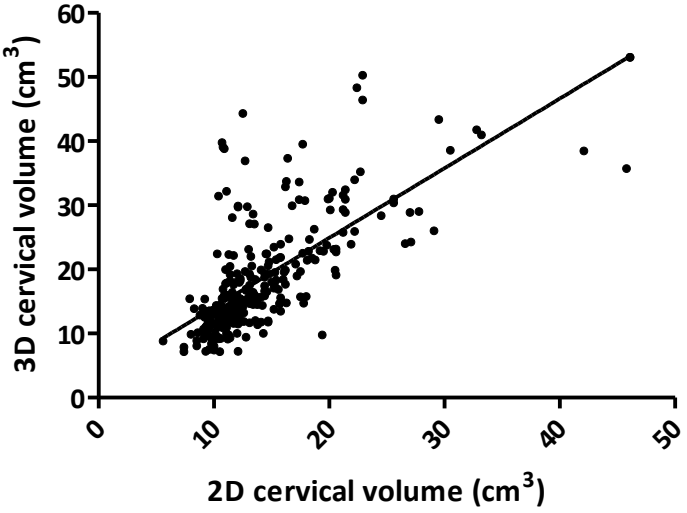
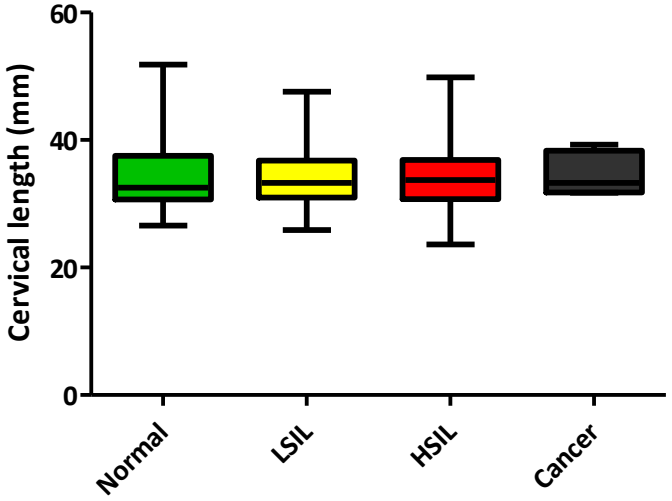


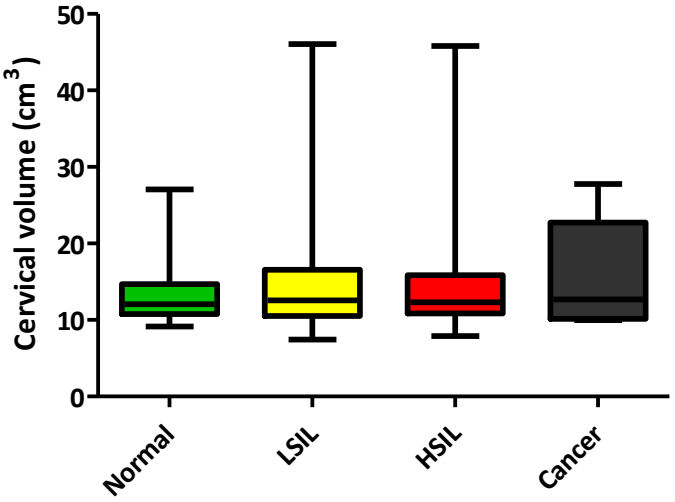
Figure 8.3. Positive correlation between 2D and 3D volume

Pearson correlation coefficient = 0.7153

A



B



C

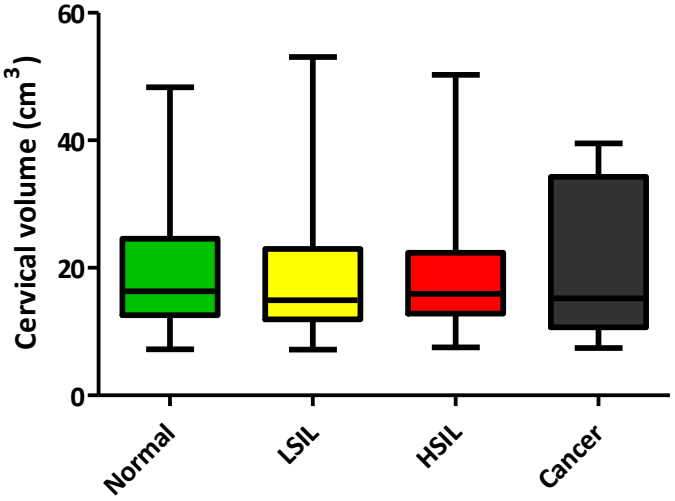


Figure 8.4. Cervical length (A), 2D volume (B) and 3D volume (C) do not differ with disease severity. Kruskall-Wallis test.

8.3.2 Cervical length and volume are significantly decreased after treatment

One hundred and fifty-two women underwent treatment and completed follow-up. After treatment, the mean cervical length was 30mm (range 19 – 37mm, SD) and volume was 12.6 cm^3 (range 5.9 – 29.8, SD 4.4cm^3) (2D)/ 16.9cm^3 (range 5.4 – 44.43, SD 7.7cm^3) (3D). Both cervical length and volume (2D and 3D) were significantly less at 6 months after treatment, compared to immediately before (cervical length, 2D volume, 3D volume – $p<0.0001$) (paired t-test) (Table 8.2, Figure 8.5), and also compared to all untreated women in the study cohort (cervical length, $p<0.0001$; 2D volume, $p=0.083$; 3D volume, $p=0.046$) (Mann Whitney test).

Table 8.2. Characteristics of treated cohort, n=152

Age	31.4 years (range 25 – 45 years, SD 5.1 years)		
Final histology of cone Normal LSIL HSIL Cancer	 1 (0.7%) 20 (13.2%) 125 (82.2%) 6 (3.9%)		
Cone length	11.4mm (range 2 – 22mm, SD 3.5mm)		
% cervical length excised	34.6% (range 5.5 – 72.1%, SD 11.6%)		
Cone volume	2.8cm^3 (range 0.7 – 9.0 cm^3, SD 1.5cm^3)		
% cervical volume excised	21.9% (range 4.9 – 73.0%, SD 13.2%) (2D) 18.6% (range 2.3 – 61.9%, SD 12.7%) (3D)		
	Pre-treatment	**Post-treatment**	**p-value**
Cervical length	34.0mm (range 24.7 – 45.8 mm, SD 4.4mm)	30mm (range 19 – 37mm, SD)	<0.0001
Cervical volume (2D)	13.5cm^3 (range 7.7 – 45.4cm^3, SD 5.5cm^3)	12.6 cm^3 (range 5.9 – 29.8, SD 4.4cm^3)	<0.0001
Cervical volume (3D)	19.0cm^3 (range 7.5 – 50.1cm^3, SD 8.3cm^3)	16.9cm^3(range 5.4 – 44.43, SD 7.7cm^3)	<0.0001

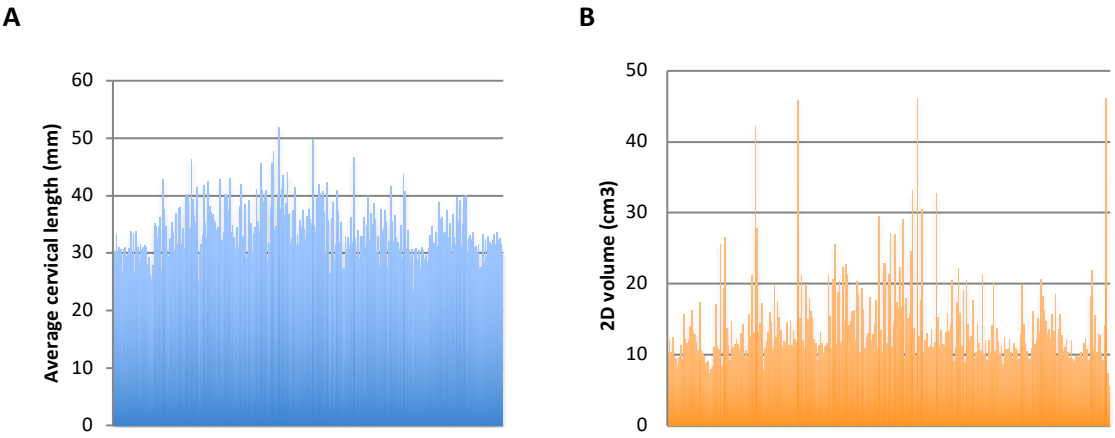


Figure 8.5. Variation in cervical length (A) and volume (B) in the study cohort at baseline

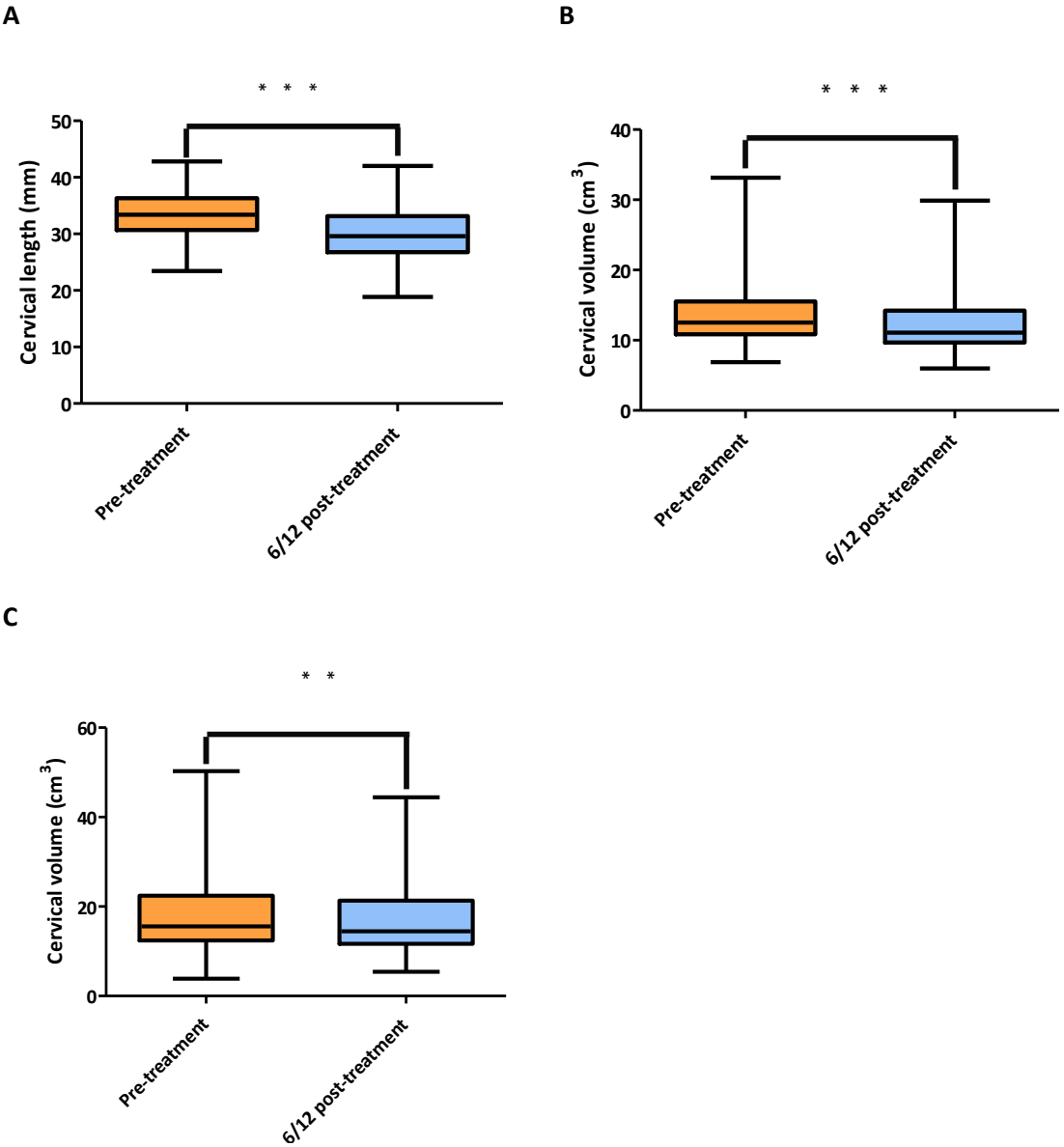


Figure 8.6. Cervical length (A), 2D volume (B) and 3D volume (C) are significantly less at 6 months post-excisional treatment (blue) than before treatment (orange).
Mann Whitney test, ***p<0.001.

8.3.3 Cervical length and volume deficit at 6 months post-excision correlates with proportion of excision

The mean cone length in the treated population in those who completed follow-up was 11.4mm (range 2 – 22mm, SD 3.5mm) and the mean proportion of cervical length excised was 34.6% (range 5.5 – 72.1%, SD 11.6%). The mean cone volume was 2.8cm^3 (range 0.7 – 9.0 cm^3, SD 1.5cm^3) and mean proportion of original cervical volume excised was 21.9% (range 4.9 – 73.0%, SD 13.2%), and 18.6% (range 2.3 – 61.9%, SD 12.7%), of 2D and 3D volume respectively (Table 8.2).

At six months follow-up the mean deficit in cervical length was 11% (range 0 – 35%, SD 8%), in 2D volume was 9% (range 0 – 30%, SD 7%), and 3D volume 7% (range 0 – 33%, SD 5%). The proportion of cervical length or volume excised correlated with the deficit in length and volume at six months follow-up (Pearsons r = 0.4550 (cervical length), 0.3908 (2D volume), 0.6132(3D volume)) (Table 8.2, Figure 8.7)

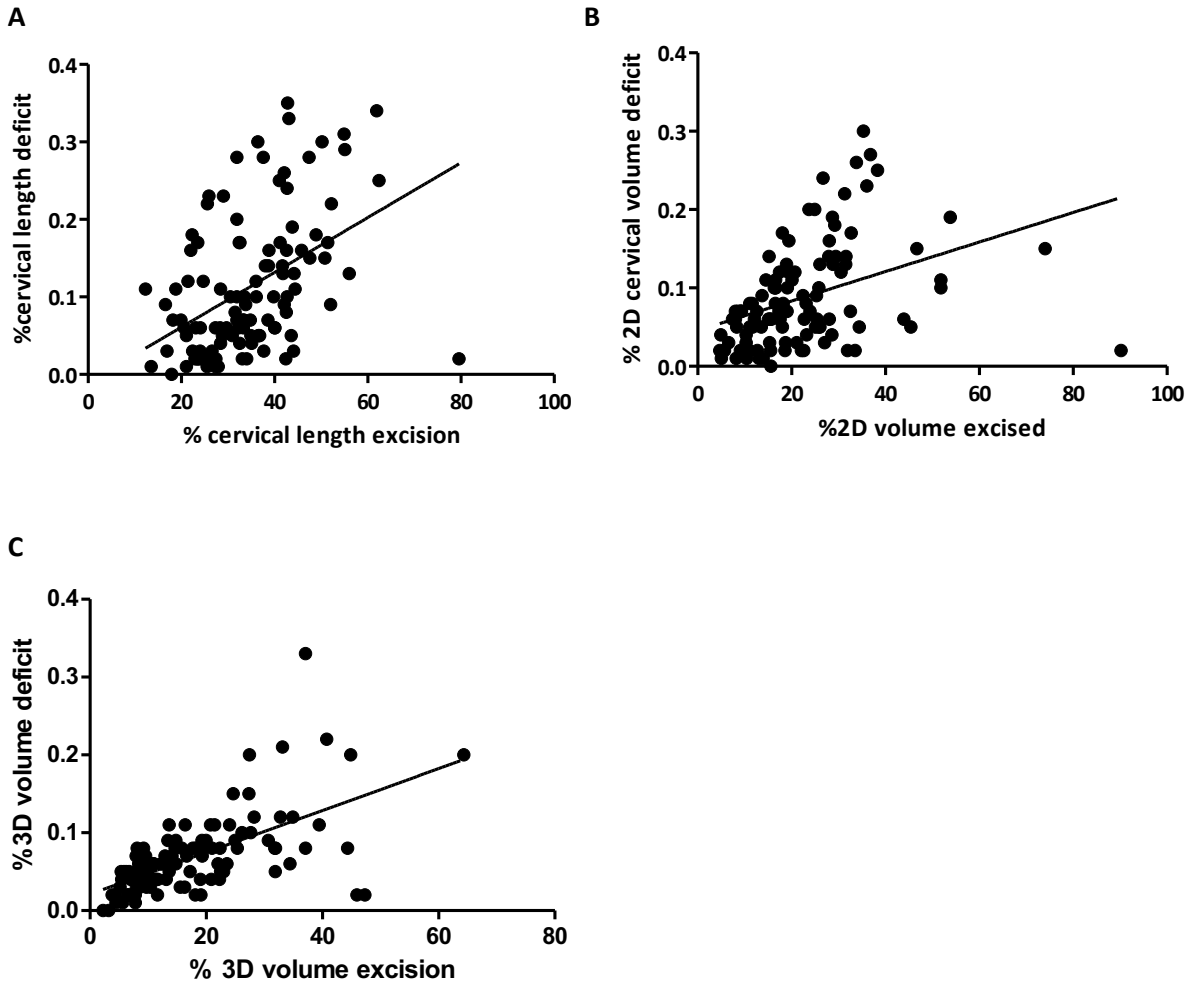


Figure 8.7. Proportion of cervical length (A), 2D volume (B) and 3D volume (C) excised correlated with proportional deficit at 6 months post-treatment.

Pearsons correlation coefficient, r = 0.4550 (A), 0.3908 (B), 0.6132 (C)

8.4 Discussion

These data support the hypothesis that the non-gravid uterine cervix is not of uniform length or volume within the general, premenopausal population. There was no difference in cervical length or volume according to disease severity, which is unsurprising, because CIN is confined to the epithelial layer of the cervix, so is unlikely to have any impact on the underlying stroma.

There was a significant difference in the 2D and 3D volumes (Table 8.2, Figure 8.2). There are relatively few published studies assessing the accuracy of cervical length and volume assessment of the non-gravid uterus. Farrell *et al.* measured transvaginal cervical length and 3D volume measurements immediately prior to hysterectomy, and compared these to actual length and volume of the histological specimen prior to fixation [408]. They showed a good correlation between ultrasound and actual cervical length, but conversely a poor correlation between 3D cervical volume and actual volume of the cervix once amputated from the body of the uterus, with good inter- and intra-observer reliability for both measurements. One of the strengths of the study described in this chapter is that over 90% of the scans were performed and 100% of scans analysed offline by a single operator (AM), drastically reducing the risk of inter-observer bias in this study. However, the significant difference between 2D and 3D cervical volumes in our patient cohort, along with the results of the aforementioned study suggests that 2D cervical volume assessment is the most reliable form of volume assessment. Furthermore, the technique is very simple and quick to perform by the bedside, requiring only two measurements, unlike 3D volume assessment, which requires several minutes of offline analysis.

A further strength of the study is that the dimensions and volume of the excised cervical cone biopsy were measured prior to formalin fixation. This step in tissue processing prior to histological analysis has been estimated to result in cervical tissue shrinkage of about 2.7%, and an average of 12.6% of the original tissue size following dehydration, clearing and paraffin wax embedding [407]. Previous studies, particularly using retrospective cohorts, have used tissue dimensions post-fixation, or from sectioned, paraffin-embedded slides, which will not only underestimate the size of the biopsy, but also add a degree of imprecision as the degree of shrinkage is likely to differ between individuals.

Follow up of the treated cohort was performed at six months post-treatment, which is the time of routine post-treatment follow-up, as described in the National Health Service Cancer

Screening Programme (NHSCSP) Document 20 guidelines. Due to ethical approval restrictions, it was not possible to invite participants for extra hospital visits in order to assess cervical length and volume regeneration at further time points, either earlier or later than six months. There are however several published studies that suggest cervical length regeneration is complete by six months using both TVUS [114] and magnetic resonance imaging (MRI) [115]. Further, a recent study that performed TV ultrasound examinations at one, three, six, nine and 12 months post-operatively found no further regeneration at either nine or 12 months [409]. Previous studies have also demonstrated a positive correlation between the cervical length prior to treatment and at six-month follow-up [409, 410]. The results presented in this chapter demonstrate there is a wide variation in baseline cervical length and volume within the premenopausal population, and we further show a linear correlation between the proportional cervical length and volume deficit at six months. Founta *et al.* have previously reported that only proportional volume excision, and not length, correlate with the proportional deficit at six months [115]. Together, the findings collectively support that the proportional deficit, rather than absolute length or absolute volume excised has the greatest influence on the final degree of cervical regeneration after excisional treatment.

Women with a history of previous LLETZ have been shown to have a significantly shorter cervical length in the second trimester of pregnancy, compared to low risk controls, and women with a previous history of spontaneous PTB [377], which was reflected in the cohort described in this chapter. These data further show that the cervical volume is also reduced. As previously described, the proportion of length or volume excision correlated with the deficit in cervical length or volume at follow-up. This not only supports the theory that women with a larger excision are likely to have a greater cervical deficit, but also supports the argument for ultrasound assessment of volume, as well as depth, prior to cervical treatment, and at follow-up. This will better inform the obstetric team managing any subsequent pregnancies, and help to support individualized healthcare decisions, to ensure interventions, such as cervical cerclage, in pregnancy are given to the women who are most likely to benefit, thus reducing the potential risk of such invasive procedures [156]. Women with a history of cervical treatment represent an increasing number of referrals to high-risk surveillance antenatal clinics, and were shown to account for over 40% of referrals made in 2012 [411]. TVUS assessment of cervical length in the second trimester has been shown to be a good predictor of preterm birth in the general population, with a 25mm threshold used to select the women in whom treatment such as progesterone or cervical cerclage could prevent a

preterm delivery [412]. The same has also been demonstrated in women post-excisional cervical treatment with a cervical length of less than 25mm identifying those at risk of preterm birth with a sensitivity, specificity, and positive and negative predictive values or 64%, 78%, 30% and 94% respectively [413].

Recently published work from our group has shown that the vaginal microbiome structure has an impact on cervical remodelling in pregnancy with community state type (CST) III and IV, being associated with mid-trimester cervical shortening in women with a history of pre-term birth [414]. Therefore, correlation of the data generated in this chapter with vaginal microbiome structure, and also HPV status may help determine whether there is an interaction between the cervical structure and biochemical microenvironment.

8.5 Conclusion

The results presented in this chapter demonstrate a wide variation in baseline cervical length and volume in untreated women, which is in agreement with previous studies showing that cervical length and volume are reduced at six months post-treatment, and demonstrate that the proportion of length and volume excision is an important factor in determining the degree of regeneration at six months. These factors are likely to play a role in the mechanism of preterm birth following excisional treatment for CIN. The following chapters will aim to correlate this information with changes in the vaginal microbiome and cervical antimicrobial peptides at six months.

CHAPTER 9. The vaginal microbiota before and after excisional treatment

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Content from this chapter is currently under preparation as:

Mitra A, MacIntyre DA, Lee YS, Smith A, Marchesi JR, Bennett PR, Kyrgiou M. The vaginal microbiota after excisional treatment for cervical intraepithelial neoplasia. (*Under preparation)*

9 The vaginal microbiota before and after excisional treatment

9.1 Background

The data presented in Chapter seven verified the previously held theory that excisional treatment for CIN is associated with adverse pregnancy outcomes including preterm birth, and now also second trimester miscarriage [86, 94]. It also demonstrated that the depth of excision correlates with risk of preterm birth, and it is likely that the same phenomenon also extends to second trimester loss. There is evidence to suggest that women with high-grade CIN have an increased risk of preterm birth above that of the general population, as also demonstrated in Chapter seven. Chapters three and five suggest that vaginal microbiota of women with CIN is markedly different to that of healthy controls and may play a role in the natural history [229, 415, 416], however it is currently difficult to determine whether this change is a cause or consequence of cervical disease [275].

One hypothesis is that the cervicovaginal microbiota is different in women with disease, due to the different metabolism within the tissue as a consequence of the disease process. This in turn alters the cervical micro-envionment making it less hospitable to *Lactobacillus* spp. resulting in overgrowth of pathogenic bacteria. Therefore, removal of disease might result in recolonisation of *Lactobacillus* spp. in the more hospitable, disease-free environment. A counter hypothesis is that a pre-existing altered vaginal microbiota is the cause of the disease, and predisposes a woman to increased risk of HPV transmission and persistence [202], which then results in disease progression and removal of the disease in the form of excisional treatment may not alter the structure if it is for example, genetically determined.

Recently published evidenced has suggested that the vaginal microbiota composition can have an influence on wound healing, with *Lactobacillus iners* shown to be associated with superior epithelial cell wound healing compared to *Gardnerella vaginalis* [417]. The ultrasound data presented in Chapter eight has shown that treated women have a significantly shorter cervix at six-month follow-up compared to untreated women and that there is a significant correlation between regeneration and the proportion of length and volume excised. This chapter will therefore further examine whether the VMB has an influence on cervical regeneration.

9.2 Aims

- Perform a prospective comparison of the vaginal microbiota before and at six months after excisional treatment for cervical intraepithelial neoplasia
- Compare the vaginal microbiota composition of treated women who are subsequently cytologically normal at 6 months, with that of healthy, untreated controls with normal cytology to determine whether removal of disease results in reversion to the same composition as untreated controls
- Determine whether vaginal microbiota composition has an impact on cervical regeneration

9.3 Results

One hundred and six patients underwent excisional treatment and attended follow-up at six months and had microbiota swabs collected at both visits. Three patients became pregnant between excision and follow-up and were excluded from the analysis leaving a total of 103 patients. Forty-two healthy untreated women were used as a control group, and were sampled once only at a single visit. Patient characteristics are detailed in Table 9.1.

Table 9.1. Patient characteristics

	Control, n=42	Pre-treatment, n=103	Post-treatment, n=103	p value
Age, years				**0.002**
Mean (SD, range)	29.9 (4.4, 20 – 43)	31.9 (5.2, 25 – 45)	32.5 (5.3, 25 – 46)	
Ethnicity , n/N (%)				0.21
Caucasian	32/42 (77)	87/103 (85)	87/103 (85)	
Asian	3/42 (7)	10/103 (10)	10/103 (10)	
Black	7/42 (16)	6/103 (5)	6/103 (5)	
Parity, n/N (%)				0.78
Nulliparous	32/42 (77)	73/103 (71)	73/103 (71)	
Parous	10/42 (23)	30/103 (29)	30/103 (29)	
Smoking status, n/N (%)				0.28
Current smoker	6/42 (14)	27/103 (26)	26/103 (25)	
Non-smoker	36/42 (86)	76/103 (74)	77/103 (75)	
Phase of menstrual cycle, n/N (%)				0.72
Luteal	24/42 (57)	55/103 (53)	50/103 (48)	
Follicular	15/42 (36)	38/103 (37)	46/103 (46)	
Unknown	3/42 (7)	10/103 (10)	7/103 (7)	
Contraception, n/N (%)				0.93
Nil	21/42 (50)	35/103 (34)	34/103 (33)	
Condoms	4/42 (10)	17/103 (16)	18/103 (17)	
COCP	13/42 (31)	37/103 (36)	37/103 (36)	
POP	1/42 (2)	5/103 (5)	5/103 (5)	
Copper IUD	0/42 (0)	2/103 (2)	1/103 (1)	
Mirena IUS	2/42 (5)	5/103 (5)	6/103 (6)	
Contraceptive implant	1/42 (2)	2/103 (2)	2/103 (2)	
Time since last intercourse, n/N (%)				0.80
>48 hours	36/42 (86)	92/103 (89)	92/103 (89)	
<48 hours	6/42 (14)	11/103 (11)	11/103 (11)	

CIN: Cervical intraepithelial neoplasia; COCP: Combined oral contraceptive pill; IUD: Intrauterine device; IUS: Intrauterine system; POP: Progesterone-only pill

The average time to follow-up was six months (range: 5–12 months). The control group were found to be significantly younger than the treated women (p=0.002). Other characteristics were not significantly different between the control and treated cohort. In the treated cohort, parity remained unchanged because treatment is not generally performed in pregnant patients, and those who had subsequently become pregnant were excluded. Of note, no patients reported a pregnancy loss at any gestation between visits. One patient reported smoking cessation between the time of treatment and follow-up. One patient had her copper IUD removed and was instead using condoms at follow-up, and one patient who was not using any contraception at the time of treatment subsequently had a Mirena IUS inserted. There was equal distribution of samples collected in the follicular or luteal phase of the cycle and in the rate of women that had intercourse within 48 hours from sample collection.

Table 9.2. Histology, cytology and HPV status

	Control, n=42	**Pre-treatment, n=103**	**Post-treatment, n=103**
Histological severity of lesion			
Normal	-	0/103 (0)	-
CIN1	-	15/103 (15)	-
CIN2	-	40/103 (39)	-
CIN3	-	48/103 (46)	-
Cytological severity of lesion			
Normal, HPV -ve	22/42 (57)	-	70/103 (69)
Normal, HPV +ve	13/42 (31)	-	11/103 (10)
Normal, HPV status unknown	5/42 (12)	-	0/103 (0)
BNC, HPV -ve	0/42 (0)	-	12/103 (11)
BNC, HPV +ve	0/42 (0)	-	5/103 (5)
LSIL, HPV -ve	0/42 (0)	-	0/103 (0)
LSIL, HPV +ve	0/42 (0)	-	3/103 (3)
HSIL	0/42 (0)	-	2/103 (2)

BNC: Borderline nuclear changes, CIN: Cervical intraepithelial neoplasia, HSIL: high-grade squamous intraepithelial neoplasia, LSIL: low-grade squamous intraepithelial neoplasia.

Histology was used to classify all patients in the pre-treatment group and cytology in all patients in the post-treatment group. Two patients were found to have severe dyskaryosis on follow-up cytology at six months and subsequent histology revealed CIN2 in both cases.
In the normal healthy control population 31% were HPV positive, compared to 14% of those who had negative cytology at six months after treatment.

9.3.1 The vaginal microbiota is not significantly altered after excisional treatment

In total, 2977082 reads were obtained from 248 samples with an average number of reads per sample of 11,400 and the mean and median read lengths of 513 and 520 bp respectively. To avoid sequencing bias, operational taxonomic units (OTUs) were randomly sub-sampled to the lowest read count of 244, which retained 99.7% of OTU counts and still provided coverage of >96% for all samples. Following removal of singletons and rare OTUs, a total of 51 taxa were identified in the vaginal microbiota of the study cohort.

Table 9.3. Rates of each CST according to disease state

	Control, n=42	**Pre-treatment, n=103**	**Post-treatment, n=103**	**p value**
CST I (*L. crispatus*)	20/42 (48)	44/103 (43)	41/103 (40)	0.6851
CST II (*L. gasseri*)	0/42 (0)	4/103 (4)	4/103 (4)	0.4305
CST III (*L. iners*)	19/42 (45)	31/103 (30)	39/103 (38)	0.1948
CST IV (*Lactobacillus* spp. deplete, high diversity)	3/42 (7)	22/103 (21)	16/103 (16)	0.1056
CST V (*L. jensenii*)	0/42 (0)	2/103 (2)	3/103 (2)	0.5257
	Control, n=42	**-**	**Post-treatment, normal cytology, n=81**	**p value**
CST I (*L. crispatus*)	20/42 (48)	-	33/81 (41)	0.5651
CST II (*L. gasseri*)	0/42 (0)	-	3/81 (4)	0.5503
CST III (*L. iners*)	19/42 (45)	-	31/81 (38)	0.5618
CST IV (*Lactobacillus* spp. deplete, high diversity)	3/42 (7)	-	12/81 (15)	0.2597
CST V (*L. jensenii*)	0/42 (0)	-	2/81 (2)	0.5466
	Normal, HPV –ve, n=24	**-**	**Post-treatment, HPV –ve, n=70**	**p value**
CST I (*L. crispatus*)	12/24 (50)	-	28/70 (40)	0.4751
CST II (*L. gasseri*)	0/24 (0)	-	2/70 (3)	>0.9999
CST III (*L. iners*)	10/24 (42)	-	27/70 (38)	0.8124
CST IV (*Lactobacillus* spp. deplete, high diversity)	2/24 (8)	-	11/70 (16)	0.5040
CST V (*L. jensenii*)	0/24 (0)	-	2/70 (3)	>0.9999

CST: Community state type, L.: Lactobacillus

The rates and frequency of the different CSTs (I, II, III, IV & V) were compared between healthy controls and the treated cohort (Figure 9.1, Table 9.3). CST I was the most frequently observed in the study cohort (105/248, 42% of all patients), and in all three groups individually, being highest in the control group as previously described in Chapter three. Prior to treatment the

rate of CST IV was higher in the pre-treatment group compared to normal controls which was approaching significance (7% control vs 21% pre-treatment, p=0.0515, Fishers exact test). CST III rates were higher in controls compared to the pre-treatment cohort although again this was approaching, but did not reach significance (45% control vs 38% pretreatment, p=0.0875, Fishers exact test).

After treatment, there was no significant difference in the distribution of the five CST's (p= 0.7050, Chi-squared test). Similarly, compared to the control group, there was no significant difference in the distribution of CST's after treatment (p=0.2372, Chi-squared test). Twenty-two treated women (30% treated cohort) had abnormal cytology at follow-up; 17 borderline nuclear changes, three mild dyskaryosis and two severe dyskaryosis. In order to test the hypothesis that removal of disease shifts the composition of the vaginal microbiome toward that of the healthy control population, 22 women with abnormal cytology were excluded, leaving 81 women (70%) with normal follow-up cytology for analysis (Table 9.3). There was no significant difference in distribution of CST's (p=0.3137, Chi squared test) although there was twice the rate of CST IV after treatment compared to controls but this was not significant (7% control vs 15% post-treatment, p=0.2597, Fisher's exact test).

There were higher rates of HPV positivity in the control population compared to the 31 treated women with negative cytology (31% control, vs 12% cytology negative post treatment, p=0.0302, Fishers exact test), and we therefore further restricted the analysis to only those who were HPV negative in both groups, leaving 24 controls and 70 with negative cytology post-treatment. Once again, there was no significant difference in CST distribution (p=0.2915, Chi-squared test). In treated women, the rate of CST IV remained twice that of healthy controls (8% control vs 15% post-treatment, p=0.5040, Fishers exact test).

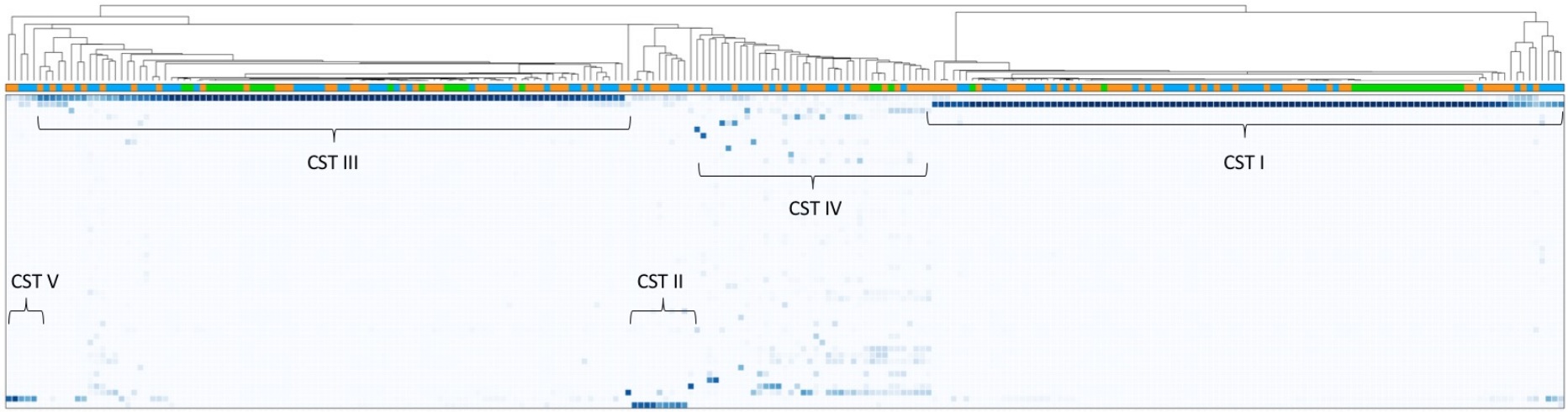


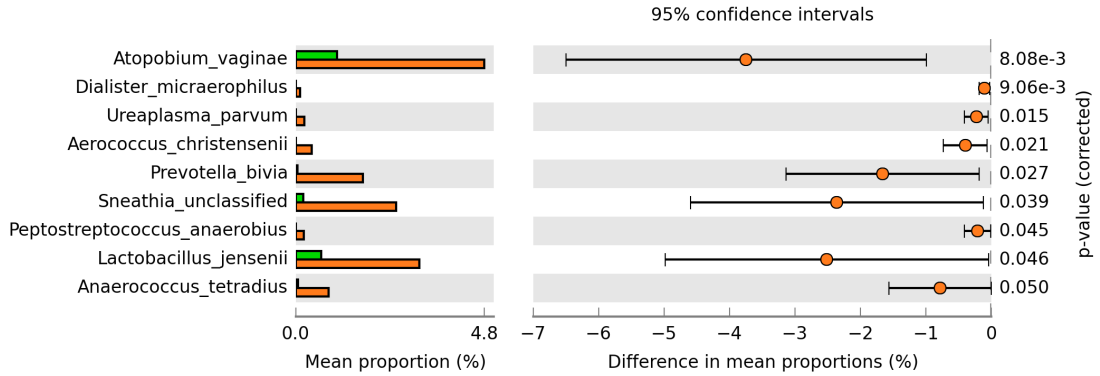
Figure 9.1. Vaginal microbiota composition before (orange) and after (blue) excisional treatment and in healthy untreated controls (green)

Higher levels of the *Lactobacillus spp.*-deplete CST IV was seend in women both pre- and post-treatment compared to untreated controls which was approaching significance (p=0.0515 & 0.0875 respectively). *CST: community state type*

9.3.2 Differential taxa between groups

Figure 9.2 shows bacterial species that were significantly different in the treated cohort compared to normal, untreated controls. Eight species known to be associated with a high-diversity, bacterial vaginosis-type VMB composition were significantly enriched in pre-treatment samples compared to controls (Figure 9.2A), and a further six high-diversity associated species were significantly higher post-treatment compared to controls (Figure 9.2B). *Lactobacillus jensenii* was higher at both timepoints in the treated group compared to controls (p=0.046 pre-treatment, p=0.0017 post-treatment, Welch's t-test) (Figure 9.2). *Lactobacillus gasseri* was found at greater levels in post-treatment samples compared to controls (p=0.03, Welch's t-test) (Figure 9.2B). No significantly differential taxa could be found when comparing the paired samples before and after treatment.

A



B

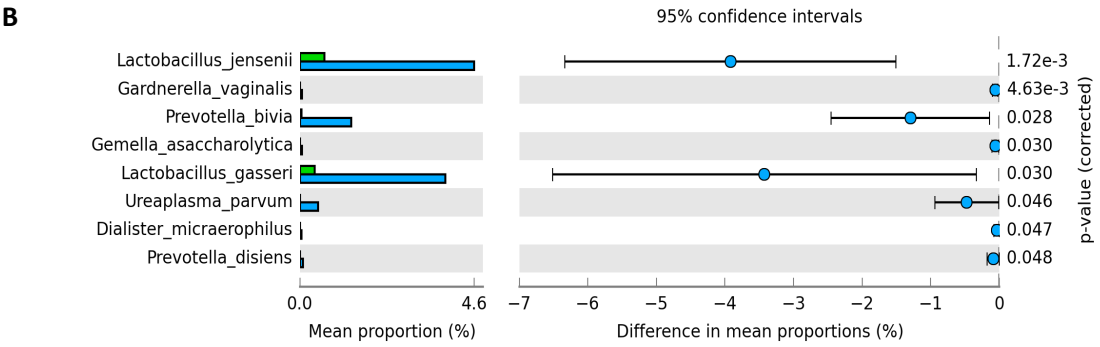


Figure 9.2. Differential species in normal controls compared to the pre-treatment cohort (A) and those women after treatment (B)

Green: control; orange: pre-treatment; blue: post-treatment.

9.3.3 Dynamics of the vaginal microbiota after treatment

Figure 9.3 is a Sankey plot showing the dynamics of the distribution of CST's pre- and six-months post-treatment. Of the 103 treated patients, 58 (56%) has the same vaginal microbiota CST on the day of treatment and at their six-month follow-up appointment. Women with CST I were most likely to have the same CST before and after treatment (28/44, 64%), followed by CST III (19/31, 60%), then CST II (2/4, 50%). Women with CST IV on the day of treatment were least likely to have the same CST after treatment, with only 41% having CST IV at both time points (9/22).

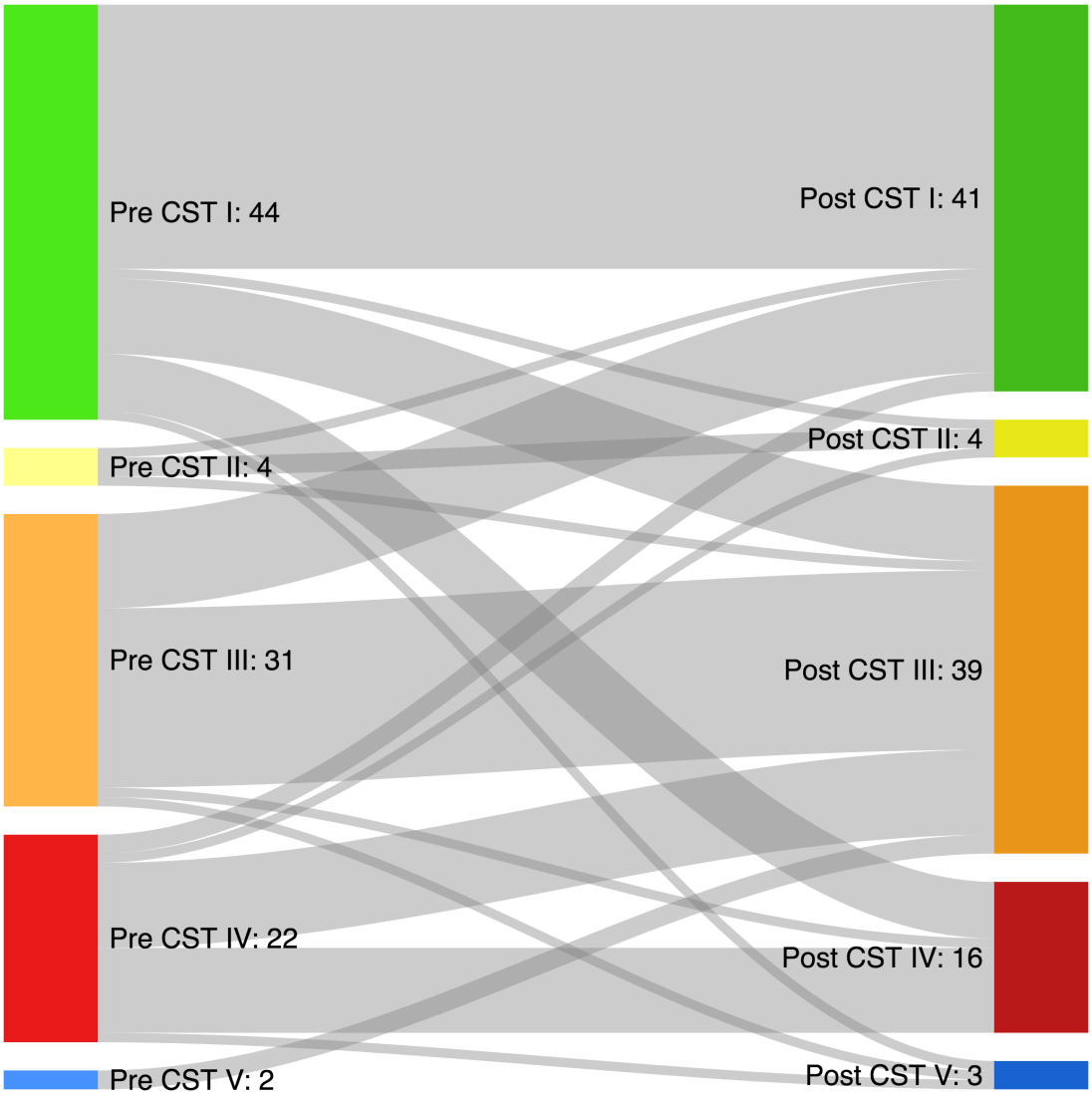


Figure 9.3. Sankey plot showing dynamics of vaginal microbiota composition pre- and post-treatment

Of the 45 women who switched to another CST, the majority switched between *Lactobacillus* spp.-dominant states (25/45, 56%). Seven women switched from a *Lactobacillus* spp.-dominant CST to CST IV (7/45, 16%) and 13 changed in the opposite direction from CST IV to a *Lactobacillus* spp.-dominant CST (13/103, 13%).

The control group were only sampled at a single time point, and therefore no similar longitudinal comparison can be made.

9.3.4 Richness and diversity are not altered after excision

Consistent with increased rates of CST IV in the treatment cohort compare to controls, vaginal microbiota richness as measured by number of species observed, and diversity, represented by the inverse Simpson index were also found to be higher in women with women both pre- and post-treatment compared to healthy, untreated controls. There was no difference between richness or diversity between pre- and post-treatment samples in the 103 matched women (Figure 9.4).

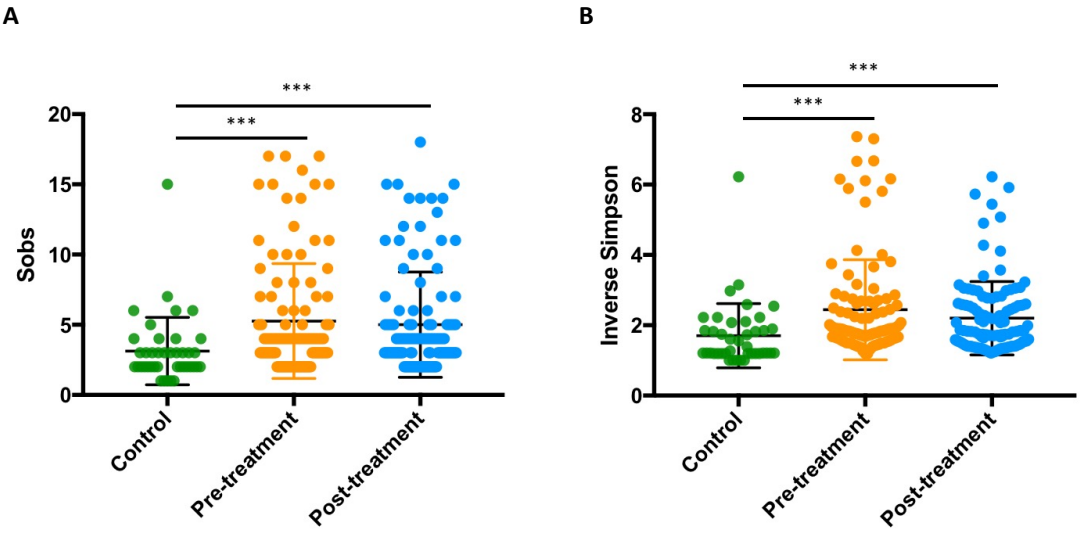


Figure 9.4. Vaginal microbiota richness (A) and diversity (B) indices associated with disease status
Kruskall-Wallis test, ***p<0.001. *Sobs: Species observed.*

9.3.5 Cervical regeneration does not correlate with microbiome composition

Comparisons were made between VMB CST pre- and post-excision to determine whether this may have any impact on cervical regeneration. Chapter eight has shown that regeneration correlates with proportion of excision so this was also examined alongside absolute length regeneration, however neither classification revealed an apparent relationship with either pre- or post-excision CST. The initial absolute and proportional length deficit did not differ between groups. The same analysis was performed for volume of excision and regeneration and again, did not show either a trend or any significant relationships.

Table 9.4. Cervical length regeneration according to pre- and post-treatment CST

Regeneration according to pre-treatment CST				
	Depth of cone excised, mm Average (range, SD)	**Cervical length at follow-up, mm** Average (range, SD)	**Proportional length excised, %** Average (range, SD)	**Proportion length regeneration, %** Average (range, SD)
CST I, n = 44	11.2 (7 – 22, 3.3)	29.8 (23 – 42, 6.9)	33.4 (12 – 80, 12.6)	62.1 (14 – 98, 24.9)
CST II, n = 4	10.50 (7 – 17, 5.1)	30.9 (24 – 38, 6.6)	32.3 (17 – 51, 18.2)	65.5 (44 – 81, 15.7)
CST III, n = 31	12.00 (7 – 21, 3.6)	28.4 (24 – 39, 6.7)	36.8 (18 – 62, 11.0)	70.4 (12 – 97, 21.3)
CST IV, n = 22	11.6 (7 – 18, 3.8)	30.1 (22 – 36, 4.0)	32.4 (11 – 52, 11.6)	70.7 (12 – 98, 22.4)
CST V, n = 2	10.5 (8 – 13, 3.5)	29.7 (29 – 30, 0.7)	32.8 (26 – 40, 9.8)	82.6 (74 – 91, 12.8)
p value	0.8490	0.8253	0.7027	0.3828
Regeneration according to post-treatment CST				
	Depth of cone excised, mm Average (range, SD)	**Cervical length at follow-up, mm** Average (range, SD)	**Proportional length excised, %** Average (range, SD)	**Proportion length regeneration, %** Average (range, SD)
CST I, n = 41	10.8 (7 – 21, 3.6)	30.0 (24 – 42, 8.1)	31.8 (12 – 62, 11.8)	67.5 (15 – 94, 22.1)
CST II, n = 4	9.9 (8 – 22, 5.8)	30.1 (23 – 29, 3.1)	29.2 (27 – 80, 22.8)	54.6 (40 – 98, 25.8)
CST III, n = 39	12.1 (7 – 17, 2.8)	29.2 (22 – 39, 4.8)	35,8 (18 – 55, 9.0)	68,0 (12 – 97, 24.9)
CST IV, n = 16	12.2 (7 – 18, 3.8)	28.2 (22 – 36, 4.5)	37.7 (11 – 56, 13.0)	62.5 (12 – 98, 23.0)
CST V, n = 3	11.0 (11 – 13, 1.4)	29.1 (29 – 34, 2.8)	35.0 (33 – 37, 2.4)	78.8 (71 – 87, 10.9)
p value	0.3418	0.8679	0.3301	0.6048

9.4 Discussion

This chapter describes the vaginal microbiota in a cohort of 103 women treated for CIN who were sampled on the day of their excisional treatment, and at six months post-treatment. The prevalence of vaginal microbiota CST's did not change significantly following treatment, despite successful removal of disease, which has been hypothesised to be the driver of the higher diversity, *Lactobacillus* spp. deplete VMB that is seen more commonly in women with HSIL compared to healthy controls. Higher levels of CST IV were observed in the treated women both prior to and after treatment and compared to healthy controls. The difference in CST IV rates was not significant, but this may be due to the relatively modest size of the control group. This increased prevalence of CST IV is also in keeping with the observation that microbiota richness and diversity were both significantly higher in the treated women at pre- and post-treatment visits compared to controls.

Seventy nine percent of treated women had negative cytology at follow-up and these were compared to the control group, in order to give a comparison of two groups who were cytologically the same, in order to try and tease out the true effect of treatment in the cohort. The same pattern of CST distributions was seen. There were higher rates of HPV positivity in the control population compared to those who had negative cytology after treatment. Further subgroup analysis was therefore performed to compare only women who had negative cytology and a negative HPV DNA test. Yet again, the same pattern was found, with treated women having a higher rate of CST IV compared to untreated controls, although did not reach significance. Data suggests that cervical length regeneration is complete at six months, and therefore it is unlikely that wound healing and the concomitant immune disruption caused by excision, would still be taking place which could potentially alter the vaginal microbiota composition.

It is feasible that scar tissue present on the cervical surface plays host to a different bacterial population compared to the native cervical epithelium, which is known to contain high glycogen levels. Host α-amylase, present in vaginal mucosa, metabolise the glycogen to simple sugar products such as maltose, maltotriose, and maltotetraose that appear to preferentially support *Lactobacillus* spp. colonization [183]. Whilst there are no published studies comparing glycogen levels of scared and naïve cervical epithelium, the application of iodine during colposcopy gives a very rough indication that the scar tissue probably contains less glycogen because it does not turn dark brown with the same intensity as the normal naïve cervical and

vaginal epithelium (Schillers test). This could explain the observed higher diversity microbiome even after removal of CIN.

A further explanation for this lack of alteration of the VMB following removal of CIN, may be due to a genetic predisposition to the presence of particular bacteria in the lower reproductive tract. Genetic polymorphisms have been found in women with recurrent bacterial vaginosis in a variety of genes including those encoding mannose binding lectin [418, 419] and IL-1β [420], both of which play key immune roles. Interestingly, there are particular gene polymorphisms that have been shown to be associated with a higher risk of PTB exclusively in the presence of BV, including the gene encoding methylenetetrahydrofolate reductase [421], interleukin-6 [422] and tumour necrosis factor-2 [423]. Not only does this hint at a connection between genetic risk and the microbiota, but it also suggests women predisposed to having HSIL, may also be predisposed to preterm birth. The data presented here compliment the findings of a higher baseline risk for PTB in women with HSIL as described in Chapter seven and suggest that a predisposition to an altered VMB, even after treatment, may contribute to this risk.

Sneathia spp., *Anaerococcus tetradius* and *Peptostreptococcus* were identified in Chapter three to be biomarkers of high-grade disease and were significantly enriched in pre-treatment samples compared to control. Whilst none of these species were enriched in the post-treatment samples, both pre- and post-treatment samples were significantly enriched with other strict anaerobic species known to be associated with the bacterial vaginosis-type states that have been previously associated with negative outcomes. Borgdorff and colleagues proposed modulation of the vaginal epithelial barrier by a high-diversity, *Lactobacillus* spp. deplete microbiota as an important driver of infection, with such a state resulting in disruption of key vaginal epithelial cytoskeletal proteins and increased cell death, which implies epithelial cell damage and desquamation [277]. This change would potentially facilitate entry of HPV into the basal epithelial cells of the cervical transformation zone where the virus thrives and CIN develops [424]. The next stage in viral persistence is replication and shedding of viral particles. BV is associated with higher shedding of HIV and HSV-2 [425], and *G. vaginalis* in particular, which was significantly enriched after treatment, has been shown to induce HIV replication *in vitro* [426]. It is therefore plausible that a similar mechanism may exist for HPV, and that dysbiosis, paucity of *Lactobacillus* spp. or a combination of the two, creates an environment that promotes the viral life cycle, persistence of the infection, and ultimately

development of dysplasia. BV is commonly diagnosed based on the presence of a characteristic thin, watery foul-smelling vaginal discharge [427], which is thought to arise from squamous cell exfoliation in response to amines produced by particular bacterial species [428] and mucus breakdown. Sialidases are a group of mucin-degrading enzymes produced mainly by *Prevotella* and *Bacteroides* spp. and are found at significantly higher levels in women with BV [429], and the former being higher in the treated cohort at both time points. Evidence also suggests that inflammation plays an important role in dysbiosis-induced cervical disease. Clinical studies have shown that levels of vaginal pro-inflammatory cytokines are higher in women with dysbiosis [302, 303] which can result in chronic inflammation; a well-known factor in carcinogenesis of numerous tissues in the body [306]. All of these mechanisms contribute to higher risk of subsequent cervical cancer in treated women compared to the general population [405, 430, 431].Studies following treated cohorts over a number of years are needed in order to determine whether this is the case.

We have previously shown lower levels of *L. jensenii* to be found in women with HSIL compared to LSIL [229], yet interestingly in this analysis we have shown *L. jensenii* to be enriched in women both prior to treatment, when the majority had HSIL, and also after treatment, when compared to controls. This is a slightly unexpected observation and whilst there is little work published on the specific characteristics of L. jensenii, it has been shown to have inhibitory effects on the growth of cervical cancer cell lines *in vitro* with its ability to regulate expression of HPV oncogenes and cell cycle-related genes as a postulated mechanism [432]. It has also been shown to produce bio-surfactants capable of breaking down the biofilms that may be seen in the vagina and usually associated with CST IV [433]. Thus, it appears there are likely to be more protective mechanisms of *L. jensenii* than reasons for it to be associated with a disease such as CIN that is largely now accepted to be associated with higher microbiota diversity. A likely explanation may be that CST V that is dominated by *L. jensenii* was not seen in the control population, and whilst this may be a true biological phenomenon, it may also be an artefact of the relatively small sample size of the healthy control group.

Pregnancy is known to be associated with a higher rate of *Lactobacillus* spp. colonisation and VMB stability [158]. The absence of *Lactobacillus* spp. likely results in and increased risk of ascending infections which are known to be associated with at least 40% of cases of PTB [117]. The presence of BV-like bacterial species are also known to release small metabolites

including the short-chain fatty acids succinate and butyrate that are able to modulate the immune responses [434] and inflammatory pathways known to be responsible for both term, and preterm parturition [434]. Changes in the character of cervical mucus, which is produced by the glandular endocervical epithelium, may also be associated with pre-term birth [118], which as previously discussed may also occur as a result of overgrowth of BV-like, strict anaerobes. There are therefore numerous plausible mechanisms that could associate an altered VMB with pregnancy complications. Ascending infections commonly result in infection between the amnion and chorion or in the choriodecidual space, and although the exact timing of such bacterial invasion is unclear, it has been posulated that this may arise early in pregnancy and go undetected for several months [435]. Once the endometrial cavity has been sealed off around 20 weeks, the likelihood of ascending infections is decreased, which suggests intrauterine infections may in fact be the result of a chronic infection that began in early pregnancy, or even prior to concepteion [435], and therefore the preconception VMB composition likely dictates important outcomes.

The structure of the vaginal microbiota (VMB) is dynamic and transitioning between CSTs can occur, particularly in the non-pregnant population [158]. The most common transition observed is from CST III to CST IV, which suggests *L. iners* may be less able to inhibit colonisation of strict anaerobes and pathobionts compared to other *Lactobacillus* spp. [158] or because *L. iners* appears more capable of surviving and adapting to a wide range of pH and other metabolic stress-related conditions due to the constitutive and inducible expression of genes not seen in other lactobacilli [159, 160]. *L. crispatus*-dominant CST I is the most stable of the five CST's [186] and this was reflected in our data with 60% of women with CST I at baseline having CST I at follow-up. This microbiota structure demonstrates the lowest pH of all five CST's [153], which is known to protect against many bacterial and viral pathogens including bacterial sexually-transmitted infections (STIs), Herpes simplex virus (HSV)-2, HIV, as well HPV [436]. It is thus unsurprising that the presence of *L. crispatus* has been negatively correlated with CIN [269] and is associated with better pregnancy outcomes being highly predictive of term birth when found at 16 weeks gestation (positive-predictive value, PPV = 98%) [414]. Although we do see a relatively high presence in our women undergoing treatment for high-grade disease, it is tempting to speculate that these women may be at the lowest risk of subsequent complications of pregnancy, and of cervical disease recurrence, in light of its numerous well-established protective characteristics.

Little is known about the exact cellular mechanisms of cervical regeneration that take place following excisional treatment. There are two main tissues that regenerate; the cervical epithelium, and the underlying stroma, which is composed of fibroblasts, smooth muscle, elastic fibres and collagen. Zevin *et al.* published a recent study in which they performed an *in vitro* wound assay to show that presence of *G. vaginalis* was associated with lower rates of healing in HeLa cell monolayers compared to in the presence of *L. iners* [417]. A recent study within our group has shown that CST III in combination with a short cervix (<25mm) at 16 weeks gestation was a significant risk factor for delivery before 34 weeks gestation [414] and suggests a possible interaction between the cervical structure and microbial population. We did not however find any difference in the cervical length or volume regeneration in relation to pre- and post-treatment CST. One of the limitations of this analysis is that we have no information about the patients CST in between the two time points. Furthermore, the vaginal microbiota is not in direct contact with the underlying stroma, which is the main tissue type that regenerates to contribute to the final cervical length and volume, and therefore it is plausible that the VMB composition is unable to have any impact. The following chapter will examine the impact of excision on the functional quality of the regenerated cervical epithelium.

9.5 Conclusion

Cross-sectional studies exploring the associations between the VM, HPV infection and cervical pre-invasive and invasive disease have shown that a high-diversity VM is associated with higher grades of cervical disease in Chapter three and other published cohorts [229, 269, 270], and in acquisition and persistence of its causative agent HPV [202]. To our knowledge, this is the first study to examine the impact of the surgical removal of a disease known to be associated with an altered vaginal microbiome compared to healthy controls. We have shown that a high-diversity, *Lactobacillus* spp. deplete vaginal microbiota remains in spite of successful removal of the disease, and of HPV infection. We suggest this may partly explain adverse pregnancy outcomes, and disease recurrence following treatment for CIN. If proven, manipulation of the VMB using pre- and probiotics may represent a novel therapeutic method to prevent these unfavourable obstetric and oncological outcomes. It is possible that the microbiota may also have an impact on the innate immune system after treatment which will be examined in the following chapter.

CHAPTER 10. Antimicrobial peptides and cervical inflammatory cytokines before and after excisional treatment

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Content from this chapter is currently under preparation as:

Mitra A, MacIntyre DA, Whelan E, Lee YS, Smith A, Marchesi JR, Lyons D, Bennett PR and Kyrgiou M. Cervical intraepithelial neoplasia is associated with an altered vaginal microbiome and innate immune disruption that is not altered following excision of disease. (*Under preparation)*

10 Antimicrobial peptides are decreased after excisional treatment

10.1 Background

There is a vast array of causes of spontaneous preterm birth (PTB) including infection, antepartum haemorrhage, uterine overdistension and cervical incompetence [104]. Around 40% of PTB is thought to result from infection, most likely ascending from the vagina, which prematurely activates inflammatory pathways in the reproductive tract leading to chorioamnionitis and preterm prelabour ruture of membranes (PPROM) [117] [437], cervical remodeling and uterine contraction. Prevention of ascending infection is therefore paramount for the development and maintenance of a healthy, term pregnancy. The innate immune system is the first components for defense against pathogens [438], lying at the interface between the external environment and the mucosal surface of the reproductive tract as well as the fetal tissues and membranes. In Chapter six we showed that the expression of two AMPs; Human beta-defensin-1 (hBD-1) and Secretory leucocyte protease inhibitor (SLPI) is increased with increasing severity of CIN.

Human beta-defensin-1 has been shown to have broad-spectrum antimicrobial activity, particularly against Gram-negative species known to be associated with PTB including *Ureaplasma* spp. [215] and *Escherichia coli* [216] and indeed upregulated defensin expression has been observed in infection-associated cases of PTB [218]. Secretory leucocyte protease inhibitor (SLPI) has been found at high concentration in the mucus plug and may be 10-12 times higher in cervicovaginal secretions of pregnant women compared to non-pregnant [226]. Expression increases steadily throughout gestation but is significantly higher in women who subsequently deliver preterm compared to those with threatened preterm labour who delivered at term, and normal term deliveries [219]. SLPI also appears to play a role in term parturition, with concentrations found to be elevated in cervical mucus of women samples within seven days prior to delivery in uncomplicated term pregnancy [227]. These data suggest a role for SLPI in maintenance of a pregnancy and in regulation of the cervical remodelling required for normal term parturition, as well as preterm birth.

Both AMPs therefore appear to play an important role in both maintaining a pregnancy to term, and in protection specifically against infection-related preterm births. Excisional treatment for CIN is known to cause a scar on the cervix, and it is unlikely that scarred epithelium is able to produce the same level of AMPs as untouched epithelium, which may

further disrupt the innate immune function of the cervix and present an added risk factor for preterm birth. This hypothesis has not yet been tested and will be explored in this chapter.

10.2 Aims

- Investigate changes in expression and localisation of hBD-1 and SLPI at six months following excisional treatment for CIN.

10.3 Results

A total of 114 women were included in the analysis and classified into three groups; 34 normal who were sampled at one time point and 80 women who underwent excisional treatment and were sampled at two time-points; pre- and six-months post-treatment. The average time to follow-up was six months (range – 5 – 12 months). Patient metadata were analysed as shown in table 10.1.

No differences in metadata were detected between the control and treated cohort. Characteristics of the treated cohort did not change significant between the baseline and follow-up visit, apart from two patients who changed their method of contraception. One patient had her copper IUD removed and was instead using condoms at follow-up, and one patient who was not using any contraception at the time of treatment subsequently had a Mirena IUS inserted. There was equal distribution in the samples collected at the follicular or luteal phase of the cycle and in the rate of women that had intercourse within 48 hours from sample collection.

Table 10.1. Characteristics of included patients

	Control, n=34	Pre-treatment, n=80	Post-treatment, n=80	p value
Age, years				0.0985
Mean (SD, range)	30.0 (4.415, 20-43)	31.7 (5.1, 25 – 45)	32.3 (5.2, 25 – 46)	
Ethnicity, n/N (%)				0.3541
Caucasian	26/34 (77)	70/80 (88)	70/80 (88)	
Asian	3/34 (1)	6/80 (8)	6/80 (8)	
Black	5/34 (14)	4/80 (5)	4/80 (5)	
Parity, n/N (%)				0.7881
Nulliparous	27/34 (81)	59/80 (74)	59/80 (74)	
Parous	7/34 (19)	21/80 (26)	21/80 (26)	
Smoking status, n/N (%)				0.4343
Current smoker	5/34 (15)	20/80 (25)	20/80 (25)	
Non-smoker	29/34 (85)	60/80 (75)	60/80 (75)	
Phase of menstrual cycle, n/N (%)				0.6004
Luteal	12/34 (35)	40/80 (50)	37/80 (46)	
Follicular	18/34 (55)	35/80 (44)	38/80 (48)	
Unknown	4/34 (10)	5/80 (6)	5/80 (6)	
Contraception, n/N (%)				0.9513
Nil	16/34 (47)	29/80 (36)	28/80 (35)	
Condoms	5/34 (15)	12/80 (15)	13/80 (17)	
COCP	9/34 (26)	28/80 (35)	28/80 (35)	
POP	1/34 (3)	3/80 (4)	3/80 (4)	
Copper IUD	0/34 (0)	2/80 (3)	0/80 (0)	
Mirena IUS	2/34 (6)	5/80 (6)	6/80 (8)	
Contraceptive implant	1/34 (3)	1/80 (1)	1/80 (1)	
Time since last intercourse, n/N (%)				0.9704
>48 hours	30/34 (87)	71/80 (89)	70/80 (88)	
<48 hours	4/34 (13)	9/80 (11)	10/80 (12)	

CIN: Cervical intraepithelial neoplasia; COCP: Combined oral contraceptive pill; IUD: Intrauterine device; IUS: Intrauterine system; POP: Progesterone-only pill

In the untreated control cohort, histology was available for only three patients (3/34, 11%) and the remaining 88% were classified according to cytology. In the cohort of 80 women who underwent excisional treatment, histology results for the excisional specimen were available in 100% of cases and the cytology collected at routine six-month follow-up was used for disease classification, and again was available in 100% of patients. Table 10.2 shows these pre-treatment histology results and cytology for women at six months post-treatment, and the healthy control population. A higher proportion of treated women with normal cytology were HPV negative compared to the control group but this was not significant (p=0.0832, Fishers exact test).

Table 10.2. Histology, cytology and HPV status

	Control, n=34	Pre-treatment, n=80	Post-treatment, n=80
Histological severity of lesion			
Normal	-	0/80 (0)	-
CIN1	-	9/80 (11)	-
CIN2	-	32/80 (40)	-
CIN3	-	39/80 (49)	-
Cytological severity of lesion			
Normal, HPV -ve	22/34 (65)	-	57/80 (71)
Normal, HPV +ve	9/34 (26)	-	8/80 (10)
Normal, HPV status unknown	1/34 (3)	-	0/80 (0)
BNC, HPV -ve	0/34 (0)	-	8/80 (10)
BNC, HPV +ve	0/34 (0)	-	3/80 (4)
LSIL, HPV -ve	0/34 (0)	-	0/80 (0)
LSIL, HPV +ve	0/34 (0)	-	2/80 (3)
HSIL	0/34 (0)	-	2/80 (3)

BNC: Borderline nuclear changes, CIN: Cervical intraepithelial neoplasia, HSIL: high-grade squamous intraepithelial neoplasia, LSIL: low-grade squamous intraepithelial neoplasia.

10.3.1 hBD1 and SLPI levels in are reduced at 6 months post-excisional treatment

Paired measurements of hBD1 and SLPI at baseline and at six months post-excisional treatment were available for 80 women. There was no significant difference in total protein concentration pre-or post-treatment (p=0.8105, paired t-test) (Table 10.3).

Levels of both peptides were reduced after treatment in a paired analysis of 80 women pre- and post-treatment (hBD-1 and SLPI, both p<0.0001, paired t-test) (Table 10.3, Figure 10.1). Of the 80 treated women, 65 had a normal cervical cytology test at follow-up and were compared to healthy, untreated controls, and both AMP levels were significantly less in treated women compared to the healthy control population (hBD-1 p=0.002 and SLPI, p=0.04, unpaired t-test) (Table 10.3, Figure 10.1). In a further subgroup analysis of HPV negative controls with those who were HPV negative with normal cytology after treatment, the same observations were seen (hBD-1 p=0.002 and SLPI, p=0.04, unpaired t-test).

There was no correlation between levels of hBD1 or SLPI and change in cervical length, volume, or according to proportion of length or volume excision.

Table 10.3. Total protein, hBD-1 and SLPI levels after treatment

	Total protein (µg/ml) (median ± IQR)	**hBD-1:total protein (pg/µg protein)** (median ± IQR)	**SLPI:total protein (pg/µg protein)** (median ± IQR)
Paired analysis			
Pre-treatment, n=80	534 ± 315	5651 ± 2693	221437 ± 94039
Post-treatment, n =80	508 ± 245	1007 ± 423	34073 ± 22164
Subgroup analysis			
Normal control, n = 34	619 ± 409	2096 ± 1164	90486 ± 41324
Normal post-treatment, n = 65	488 ± 345	1008 ± 423	33714 ± 23431

IQR; interquartile range

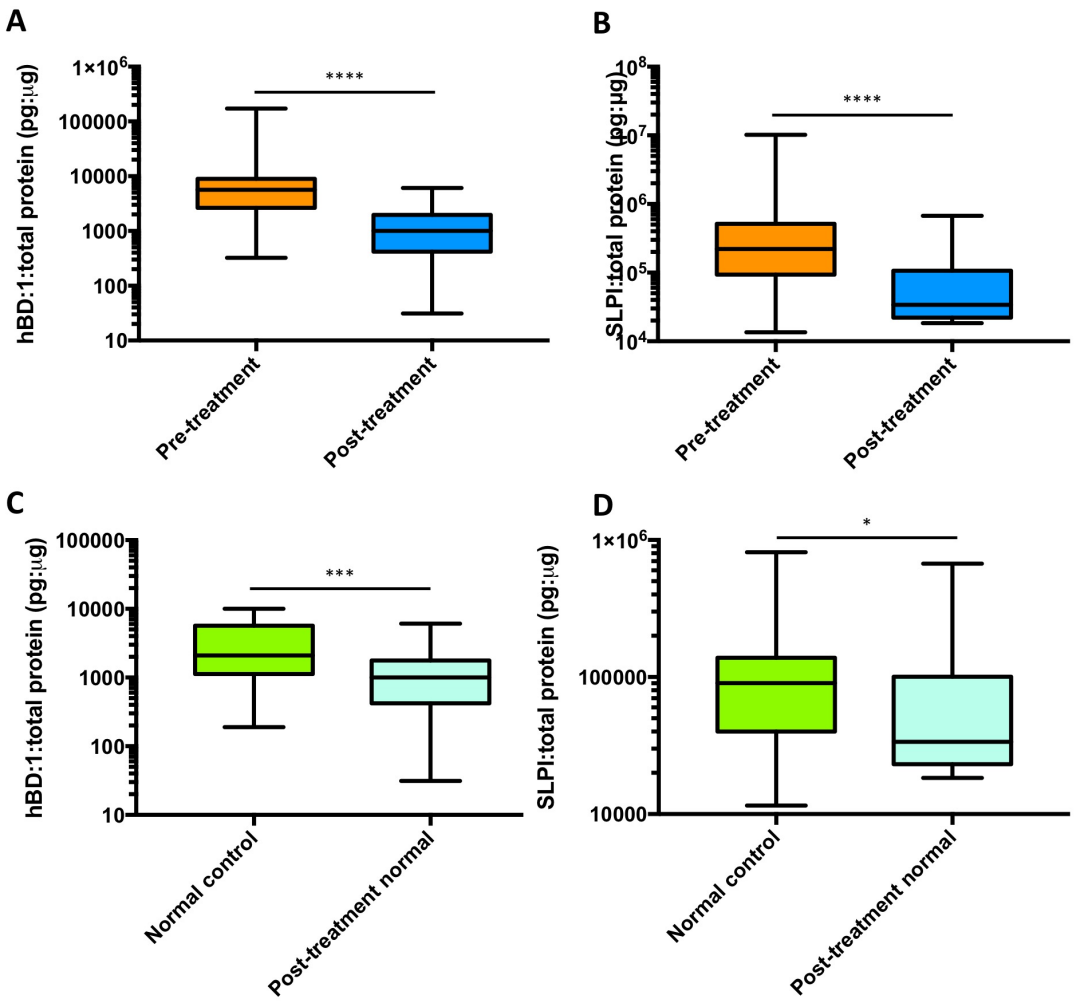


Figure 100.1. Impact of excisional treatment on hBD-1 and SLPI levels in paired samples (A&B) and compared to normal, untreated controls (B&D).

Levels of both hBD-1 and SLPI were significantly lower after treatment compared to before in a paired cohort, Paired t-test. Post-treatment levels of both peptides were also significantly lower than those seen in untreated, normal controls, Unpaired t-test. *p<0.05, **p<0.01, ***p<0.001, p<0.0001.

10.3.2 hBD1 and SLPI expression is reduced in the scarred cervix after excisional treatment

Two women within the recruited cohort underwent repeat excisional treatment for residual high-grade disease at follow-up, according to current BSCCP guidelines. Sections from the excisional biopsies were obtained from the NHS histopathology department and stained with hBD1 and SLPI antibodies, followed by haematoxylin counterstaining. Figure 10.2 shows the distribution of hBD1 and SLPI in a cervical cone containing CIN3. Staining for both peptides was strongest in the glandular epithelium and areas of high-grade CIN with weak staining of the normal squamous epithelium.

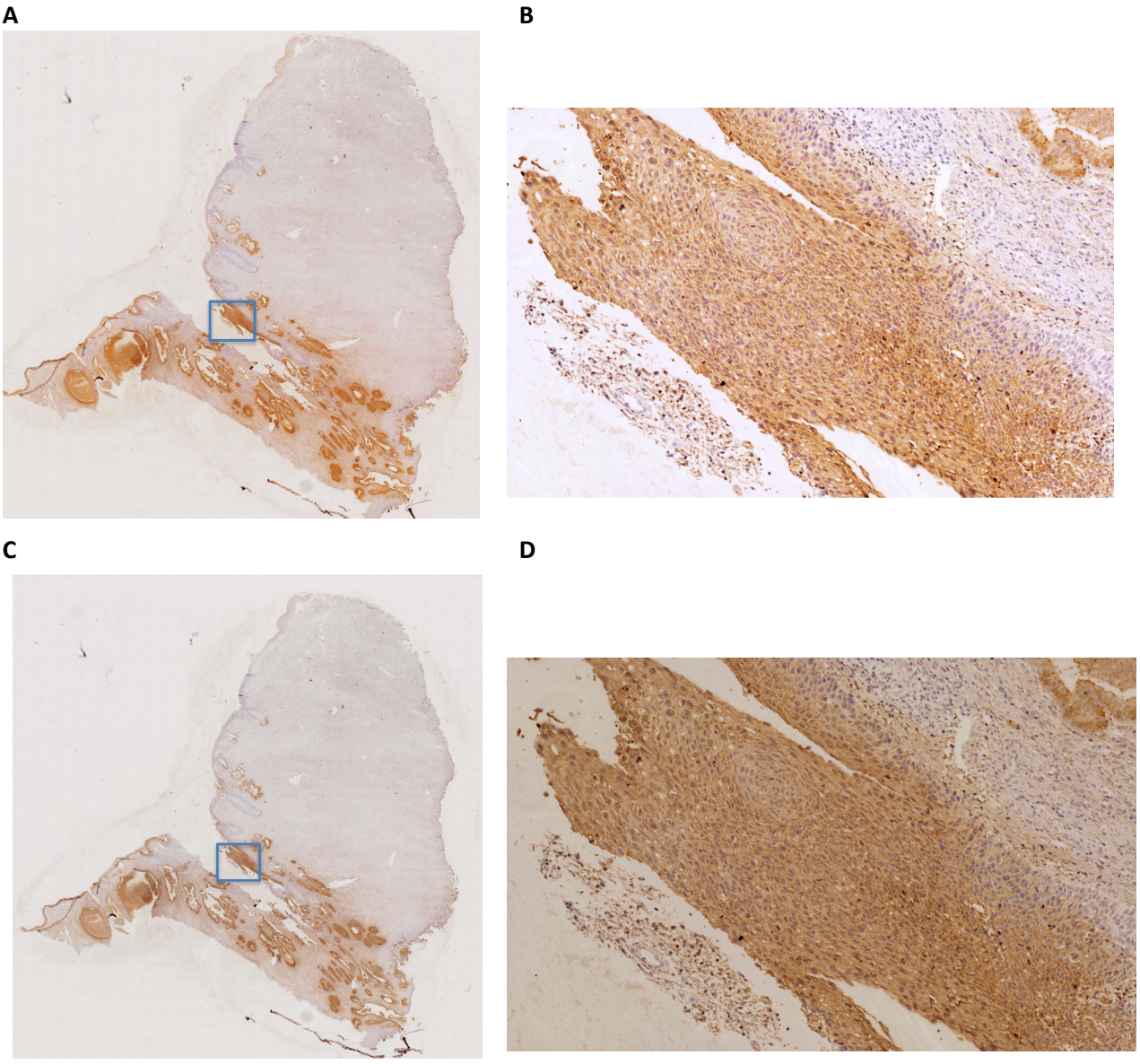
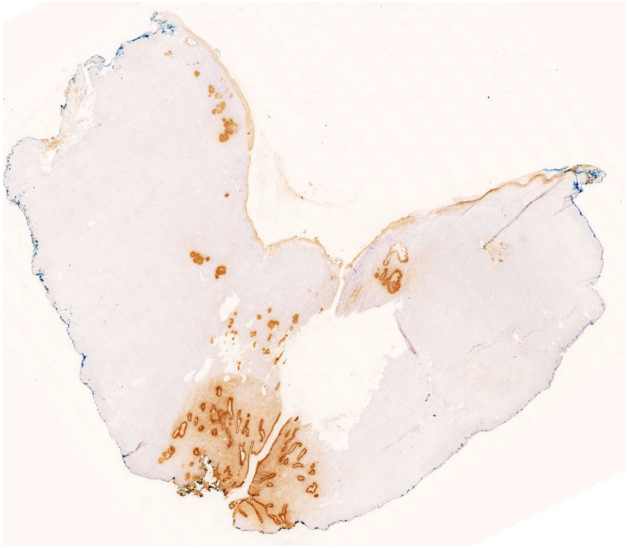


Figure 100.2. hBD1(A) and SLPI expression (C) in a cervical cone, and in an area of CIN3 (B:hBD1, C:SLPI)

Both peptides were strongly expressed in the glandular epithelium and areas of high-grade CIN

Figure 10.3 shows a section for the second cone performed due to residual CIN2 at the endocervical margin. Although this residual disease is not present in this particular section, we can see expression remains highest in the glandular epithelium with minimal staining seen in the squamous ectocervical epithelium. The crater from the previous excision is visible and the squamous epithelium here exhibits the weakest staining for both peptides.

A



B

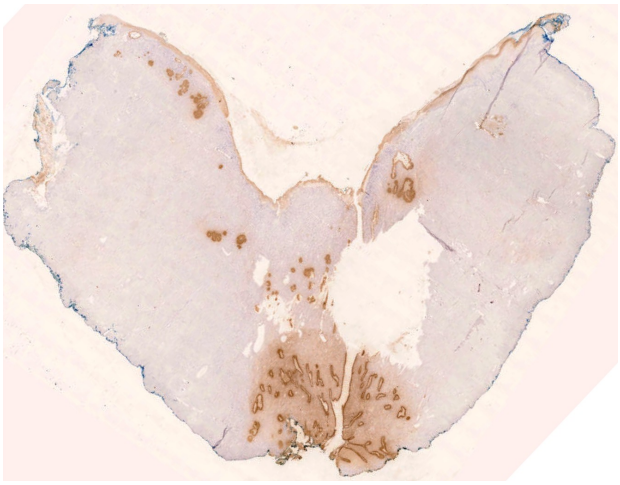


Figure 100.3. Distribution of hBD1 (A) and SLPI (B) after repeat excision

The scarred epithelium appears to exhibit weak staining for both peptides compared to the untouched cervical and glandular epithelial surfaces.

10.3.3 Proinflammatory cytokine levels remain elevated after treatment

Figure 10.4 and Table 10.4 show the impact of treatment on cytokine levels. The proinflammatory cytokines IL-1β and IL-8 were both significantly elevated prior to treatment compared to healthy controls ($p<0.0001$ and $p=0.0036$ respectively, Kruskal-Wallis test) and whilst they dropped slightly after excision they remained significantly higher than healthy controls ($p=0.0002$ and $p=0.0114$ respectively, Kruskal-Wallis test). TNF-α was also elevated prior to treatment compared to controls but this was not significant, and after treatment levels fell and this was approaching significance when compared to pre-treatment levels ($p=0.0997$, Kruskal-Wallis test). IFN-γ was significantly lower prior to treatment compared to controls ($p=0.0146$, Kruskal-Wallis test). Levels were significantly higher after treatment ($p=0.002$, Kruskal-Wallis test), and were similar to levels seen in healthy controls.

Table 10.4. Impact of treatment on levels of Levels of IL-1β (A), IL-8 (B), IFN-γ (C) and TNF-α (D)

	IL-1β:total protein (pg/µg protein) (median ± IQR)	**IL-8:total protein (pg/µg protein)** (median ± IQR)	**Interferon-γ pg/µg protein)** (median ± IQR)	**TNF-α:total protein (pg/µg protein)** (median ± IQR)
Normal, n = 34	187.9 ± 284.1	199.9 ± 309.5	28.2 ± 74.5	13.1 ± 11.4
Pre-treatment, n=80	463.9 ± 714.2	408.8 ± 1085.2	13.7 ± 13.3	14.9 ± 39.3
Post-treatment, n=80	421.7 ± 1103.5	374.4 ± 1302.3	24.7 ± 58.2	8.9 ± 13.7

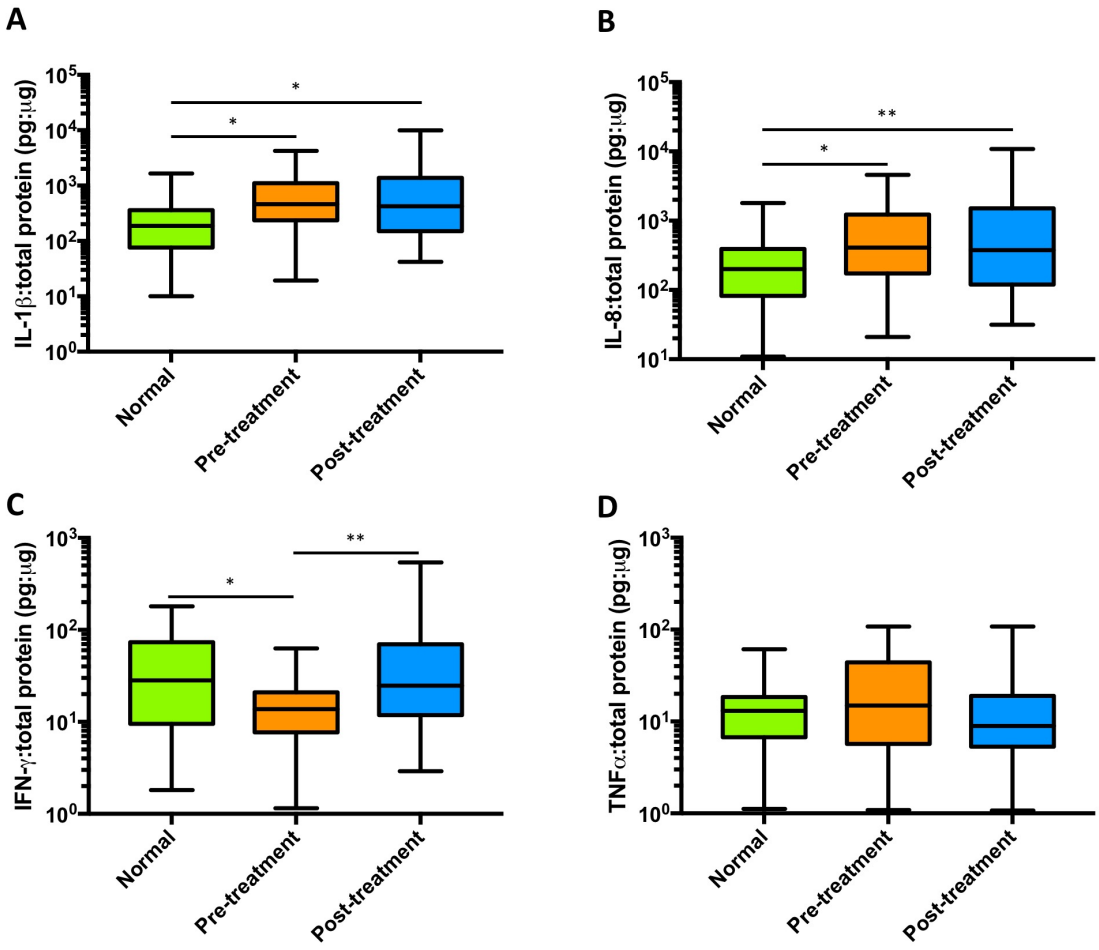


Figure 100.4. Impact of treatment on levels of Levels of IL-1β (A), IL-8 (B), IFN-γ(C) and TNF-α(D)

Kruskall-Wallis test, *p<0.05, **p<0.01.

10.4 Discussion

AMPs play a key role in the innate immune system of the cervix and are inducible in the presence of infection and inflammation, which is important for protection against viral and bacterial pathogens, both of which play a role in cervical carcinogenesis and preterm birth. Levels of hBD1 and SLPI detected in cervical secretions both increase with increasing disease severity in our study population as described in Chapter six. The data presented in this chapter explores the impact of excisional treatment in 80 women who provided samples before and after treatment for CIN, and compares the levels to those seen in healthy untreated controls. Levels of both AMPs were found to be significantly lower after treatment compared to before. Two women underwent repeat treatment, and both cones were obtained for assessment. Using immunohistochemistry, we have also shown that the peptides are heavily expressed in areas of high-grade disease in sections from cervical cone biopsies. Therefore, with removal of the high-grade disease it is not surprising that the levels of these peptides drop, as the disease may be the driver of their expression, which may be one of the mechanisms that the virus or the disease itself employs as a way of persistence. Most strikingly however, we have also demonstrated that the peptide levels in the cervical secretions of treated women are significantly lower than healthy untreated controls. Both of these groups are free of disease, and this suggests that it is the treatment that is leading to these significantly reduced AMP levels. Staining of sections in the two patients who have previously undergone conisation have suggest the scarred epithelium overlying the previous excisional crater does not produce such high levels of these AMPs compared to the untouched squamous epithelium.

Both hBD1 and SLPI appear to play a role in protecting pregnancy and therefore the lack of these peptides may be one of the mechanisms though which treatment increases the risk of subsequent late miscarriage and preterm birth. Human beta-defensin-1 is known to possess particularly strong antimicrobial activity against microbes that are associated with PTB [215, 216] and other types of defensins may be upregulated in PTB where an infective aetiology can be specifically identified [218]. SLPI is known to be present in high concentrations in the mucus plug, and pregnant women have been found to have 10-12 fold higher levels than their non-pregnant counterparts [226]. Expression progressively increases throughout gestation, although is significantly higher in women who experience PTB compared to those with threatened preterm labour who delivered at term, and normal term deliveries [219]. Furthermore, SLPI is known to inhibit Nuclear factor kappa-B activation [439], which is known

to activate both preterm and term labour [440]. However, alongside an antibacterial role, protease inhibitors in particular may be upregulated at the time of parturition as a regulator to prevent excessive tissue remodeling. Converesely, downregulation of SLPI has been observed in the cervix of pregnant [441] and non-pregnant [228] women with bacterial vaginosis, and in the fetal membranes of women with PPROM, which may occur as a result of their inability to inhibit tissue degradation by proteases secreted by invading pathogens [218]. It seems that there is a complex interplay between the expression of AMPs and interactions with other factors of the innate immune system, as well as the adaptive immune system and subsequent activation of inflammatory pathways that may precipitate PTB. However, this increase in levels in those who deliver preterm may be as a result of an infective stimulus which is known to both induce expression of AMPs [208, 220] and cause a significant proportion of PTB [117]. If the cervix is less able to produce these peptides, this may mean that these women are less likely to make a sufficient innate response in an attempt to protect the pregnancy from infective threats, resulting in ascending infection and the resulting sequelae.

SLPI and hBD1 both appear to be strongly expressed by the glandular epithelium as demonstrated in this chapter using immunohistochemistry. The aim of loop excision is to remove the entire transformation zone [406] in order to remove all CIN including the visible lesion and also smaller areas of disease that may be less apparent to the naked eye, and also removes the underlying HPV infection. Whilst removing the transformation zone, a varying amount of healthy glandular tissue will invariably be removed due to the morphology of the cervix. The immunohistochemistry experiments performed in this chapter have shown that AMP expression is greatest in the healthy glandular tissue compared to the normal squamous or metaplastic cells of the transformation zone, which is in keeping with what has previously been shown with regards to hBD1 [211] and SLPI expression [442] in the healthy cervix. Whilst there was no correlation between the drop in AMPs and the length or volume of tissue removed in the data presented in this chapter, it is difficult to accurately estimate the amount of glandular epithelium contained in a specimen using these measurements. It is possible that the women who had the greatest drop in levels between visits would be those who had the largest amount of glandular epithelium removed and this may subsequently translate into those who are at greatest risk of subsequent pregnancy complications.

Some colposcopists have previously advocated using a top-hat procedure for excising deep

lesions, particularly in women with a type three transformation zone in an attempt to ensure complete excision of deeper lesions, without removing excess healthy tissue and stroma. The technique involves performing a routine LLETZ to remove the entire transformation zone, followed directly by a further excision deeper into the endocervical canal with a narrower base [443]. Anecdotal evidence suggests the technique appears to have fallen out of favour amongst UK-based colposcopists following a comparative study of 513 patient treated with conventional and top-hat LLETZ that has shown top-hat not only removed significantly more tissue, it did not significantly decrease the number of women with clear endocervical margins, and also resulted in higher rates of inadequate post-treatment colposcopy and cytology due to a greater degree of scarring [443]. With this in mind, the data presented here reinforce why this procedure should not be carried out amongst women of childbearing age, most of whom will have either type one or type two transformation zone, which will thus result in a large amount of glandular tissue being removed, which likely results in a functional deficit of the cervix. Such a functional deficit of the cervix, along with the anatomical deficit described in Chapter eight, may further increase a woman's risk of second-trimester miscarriage and preterm birth.

Inflammation is known to induce AMP expression [208], and we therefore explored the changes in levels of the four profinflammatory cytokines known to be altered in high-grade disease, as shown in Chapter six. After treatment, TNF-α levels fell and became similar to levels seen in the control group, which is likely due to successful removal of disease because elevated TNF-α levels are associated with HPV persistence and disease progression [444]. INFγ levels were lower in women with high-grade disease, and were elevated to levels similar to controls after treatment, which may be a result of the removal of HPV which probably uses its oncogenes E6 and E7 to suppress expression of the antiviral cytokine. We have however demonstrated that levels of the proinflammatory cytokines IL-1β and IL-8 remained elevated after treatment, relative to untreated controls. These findings are broadly in keeping with a previous study of cytokine levels after LLETZ published by Saftlas *et al.* [445] and are interesting for several reasons. Firstly, IL-1β, TNFα and IL-8 have all been demonstrated to promote cervical ripening in a guinea pig model [446], and thus ongoing baseline inflammation in treated women could also be a predisposing factor to adverse obstetric outcomes. Secondly chronic cervical inflammation can predispose to further HPV infections, persistence and disease progression [447], which may present a molecular mechanism explaining increased risk of future invasive cancer [405, 430]. Thirdly, if these women have

ongoing inflammation that is greater than in healthy controls, one would expect the cervical epithelium to respond by increasing AMP expression, which is not seen. Therefore, we suggest that treated women may have a functional cervical deficit, and may therefore be unable to mount such a strong response to cervical inflammation. One potential reason for an ongoing elevation of certain proinflammatory cytokines may be due to ongoing tissue regeneration. It is well documented that cervical length and volume regeneration are complete at six months when these women had their follow-up appointment [114, 409, 410], although there is no published evidence to confirm that regeneration is complete at an epithelial level.

The main limitation of this study is that we do not have longitudinal data for the healthy control population so we do not know how these peptides change with time. We do know from the published literature that there may be changes in AMP expression throughout the menstrual cycle [442], however, we did not detect any significant differences according to phase of the cycle (data not shown), and the same proportion of samples were collected in the follicular and luteal phases in each group. Patients often do not accurately record the date of their last menstrual period, and not all women have a standard 28-day cycle therefore there may be some imprecision in their phase of the menstrual cycle, as well of the use of hormonal contraceptions, which could all account for why a difference was not detected.

AMPs may also be secreted by immune cells as well as the epithelia of the female reproductive tract. To study the impact of CIN and its treatment on the populations of immune cells, and also other AMPs, will help us to gain a better understanding of the very complex interplay between cervical disease, the cervical epithelium, and the innate, as well as adaptive immune response in HPV-related disease, and subsequent pregnancy complications. It will be useful to carry out longer follow-up to determine whether inflammation subsequently subsides, or AMP expression is recovered, and further follow them into pregnancy to determine how these changes affect pregnancy outcomes.

10.5 Conclusion

As reported in Chapter eight, the cervix is capable of regenerating in length after treatment, particularly with smaller excisions. This involves the cervix healing with a scar, which is frequently observed at colposcopy. In this chapter, ELISA was used to show that levels of both AMPs are significantly lower after treatment and immunohistochemistry experiments suggest

that whilst the healed scar tissue is capable of AMP expression, the glandular tissue is the most prominent source of these peptides in the cervix. Excision of glandular tissue therefore is the likely reason for decreased AMP levels observed following treatment. To the best of our knowledge, these data are the first to show that in spite of good anatomical regeneration, there is inferior functional capacity of the cervix after excisional treatment for CIN. The question therefore arises as to whether these women are capable of mounting a sufficient immune response in pregnancy in the case of infection, which may predispose to adverse obstetric outcomes such as second trimester miscarriage and preterm birth.

CHAPTER 11. Discussion

11 Discussion

11.1 Summary of hypotheses

It is well-established that HPV is the causative agent of cervical cancer and its preinvasive precursor lesion CIN [12, 448]. HPV is known to infect at least 80% of sexually active women by the age of 50 [15], however the lifetime risk of developing invasive cervical cancer is much lower at 0.6% [16]. Despite infecting the majority of the female population, it causes significant disease in only a minority through a largely unknown mechanism. Increasing evidence suggests the cervicovaginal microenvironment including the vaginal microbiome has an influence on the natural history of HPV and subsequent disease [202]. As part of the studies described in this thesis, it was hypothesised that the vaginal microbiome in women with high-grade disease would be different to that of normal controls. To test this the vaginal microbiome was characterised in a cross-sectional cohort of women with CIN and compared to healthy controls. This data was integrated with analyses of antimicrobial peptides and inflammatory cytokines thought to play a role in predisposing a woman to persistent HPV infection and progressive cervical disease. Additional study of the role of the microbiome in disease progression was undertaken using 607 samples collected longitudinally from 95 women with CIN2 who did not receive treatment.

Excisional treatment is the preferred method in the UK for treatment of cervical-screening detected, high-grade CIN and is very effective at preventing cervical cancer. A common concern of patients undergoing treatment is its impact on fertility and miscarriage risk. However a lack of mechanistic evidence makes the provision of informed, objective advice difficult. It is plausible that the depth of excision partly predicts the risk of preterm birth following treatment, and the use of different control populations may affect the calculated risk. Systematic reviews and meta-analyses were therefore performed to address these issues. It was further hypothesised that there may be a wide range of cervical lengths in the untreated population and therefore proportion of cervical excision may more accurately predict an individuals risk of preterm birth.

Aside from structural changes it was hypothesised that an intrinsic difference in the cervicovaginal environment in women with high grade disease may exist that is further altered by treatment, which has not previously been considered. We therefore investigated the impact of treatment on the vaginal microbiome, antimicrobial peptides and inflammatory cytokines as the first step to addressing this hypothesis.

11.2 Summary of findings

11.2.1 Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity

Chapter three was the first published study to describe the vaginal microbiome of women with varying grades of CIN compared to healthy controls and a small number of women with invasive cancer. The data showed that increasing disease severity was associated with greater vaginal microbial diversity characterised by lower levels of *Lactobacillus* spp. Five bacterial species were identified that could differentiate low- and high-grade disease. With further research these may improve our understanding of the role of the bacterial microenvironment in HPV persistence, development of CIN and progression to cancer. These are the first published findings to suggest that the presence and prevalence of specific vaginal microbiome CSTs may be involved in the pathogenesis of CIN and cervical cancer, however longitudinal assessment is required to further evaluate whether this association relates to disease causality. This was addressed in the following two chapters.

11.2.2 Comparison of vaginal microbiota sampling techniques: cytobrush versus swab

The current literature describes largely cross-sectional data, and the use of serial samples will help researchers to separate association from causality, and increase understanding of the molecular mechanisms associating microbiome composition with disease outcomes. In the field of cervical pathology, we are very fortunate that biobanks of longitudinal samples exist, collected both by individuals, and as part of cervical screening programmes. Such samples are usually collected using cervical cytobrushes, rather than the swabs most commonly reported in the current literature as well as in Chapter three, and therefore it is important to determine whether sample collection technique impacts on sequencing results.

Chapter four described a small pilot study of 30 women with high-grade disease to compare these two techniques and showed that rayon swabs and polyethylene cervical cytobrushes produce comparable results when comparing the vaginal microbiota composition at species level. It was hypothesised that cytobrushes may be able to disrupt biofilms and thus uncover a more diverse vaginal microbiome structure compared to swabs. In 10% of the sampled cohort, cytobrushes detected a CST IV microbiome structure that was not demonstrated by the corresponding swab sample, which may be due to the cytobrush having a greater

exfoliative capacity, enabling biofilm disruption. Cytobrushes were also found to collect a higher bacterial load, which may reduce the impact of potential sample contamination.

11.2.3 Untreated CIN2: the role of the vaginal microbiota in the natural history

Cross-sectional studies exploring the associations between the VM, HPV infection and cervical pre-invasive and invasive disease have shown that a high-diversity VM, and to a lesser extent *L. iners*-dominant VM's correlate with increasing cervical disease severity [229, 269, 270], and in acquisition and persistence of its causative agent HPV [202].

Chapter five described a novel longitudinal cohort of 87 young women with CIN2 recruited in California, who were observed for between five and 46 months. High rates of regression were observed and provisional analysis suggested that absence of *Lactobacillus* spp. and presence of a diverse population of strict anaerobes may be associated with higher rates of disease persistence and slower disease regression. Currently available *in vitro* and *in vivo* studies largely centred around bacterial vaginosis, both in the absence and presence of cervical disease suggest many plausible mechanisms that may explain these findings, particularly focussed on alterations in the cervical immune system and activation of proinflammatory pathways, which were examined in the following chapter.

11.2.4 Antimicrobial peptides and cervical inflammatory cytokines are altered in cervical intraepithelial neoplasia

Chapter six studied the interaction of the vaginal microbiome, cervical antimicrobial peptides and proinflammatory cytokines in women with CIN and healthy controls, to begin to investigate alterations in the innate system which are hypothesised to play a role in determining the fate of a cervical lesion. Cervical antimicrobial peptides human beta-defensin-1 (hBD1) and secretory leucocyte protease inhibitor (SLPI) were found to be increased according to disease severity, independent of HPV status. Overexpression of SLPI in particular has been seen previously in cervical cancer, as well as other epithelial cancers, and therefore it seems that excessive production may result in a negative effect. Furthermore, higher levels of hBD1 and SLPI were seen in women with CST IV, which suggests an interplay between the microbiome and the innate mucosal immune response. The current literature suggests that a CST IV vaginal microbiome may be associated with higher levels of inflammation and this was corroborated by the data presented showing IL-1β, IL-8, TNF-α,

and IFN-γ to be altered according to both disease status and VMB composition. Finally, this chapter also found hBD1 levels to be lower in smokers than in non-smokers. Smoking is a known risk factor for both HPV infection and cervical cancer, and this is the first study to show that smoking may have an impact on AMP production in the cervix, which may represent a mechanism of smoking-induced susceptibility to HPV and subsequent disease.

11.2.5 The impact of conservative treatment on fertility, early pregnancy and obstetric outcomes

A systematic review and meta-analysis was performed which showed conservative treatment does not increase the risk of fertility and first trimester miscarriage. It was noted that there was a significant increased risk of ectopic pregnancy which is likely to be confounded by sexual behaviours which correlate with both risk of ectopic pregnancy secondary to sexually transmitted infections, and to acquisition of HPV resulting in development of CIN. Conservative treatment, particularly using excisional techniques was however found to significantly increase the risk of second trimester miscarriage. A further systematic review and meta-analysis showed there is a positive correlation between the risk of preterm birth and the amount of tissue removed. The conclusions of both meta-analyses must be treated with caution however because of the observational and frequently retrospective nature of the studies included, however, the included studies are the best available evidence, due to a lack of randomised controlled trials which will never be available due to the precancerous nature of the disease being treated. This work has reinforced the importance of the choice of control group when performing meta-analyses and further supports the theory that women with high-grade CIN have an intrinsic predisposition to preterm birth, which may also extend to an increased risk of second trimester miscarriage. The following three chapters explored potential structural and biochemical mechanisms for these pregnancy-related complications.

11.2.6 Ultrasound assessment of cervical length and volume before and after excisional treatment

Transvaginal ultrasound scanning was used to assess cervical length and volume in the prospective cohort of 275 women recruited in colposcopy clinic and in a subgroup of 152 women who underwent excisional treatment and completed follow-up in Chapter eight. Most strikingly the data collected in untreated women demonstrated a wide range in cervical length and volume within the population. Proportion of excision, rather than absolute length or volume, may therefore be more important for predicting future obstetric outcomes. The results following excision were in keeping with several published studies that have

demonstrated cervical length and volume are reduced at six months post-treatment, but further shows that the proportion of length and volume excision is an important factor in determining the degree of regeneration at six months.

11.2.7 The impact of excisional treatment on the vaginal microbiota

Chapter nine has examined the impact of the surgical removal of CIN, which as shown in chapter three is known to be associated with an altered vaginal microbiome compared to healthy controls, and showed that a high-diversity, *Lactobacillus* spp. deplete vaginal microbiota remained to be more prevalent in the treated cohort even after successful removal of the disease, and of HPV infection. This may represent one of the mechanisms of adverse obstetric outcomes after treatment, but could also contribute to risk of disease recurrence and higher levels of future invasive cervical cancer observed in comparison to the untreated population, even with initial complete disease removal.

11.2.8 Cervical antimicrobial peptides and cytokines after excisional treatment

Whilst it has been shown in Chapter eight that the cervix is capable of regenerating in length and volume after treatment, Chapter ten demonstrated that significantly lower levels of hBD1 and SLPI are seen after treatment. The glandular cervical epithelium appeared to be the most prominent source of AMP expression, and in spite of the scarred epithelium producing both AMPs, immunohistochemistry experiments have suggested excision of the glandular epithelium probably accounts for the decrease in AMP levels. Therefore, in spite of good anatomical regeneration, there is inferior functional capacity of the cervix after excisional treatment for CIN. Higher levels of proinflammatory cytokines also remain after successful removal of disease.

11.3 Findings in context with the recent literature

The role of the vaginal microbiome in women's health is an exciting and fast-moving area of research, and the data presented in this thesis support the hypothesis that its composition has an impact on the natural history of cervical disease. Following the publication of data presented in Chapter three, three further groups have studied the vaginal microbiome of women with CIN, as summarised in Table 11.1. Audirac-Chalifour *et al.* described the vaginal microbiota and vaginal mucosal cytokine profiles in 32 Mexican women (20 normal, four with SIL and eight with cervical cancer) and corroborated some of our findings albeit in a smaller sample size [279]. In their study increased diversity and a greater relative abundance of *Sneathia* spp. and members of the closely related *Fusobacterium* spp. were shown to be associated with increased disease severity. This study however has some major methodological flaws, most notably that a different sample method was used for each study group, which as highlighted by the study in Chapter four, may influence the results of bacterial sequencing experiments.

A further study in 70 Korean women with CIN and 50 healthy controls used regression modeling and calculated relative excess risk due to interaction and synergy indices to determine biological interactions between vaginal microbiota and hrHPV [269]. Their results were in accordance with our study in Chapter three [229] and Audirac-Chalifour and co-workers [279], concluding that women with CIN had a higher vaginal diversity than healthy controls. The study also identified presence of *Anaerococcus vaginae, Garderella vaginalis,* and *L. iners* in the absence of *L. crispatus* to be the most high-risk combination for development of CIN, with an odds ratio (OR) of 34.1 for CIN in the presence of hrHPV, compared to hrHPV negative women. The existence of *A. vaginae* alone was associated with an OR of 29.9 in HPV positive women, compared to negative women in the cohort. Interestingly, the OR of CIN in hrHPV positive women with *L. iners* was 10.9.

Piyathilake and colleagues [270] have also studied women with CIN (cytologically-defined HSIL (n=340) versus LSIL (n=90); all women were hrHPV positive) and used the Dirichlet multinomial mixture model to partition samples into four different metacommunities (Partition 1-4), rather than the previously defined CSTs. Unlike the three previously described studies, this group did not find a high vaginal microbial diversity (CST IV/BV-like VMB) to be associated with HSIL.

Table 11.1. Characteristics of studies exploring the association of cervical pre-invasive and invasive cervical disease to the vaginal microbiome using next generation sequence techniques.

Study	Summary of findings	Study Characteristics
Mitra *et al.* 2015 (Presented in Chapter Three)	- CST IV associated with increasing disease severity (Normal=10%; LSIL=21%; HSIL=27%; ICC=40%). - CST I negatively associated with increasing disease severity (Normal=50%; LSIL=42%; HSIL=40%; ICC=20%) - Higher levels of *S. sanguinegens* (P < 0.01), *A. tetradius* (P < 0.05), *P. anaerobius* (P<0.05) associated with HSIL vs LSIL - Lower levels *L. jensenii* (P<0.01) associated with HSIL vs LSIL.	**Participants:** 169 premenopausal women attending colposcopy clinic; 20 normal, 52 LSIL, 92 HSIL, 5 ICC **Sexual history meta-data:** History of intercourse in 48 hours prior to sampling, type of contraception used **VMB sampling:** Clinician-collected, high-vaginal swab **HPV testing technique:** Abbott RealTime HR HPV assay (Abbott M2000 platform) **NGS technique:** 16s rRNA gene regions: V1-V2, Primers barcoded: 27F and 338R, Platform: Ilumina MiSeq
Oh *et al.* 2015	Higher risk of CIN for the higher vs the lower tertile of: - *A. vaginae*, *G. vaginalis*, *L. iners* predominance with a minority of *L. crispatus*: OR 5.80, 95%CI 1.73–19.4) - *A. vaginae*: OR 6.63, 95%CI 1.61–27.2). - Risky microbial pattern in presence of oncogenic HPV:OR 34.1, 95% CI 4.95–284.5.	**Participants:** 120 premenopausal women attending gynaecological oncology clinics; 70 CIN cases: CIN 1 (n=55), CIN 2 or 3 (n=15), Controls: Normal cytology (n=25), ASCUS (n=25) **Sexual history meta-data:** Not reported, use of oral contraception recorded **VMB sampling:** Clinician collected Digene Cervical Sampler Brush **HPV testing technique:** Hybrid capture II DNA Test (Qiagen, Gaithersburg, MD, USA) **NGS technique:** 16s rRNA gene regions: V1-V3, Primers barcoded: not stated, Platform: Roche/454 Genome Sequencer Junior
Piyathilake *et al.* 2016	**Summary of findings** - *L. iners* and unclassified *Lactobacillus* spp. associated with higher CIN2+ rates compared to diverse taxa unclassified *Lactobacillus* spp, *L. iners*, Bifidobacteriaceae, Clostridiales, Allobaculum (OR=3.48, 95% CI: 1.27-9.55). - Lactobacillaceae, Lactobacillus, *L. reuteri* and several sub-genus level Lactobacillus OTUs higher in women with CIN2+ vs CIN1 - DNA oxidative damage does not correlate with VM structure.	**Participants:** 430 HR-HPV positive women aged 19-50 years attending colposcopy clinics; 340 Cases: CIN2 (n=208), CIN3 (n=132), 90 Non-cases: All CIN1 **Sexual history meta-data:** Not reported, use of oral contraception recorded **VMB sampling:** Clinician collected high vaginal swabs (Merocel ophthalmic sponges) **HPV testing technique:** Roche Diagnostics Linear Array **NGS technique:** 16s rRNA gene region: V4, Primers barcoded: not stated, Platform: Illumina MiSeq
Audirac-Chalifour *et al.* 2016	**Summary of findings** - VMB diversity significantly higher in CIN and ICC compared to normal, HPV-negative women (p= .006, p= .036 respectively) - *L. crispatus* and *L. iners* predominate in normal women - *Sneathia* spp. predominate in women with CIN - *Fusobacterium* spp. in women with ICC - Highest mean levels of IL-4 and TGF-β1 mRNA in *Fusobacterium* spp. VMBs.	**Participants:** 32 women aged 22-61 years, selected from a biobank, recruited from the Gynaecological service at a National Cancer Institute; 20 normal (10 HPV-negative, 10 HPV-positive), 4 CIN (All HPV-positive), 8 ICC (All HPV-positive) **Sexual history meta-data:** Age at first intercourse, number of lifetime sexual partners, no sexual activity "in previous days of the sampling" (Number of days not stated) **VMB sampling:** Cervical scraping swabs from normal women and fresh cell biopsies from women with CIN and ICC **HPV testing technique:** Seegene Anyplex II HPV HR Detection assay **NGS technique:** 16s rRNA gene regions: V3–V4, Primers barcoded 347F and 803R, Platform: Roche/454 Genome Sequencer Titanium system

A. vaginae: Atopobium vaginae; CI: confidence interval; CIN: cervical intra-epithelial neoplasia; HPV: human papillomavirus; HR-HPV: high-risk HPV; HSIL: high-grade squamous intraepithelial lesion; ICC: invasive cervical cancer; L: Lactobacillus; LSIL: low-grade squamous intraepithelial lesion; NSG: next generation sequencing; OR: odds ratio; OTUs: operational taxonomic units; SIL: squamous intraepithelial lesion; VM: vaginal microbiome

Partition three, dominated *L. iners* and unclassified *Lactobacillus* spp. had higher HSIL levels as compared to those with diverse taxa unclassified *Lactobacillus, L. iners, Bifidobacteriaceae, Clostridiales* and *Allobaculum* (Partition one) (OR=3.48, 95% CI: 1.27-9.55). Such an observation may arise due to ethnic differences between studies, but also their use of cytological patient classification rather than histology. At present there are no exisiting studies describing longitudinal cohorts of women with CIN.

11.4 Potential mechanisms for an interplay between the cervicovaginal microenvironment and disease outcomes

Figure 11.1 summarises the mechanisms by which the vaginal microbiome composition may impact on cervical disease development and carcinogenesis. Recent observational cross-sectional studies support the concept that CST III and IV in particular, are frequently linked with the presence of HPV infection and development of preinvasive cervical disease states [201, 202, 229, 269, 279]. Colonisation by *Lactobacillus* spp. is important for maintenance of the cervical epithelial barrier function that inhibits entry of HPV to the basal keratinocytes. When BV-associated strict anaerobes are able to colonise, they produce enzymes and metabolites, which may compromise this barrier, facilitating HPV entry [277] (Figure 11.1A).

Clinical studies have shown that levels of vaginal pro-inflammatory cytokines are higher in women with dysbiosis [302, 303], which is supported by the data presented in Chapter six, and this can result in chronic inflammation; a well-known factor in carcinogenesis of numerous tissues [306]. This proinflammatory environment, as well as bacterial production of bioactive amines also result in higher levels of oxidative stress [301], which can generate reactive oxygen species (ROS) that subsequently cause double-stranded DNA breaks in the both the HPV episome and host genome, thus assisting HPV integration and ultimately neoplastic transformation (Figure 11.1B).

Vaginal *Lactobacillus* spp. prevent colonization of bacterial vaginosis-associated bacterial species through maintenance of a low pH [194-197], and bacteriocin production [294-296]. BV is commonly diagnosed based on the presence of a characteristic thin, watery foul-smelling vaginal discharge [427], which is thought to arise from squamous cell exfoliation in response to amines produced by particular bacterial species [428] and mucus breakdown. Sialidases are a group of mucin-degrading enzymes produced mainly by *Prevotella* and *Bacteroides* spp. and are found at significantly higher levels in women with BV [429]. Overgrowth of strict anaerobes may also result in decreased mucus production [277], and subsequent decreased

viral trapping through antibody capture as well as increased exposure of the cervical epithelium. *Sneathia* spp. have frequently been identified in association with HPV-positivity [201], and with CIN and cervical cancer [229, 279], as described in Chapters three and five. Furthermore, *Sneathia* spp. have also been associated with adverse obstetric outcomes including miscarriage and preterm labour [449, 450]. Previously Nawrot and colleagues used a PCR-based method to show *Leptotrichia amnionii* (now re-named *Sneathia amnii*) was associated with cervical cancer, but not HPV-infection or CIN [451]. The species was, however, not unique to women with cancer, suggesting it may play a role in carcinogenesis, rather than occurring as a consequence of the disease. *Sneathia* spp. belong to the *Fusobacterium* genus, which has been implicated in colorectal carcinogenesis [147, 257, 452] through activation of pro-inflammatory pathways, inhibition of immunocytotoxicity [453] and production of FadA, a virulence factor, capable of WNT signaling activation; a key cell survival and proliferation pathway which is dysregulated in colorectal carcinogenesis [452] and cervical cancer [304]. Fusobacteria also act on immunomodulatory pathways and *F. nucleatum* DNA levels have been shown to be inversely proportional to CD3+-T cell counts in colonic mucosa [454], which is interesting given that aberrant CD3+-T cell signaling and function has been observed in cervical cancers [305] and relapsing disease [455] (Figure 11.1C).

Lactobacillus iners appears quite dissimilar to many other vaginal *Lactobacillus* species, which may underlie the reason that this species seems intermediate in its protective nature from the data presented in this thesis, for several potential reasons. Firstly, lactic acid is a chiral compound with a D- and L-isomer, with the former being produced by *L. jensenii, L. crispatus* and *L. gasseri*. However, the L-isomer of lactic acid is produced by the vaginal epithelium, *L. iners* and various anaerobes associated with dysbiosis [456], which appears less able to trap viruses such as HPV [457], and is associated with increased expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and activation of matrix metalloproteinase (MMP-) 8 which could alter cervical integrity [456], and facilitate entry of HPV to the basal keratinocytes, where the virus thrives. *L. iners* is known to be less stable in comparison to other *Lactobacillus* spp.-dominant CSTs [158], allowing growth of strict anaerobes resulting in transition to CST IV. There is a notable lack of published evidence to suggest that *L. iners* produces many of the bacteriocins and biosurfactants that prevent bacterial adhesion and biofilm formation, which are associated with overgrowth of pathogenic anaerobes, in particular *G. vaginalis* [294-296, 458]. This may account for the comparatively high rates of transition observed between *L. iners*-dominant microbial communities and CST IV [186].

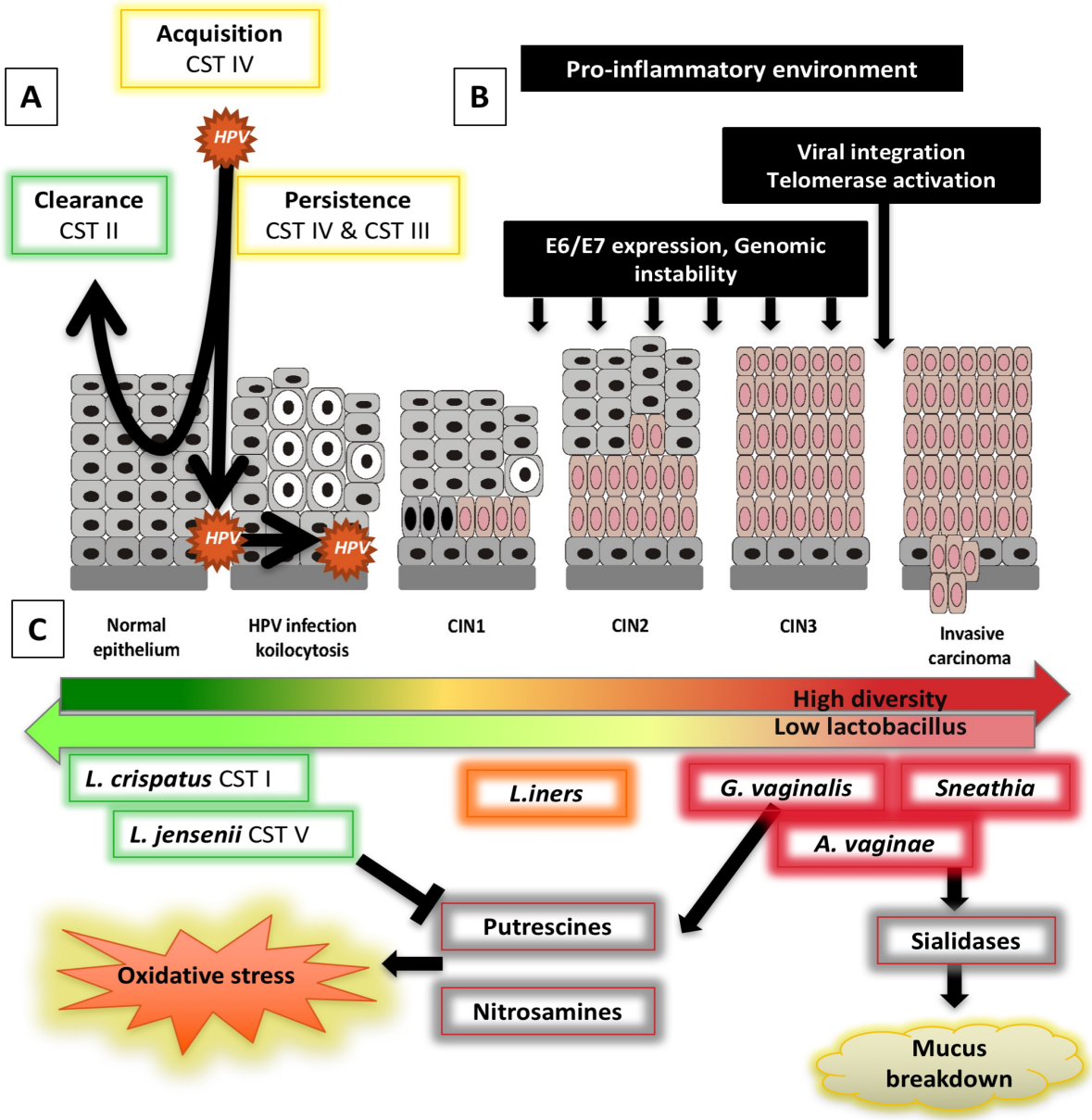


Figure 11.1. Summary of potential vaginal microbiome-mediated mechanisms of cervical carcinogenesis

A. VMB structure appears to be associated with acquisition and persistence of HPV infection, and CST II in particular is associated with most rapid clearance of an acute HPV infection.

B. Dysbiosis can result in a pro-inflammatory environment, which can facilitate several of the necessary steps in viral transformation including E6 and E7 expression, genomic instability, viral integration and telomerase activation, which are necessary for carcinogenesis.

C. Higher diversity with lower *Lactobacillus* spp. content has been associated with increasing severity of CIN. Particular species associated with high diversity VMB's may produce sialidases which cause mucus breakdown, predisposing the cervical epithelium to tissue damage, as well as producing biological amines which are responsible for oxidative stress; a key mechanism in carcinogenesis. Certain species *Lactobacillus* spp. have been shown to mop up these amines, and therefore their presence may reduce the risk of oxidative damage. *L. iners* does not appear to share many of the protective mechanisms of other *Lactobacillus* species, and therefore appears intermediate in its ability to prevent cervical disease.

It is also interesting to note that particular strains of *L. iners* have been shown to be more susceptible to AMP induced-death compared to *L. crispatus* and *L. jensenii in vitro* using LL-37, an intrinsic AMP belonging to the cathelicidin family [321]. This supports the concept of *Lactobacillus* spp. having a protective niche in the human vagina, and also suggests that certain strains of *L. iners* are better tolerated in the vagina than others. The data presented in Chapters six and ten also suggest an interplay between the vaginal microbiota, AMP expression and cytokine profiles, and further investigation is required, because the composition of the vaginal microbiota has the potential to be used as a surrogate marker of the underlying mucosal environment.

Given that the vaginal microbiome does not change significantly after treatment as demonstrated in Chapter nine, it may be that an innate abnormality in women with high-grade CIN predisposes them to acquiring persistent HPV infections that they are unable to clear due to higher levels of inflammation, and an alteration of the innate mucosal immune system, as described in Chapter six. Much of the data presented in this thesis support the concept that the presence of CIN itself, as well as the subsequent healing after excisional treatment, results in an altered cervical immune system, which may predispose to adverse pregnancy outcomes. The absence of *Lactobacillus* spp. likely results in and increased risk of ascending infections which are known to be associated with at least 40% of cases of PTB [117], and CST IV microbiome structure is known to be inversely associated with gestational age at delivery [459]. *L. crispatus* specifically is associated with better pregnancy outcomes and has been shown to be highly predictive of term birth when found at 16 weeks gestation (positive-predictive value, PPV = 98%) [414]. The same study also showed that presence of CST III was predictive of PTB at 16 weeks, and was also associated with a short cervix in the second trimester [414]. The cohort in Chapter nine showed slightly higher rates of CST III after treatment compared to before, and also remained to have twice the rate of CST IV after treatment compared to the control population, reinforcing that these women may be at increased risk of obstetric complications. However, we did not see any association between VM composition and cervical length. Ascending infection commonly result in infections between the amnion and chorion or in the choriodecidual space, and although the exact timing of such bacterial invasion is unclear, it has been postulated that this may arise early in pregnancy, and go undetected for several months [435]. Once the endometrial cavity has been sealed off around 20 weeks, the likelihood of ascending infections is decreased, which suggests intrauterine infections may in fact be the result of a chronic infection that began in

early pregnancy, or even prior to conception [435], and thus the composition of the pre-conception microbiome likely plays a role in outcomes.

Physical damage associated with treatment also appears to impair the function of the cervix in spite of good cervical length and volume regeneration. The production of key AMPs was significantly reduced after treatment, and this may further predispose to bacterial colonisation and pregnancy loss. This work has also highlighted that women do in fact have a shorter cervix after treatment, but give the variation in pre-excisional cervical length and volume, the proportion removed may be more important than absolute length or volume of excision in predicting cervical regeneration. Pre-excisional cervical length measurement may therefore be useful for developing personalized prediction clinical scoring tools.

11.5 Clinical relevance and impact

The work carried out in this thesis suggests that the vaginal microbiome acts as a co-factor in the HPV-mediated cervical disease process, and may also play a role in the adverse obstetric outcomes associated with treatment. These data also support the notion that women with high-grade disease may be a niche population at risk of both cervical cancer and preterm birth, due to an underlying infection or inflammation based mechanism which underlies both pathologies.

Probiotics, are defined by the World Health Organisation as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.” They have the potential to be used to manipulate the microbiome towards a healthier state that is associated with better disease outcomes, and have been used successfully as an adjunct to traditional antibiotics in BV to improve cure rates and prevent recurrence through their ability to replenish the depleted pool of *Lactobacillus* spp. [268].

Metronidazole and clindamycin are the most commonly prescribed antibiotics for BV. They target the overgrowth of anaerobes, but do not appear to have a mechanism of action that would actively promote re-establishment of *Lactobacillus spp*. Following successful metronidazole treatment for BV, classified as improvement in Nugent and Amsel’s clinical scoring systems, *L. iners* is often seen to be the predominant re-colonisation species. An oral preparation of *Lactobacillus rhamnosus* GR-1 in combination with *L. reuteri* RC-14 was shown to increase the prevalence of *Lactobacillus*-dominant vaginal microbiota, as well as improve

BV cure rates when given in combination with metronidazole [460]. As neither of these are endogenous vaginal species the study provides evidence that oral administration of *Lactobacilli* is capable of modulating vaginal microbial structure. These orally administrated bacteria are thought to reach the vagina through a poorly understood mechanism involving transition via the anus and the perineal and vulval skin [461]. A potential mechanism by which these species modulate community structure involves *L. reuteri* secretion of the bacteriocin, Gasserin [296], and *L. rhamnosus* produced Lactocin 160, a similar bacteriocin that is particularly active against *G. vaginalis*. Interestingly, this particular preparation has also been associated with increased relative abundance of *L. iners* as shown using NGS [462, 463], which may arise through expansion of an existing population of this species. In a study of bacteria isolated from pre-menopausal women, PCR-based techniques demonstrated that *L. gasseri* negatively associated with *L. iners and A. vaginae* [464]; two species which co-associate [464] and are suggested to pose an intermediate and high risk for development of CIN respectively [269]. This observation provides further evidence that *L. iners* is not always associated with health, and this should be taken into consideration when designing probiotic therapies, to ensure that they do not promote dominance of this particular *Lactobacillus*.

Probiotics have also been suggested as an intervention to promote HPV clearance, and *in vitro* and *in vivo* evidence exist to support this technique. Treatment of SiHA cells, an HPV-16-infected cervical cell line, with *Bifidobacterium adolescentis* significant reduced production of E6 and E7 mRNA, suggesting this species may represent a novel therapeutic of virally-transformed cells [465], however the efficacy of this species as a probiotic is yet to be proven in humans. As described above, *L. gasseri* is associated with rapid clearance of incident HPV infections [202]. This species, along with *L. crispatus* has also been shown to be cytotoxic to HPV-18-infected HeLa cervical cancer cells, but not to normal cervical cell lines, independent of pH or lactate concentration, suggesting a more sophisticated mechanism of action [267]. A semi-randomised, interventional study of 54 HPV-positive women with low-grade cervical lesions is the first human study to show that orally administered *L. caseii* promoted significantly greater clearance of HPV infections (29% vs 19%), and cervical lesions (60% vs 31%), compared to an untreated cohort [466].

Prebiotics are indigestible carbohydrates, including the fructo-oligosaccharide (FOS) and gluco-oligosaccharide (GOS) families, which promote the growth of healthy bacteria already present in the body. They have been most well-studied in the gastrointestinal tract, where

they have been shown to modulate microbiota composition, as well as exert immunomodulatory effects independent of the microbiota [467]. Several encouraging *in vitro* studies, and a handful of small *in vivo* studies suggest proof of concept in the vagina. FOS and GOS have been shown to promote the growth of *L. crispatus, L. jensenii* and *L. vaginalis in vitro,* but not *Candida albicans*, *E. coli* or *G. vaginalis*, with the investigators using high performance liquid chromatography (HPLC) to show that GOS and FOS could not be used as energy sources by the 3 latter pathobionts [468]. GOS, applied as an intravaginal gel, has been shown in a randomised-controlled trial of 42 women, immediately following metronidazole treatment for BV, to result in a significant reduction in Nugent scores at 8- and 16-days of treatment [469]. Konjac glucomannan hydrolysates (GMH) has similarly been shown to promote *Lactobacilli* spp. colonization in women with *C. albicans* infection [470]. Beyond promotion of bacterial growth through acting as a growth substrate, mannose and GMH have been shown to inhibit adhesion of *E. coli* to human cheek epithelial cells *in vitro* [471], suggesting additional mechanisms of pathobiont inhibition. When concomitantly administered with probiotics in a symbiotic preparation, they may enhance the growth of probiotic species as well as their bacteriocin production [472]. These results are encouraging and represent a very cheap, safe intervention with few side-effects for a disease that cannot otherwise be treated without the risk of significant reproductive and obstetric morbidity [84, 86, 93, 94, 473]. While further studies are required to both understand the mechanisms by which the vaginal microbiota plays a role in the pathophysiology of cervical disease, and to identify the most protective species or strain to defend against HPV-induced dysplasia and neoplasia, and their therapeutic doses, pre- and probiotics may offer a practical intervention for the developing world, where cervical cancer is a major cause of female cancer-related mortality [2]. They may also be useful prior to conception, and during pregnancy of treated women to manipulate the microbiome towards a composition associated with positive pregnancy outcomes.

Alternative approaches for the assessment of vaginal microbiota and its interactions with the host immune system may also offer useful means to monitor HPV acquisition, persistence and subsequent cervical dysplasia and neoplastic transformation. Metabonomics, defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [474] is emerging as a novel technique to investigate the host-microbe interaction through inspecting functional metabolic changes associated with disease phenotypes [475, 476]. Using nuclear magnetic resonance

(NMR) or mass spectroscopy (MS), it is possible to identify particular metabolites or pathways that are altered in association with the VMB structure. This approach will not only increase understanding of bacterial-host interaction, which is likely extremely complex [476], but may also present the opportunity for development of novel prognostic tests for triage of patients who are most likely to develop a high-grade or cancerous cervical lesion or to have a preterm birth, which is one of the biggest clinical challenges in current colposcopy and obstetric practices.

In the colposcopy clinics, overexpression of viral E6/E7 oncogene mRNA and p16-Ki67 are emerging as potential tools for triaging low-grade disease their use in identifying those at greatest risk of progression has not yet been proven [47-50]. Integration of these data, along with HPV status/subtype and biochemical and metabolic data, could eventually be used to develop a personalized risk score to aid decisions in colposcopy. In antenatal clinics, cervical length ultrasound in the second trimester is known to be a good predictor of preterm birth in the general population [412] as well as in women post-excisional cervical treatment, with a cervical length of less than 25mm identifying those at risk of preterm birth with a sensitivity, specificity, and positive and negative predictive values or 64%, 78%, 30% and 94% respectively [413] and is used to offer targeted cerclage to women at greatest risk [477], although such an invasive procedure is still not without risk [156]. Pre-excisional cervical length measurement, along with assessment of biochemical changes in the cervicovaginal environment could also be useful for developing personalized prediction clinical scoring tools, to improve identification of those most at risk.

The risk of PTB appears to correlate significantly with the depth of the cone, which can cause a dilemma for clinicians aiming for complete excision to ensure good oncological outcomes [106], yet balance reproductive wishes of the patient. The data presented here suggest mechanisms other that simply structural tissue deficit have an impact on this risk and it is therefore suggested that clinicians continue to offer sufficient treatments to ensure complete excision for optimum oncological outcomes.

11.6 Future work

The work presented in this thesis has identified specific immune and bacterial and environments, and also particular bacterial species that may play a role in cervical health and disease and the subsequent risk of pregnancy-related complications. The women recruited in

this study should be followed-up during pregnancy to determine how these biochemical factors impact on their pregnancy outcomes.

Further work is planned to investigate the role of significant bacterial taxa in the immune response and oncogenesis. This will involve the use of *in vitro* cell-culture models using NIKS, W12, CIN-612, HeLa and CaSki cells in two- and three-dimensional models of disease.

By definition, members of a particular bacterial species have total nucleotide identity of >70% across their genomes [478]. The remaining genome diversity gives rise to the existence of different strains, which may have different functional genes that induce different biological properties. Further evidence is required in order to determine whether only certain strains of a particular bacterial species are either protective or pathogenic with regards to HPV and cervical dysplasia and a recent study by Abdelmaksoud *et al.* [479] hints this is likely. The team compared strains of *L. crispatus* from women with, and without BV, and demonstrated considerable genomic diversity within the species, and identified several genes exclusive to the presence or absence of BV [479]. These genes require further investigation to help understand the protective mechanisms exploited by certain bacteria, and other disease-associated species should also be studies using metagenomic techniques. This work will help us to further understand the role of these bacteria and their metabolites in the disease process, which could help with the future development of pre- and probiotic therapies which represent a novel, and potentially cost-effective method of preventing disease, particularly in countries with a high volume of disease, but low coverage of HPV vaccination. This data will also be translatable to women post-excisional treatment to inform their obstetric follow-up.

Alongside the bacterial microbiota, the virome is now a new emerging area of interest. Although we have known for many years that HPV is the aetiological agent in precancerous and cancerous cervical disease, other viral genera present in the normal vagina, alongside papillomaviridae, may be involved in both disease progression [480], and pathogenesis of HPV/CIN-related preterm birth.

11.7 Final conclusions

This work has shown that the cervicovaginal microenvironment appears to play a role in cervical carcinogenesis, with relation to the vaginal microbiome, innate antimicrobial peptides and proinflammatory cytokine profile. Treatment of CIN does not appear to alter the vaginal

microbiome composition which remains of higher diversity, with depletion of protective *Lactobacillus* species. Furthermore, these women appear to have a proinflammatory environment which remains even after successful treatment of disease. These factors suggest that a preexisting intrinsic abnormality puts these women at a higher risk of disease initially compared to healthy controls, and may also be instrumental in increasing their risk of subsequent recurrence.

Whilst treatment was not shown to have a negative impact on fertility it was shown to be associated with second-trimester miscarriage, and increasing depth of excision increases the risk of future preterm birth. In addition to a structurally deficient cervix, a pre-existing abnormal vaginal microbiome and inferior cervical immune function may increase the risk of ascending infections, which are known to contribute to at least 40% of cases of preterm birth.

To better understand the mechanisms and magnitude by which these bacterial and immunological factors may impact the risk of cervical carcinogenesis and adverse obstetric outcomes after treatment could be used in the future to optimise management and ultimately health-related outcomes in this group of women.

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Appendix 1. Search strategy for fertility and early pregnancy outcomes meta-analysis

1 exp Uterine Cervical Neoplasms/
2 (cervi* and (cancer* or tumor* or tumour* or neoplas* or malignan* or carcinom*)).mp.
3 exp Cervical Intraepithelial Neoplasia/
4 CIN.mp.
5 (cervi* and (intraepithel* or epithel* or dysplasia or pre-cancer* or precancer*)).mp.
6 or/1-5
7 exp Conization/
8 (conisation or conization).mp.
9 exp Laser Therapy/
10 laser.mp.
11 exp Cryotherapy/
12 cryotherapy.mp.
13 cold coagulation.mp.
14 exp Diathermy/
15 diatherm*.mp.
16 cone biopsy.mp.
17 loop.mp.
18 LLETZ.mp.
19 LEEP.mp.
20 ablat*.mp.
21 excision*.mp.
22 transformation zone.mp.
23 (CKC or LA or LC or CC or RD or TZ).mp.
24 (conservative and (method* or treatment* or intervention* or management)).mp.
25 or/7-24
26 6 and 25
27 exp Premature Birth/
28 (preterm or premature).mp.
29 exp Infant, Low Birth Weight/
30 birth weight.mp.
31 Perinatal Mortality/
32 perinatal mortality.mp.
33 exp Intensive Care, Neonatal/
34 (neonatal and intensive care).mp.
35 exp Fertility/
36 fertil*.mp.
37 conception.mp.
38 exp Pregnancy/
39 pregnancy.mp.
40 gestation*.mp.
41 exp Abortion, Spontaneous/
42 miscarriage*.mp.
43 exp Cesarean Section/
44 (cesarean or caesarean).mp.
45 exp Obstetric Labor, Premature/
46 exp Labor, Obstetric/
47 (labor or labour).mp.
48 Fetal Membranes, Premature Rupture/
49 pPROM.mp.
50 or/27-49
51 26 and 50Key:mp=title, original title, abstract, name of substance word, subject heading word

Appendix 2. Search strategy for obstetric outcomes according to depth of excision meta-analysis

1 exp Uterine Cervical Neoplasms/
2 (cervi* and (cancer* or tumor* or tumour* or neoplas* or malignan* or carcinom*)).mp.
3 exp Cervical Intraepithelial Neoplasia/
4 CIN.mp.
5 (cervi* and (intraepithel* or epithel* or dysplasia or pre-cancer* or precancer*)).mp.
6 or/1-5
7 exp Conization/
8 (conisation or conization).mp.
9 exp Laser Therapy/
10 laser.mp.
11 exp Cryotherapy/
12 cryotherapy.mp.
13 cold coagulation.mp.
14 exp Diathermy/
15 diatherm*.mp.
16 cone biopsy.mp.
17 loop.mp.
18 LLETZ.mp.
19 LEEP.mp.
20 ablat*.mp.
21 excision*.mp.
22 transformation zone.mp.
23 (CKC or LA or LC or CC or RD or TZ).mp.
24 (conservative and (method* or treatment* or intervention* or management)).mp.
25 or/7-24
26 6 and 25
27 exp Premature Birth/
28 (preterm or premature).mp.
29 exp Infant, Low Birth Weight/
30 birth weight.mp.
31 Perinatal Mortality/
32 perinatal mortality.mp.
33 exp Intensive Care, Neonatal/
34 (neonatal and intensive care).mp.
35 exp Fertility/
36 fertil*.mp.
37 conception.mp.
38 exp Pregnancy/
39 pregnancy.mp.
40 gestation*.mp.
41 exp Abortion, Spontaneous/
42 miscarriage*.mp.
43 exp Cesarean Section/
44 (cesarean or caesarean).mp.
45 exp Obstetric Labor, Premature/
46 exp Labor, Obstetric/
47 (labor or labour).mp.
48 Fetal Membranes, Premature Rupture/
49 pPROM.mp.
50 or/27-49
51 26 and 50

key: mp=title, original title, abstract, name of substance word, subject heading word

Appendix 3. Patient Information Leaflets

Imperial College Healthcare NHS Trust

Queen Charlotte's and Chelsea Hospital
Du Cane Road, London, W12 0HS

Imperial College London

Imperial College London,
Division of Cancer Institute of
Reproductive and Developmental Biology,
Hammersmith Hospital Campus,
Du Cane Road
London, W12 0NN

Patient Information Sheet (Treatment Group)
8th June 2014, Version 5.0 - REC ref no: 13/LO/0126

Information Sheet for Patients

Study title:
Risk of preterm labour related to cervical precancer and treatment
Principal Investigator: Dr Maria Kyrgiou

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.
If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part your future care will not be affected by your decision. You are free to withdraw at any time without explanation and your future care will not be affected.
Thank you for reading this.

What is the purpose of the study?

Preterm birth is the major cause of infant disability and death. Cervical precancer and treatment increase the risk of prematurity (delivering too early) in a subsequent pregnancy. Recent evidence suggests that this increase in risk may be due to slight weakening of the cervix following treatment, although cervical precancer itself and/or treatment may also affect the ability of the cervix to fight infection that can lead to preterm labour. This research aims to study the various mechanisms that increase the risk of prematurity in women with cervical precancer and/or treatment. It is plausible that, if the proposed tests were proven accurate and measured routinely prior to treatment, they could allow specialised management of women requiring additional surveillance in pregnancy, which could delay or even prevent the onset of premature labour..
Please note that you are not taking part in a clinical treatment or drug trial.

Why have I been chosen?

You are having a procedure for removal a part of cervix (neck of the womb) for suspected precancerous disease of the cervix.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.

What do I have to do?

When you come into the hospital for your appointment or procedure, we will ask you if you would agree to have a trans-vaginal ultrasound and donate for research purposes vaginal swabs/smears, urine, blood and part of the tissue that has been removed as a part of your treatment. No extra tissue is removed for research purposes; only residual tissue will be used for research after sufficient tissue is taken for diagnosis. The ultrasound and the swabs will be repeated during the first routine follow-up visit in colposcopy 6 months after your treatment.
Taking part in this study will not affect the standard treatment that you receive.

Imperial College Healthcare NHS
NHS Trust

What will happen to me if I take part?

You will be asked to sign a consent form when you come in for your appointment or procedure, which is separate from the verbal or written consent needed to perform the procedure. We will take vaginal swabs (1-2 minutes). This will not cause any additional pain or discomfort, as this will be performed during your routine gynaecological examination. We will also perform a trans-vaginal ultrasound during your routine gynaecological examination that will take approximately 2-3 minutes in total and we may also ask you to give us a sample of urine or 10-20ml of blood. You will have your procedure as normal. We may also take a small punch biopsy (pinch of tissue) before we perform your treatment with your consent or use some of the tissue removed as part of your treatment. This will be used for research and will not interfere with the procedure you are attending for. Surplus residual tissue that has been removed as part of your treatment may also be used for research. We will repeat the ultrasound and we will collect repeat samples (swabs and perhaps urine and blood) during your first routine follow-up visit 6 months after your treatment. If you become pregnant, we collect data on the outcome of your pregnancy. We will collect repeat samples and we will measure the length of your cervix (neck of the womb) with trans-vaginal ultrasound at 2 weeks intervals from 12/14 to 22 weeks. If you move to a different hospital for your pregnancy care, we may have access to information regarding your delivery held in national databases (relating to health) or by linking different datasets ie. NHS data sources such as HES (Hospital Episodes Statistics).

What are the possible disadvantages and risks of taking part?

There are no disadvantages or major risks associated with taking part.

What are the possible benefits of taking part?

There will not be any benefit to you from taking part in this study. The information you give us may help us to look after other women with your condition more effectively in the future by devising novel improved management and treatments as mentioned above.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Your personal details will be removed from the information when it is reported and participants will not be able to find out about individual results.

Who is organising the research?

This study is being organised by the staff of the department of Obstetrics & Gynaecology, Imperial College NHS Trust and Imperial College London. This research may be part of a PhD study proposal of one of the investigators.

Who has reviewed the study?

The Research Ethics Committee of Hammersmith and QCCH Hospital has reviewed this study.

What if something goes wrong?

Imperial College London holds insurance policies, which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator **Dr Maria Kyrgiou** on 020 8383 3268. The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Office.

Contact for Further Information

If you want more information, before or after you return your form, you can phone **Dr Maria Kyrgiou** on 020 8383 3268.

The local Research Ethics Committee has approved the above statement.

Imperial College Healthcare NHS Trust

Queen Charlotte's and Chelsea Hospital
Du Cane Road, London, W12 0HS

Imperial College London

Imperial College London,
Division of Cancer Institute of
Reproductive and Developmental Biology,
Hammersmith Hospital Campus,
Du Cane Road
London, W12 0NN

Patient Information Sheet (Control Groups)
11th June 2015, Version 7.0 - REC ref no: 13/LO/0126

Information Sheet for Patients

Study title:
Risk of preterm labour related to cervical precancer and treatment
Principal Investigator: Dr Maria Kyrgiou

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.
If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part your future care will not be affected by your decision. You are free to withdraw at any time without explanation and your future care will not be affected.
Thank you for reading this.

What is the purpose of the study?

Preterm birth is the major cause of infant disability and death. Cervical precancer and treatment increase the risk of prematurity (delivering too early) in a subsequent pregnancy. Recent evidence suggests that this increase in risk may be due to weakening of the cervix following treatment, although cervical precancer itself and/or treatment may also affect the ability of the cervix to fight infection that can lead to preterm labour. This research aims to study the various mechanisms that increase the risk of prematurity in women with cervical precancer and/or treatment. It is plausible that, if the proposed tests were proven accurate and measured routinely prior to treatment, they could allow specialised management of women requiring additional surveillance in pregnancy, which could delay or even prevent the onset of premature labour.
Please note that you are not taking part in a clinical treatment or drug trial.

Why have I been chosen?

You are attending a hospital appointment due to suspected cervical precancer or other gynaecological conditions.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.

What do I have to do?

When you come into the hospital for your appointment or procedure, we will ask you if you would agree to have a trans-vaginal ultrasound and donate for research purposes vaginal swabs/smears, urine, blood and part of the tissue (if tissue is removed as a part of your assessment). No extra tissue is removed for research purposes; only residual tissue will be used for research after sufficient tissue is taken for diagnosis.

Imperial College Healthcare NHS
NHS Trust

Taking part in this study will not affect the standard treatment that you receive.

What will happen to me if I take part?

- **Women attending colposcopy**

You will be asked to sign a consent form when you come in for your appointment, which is separate from the verbal or written consent needed to perform the examination. We will take vaginal swabs (1-2 minutes). This will not cause any additional pain or discomfort, as this will be performed during your routine gynaecological examination. We may also perform a trans-vaginal ultrasound during your routine gynaecological examination that will take approximately 2-3 minutes in total and we may also ask you to give us a sample of urine or 10-20ml of blood. At the time of colposcopy you may require a punch biopsy (small pinch of tissue) taken as part of your routine clinical care. In this case, we may take an extra biopsy for use for research use with your consent. If you do not require a biopsy, with your consent we will take a punch biopsy for research purposes. Some people experience mild cramping pain like a period-pain, after a punch biopsy. Bleeding after the biopsy is a very rare complication. If any other tissue is removed routinely, surplus cervical residual tissue that has been removed may be used for research. If you continue to be seen in the colposcopy clinic we may approach you at future appointments to have further swabs, urine and blood samples collected. If you become pregnant, we collect data on the outcome of your pregnancy. We may collect repeat samples and we may measure the length of your cervix (neck of the womb) with trans-vaginal ultrasound at 2 weeks intervals from 12/14 to 22 weeks. If you move to a different hospital for your pregnancy care, may have access to information regarding your delivery held in national databases (relating to health) or by linking different datasets ie. NHS data sources such as HES (Hospital Episodes Statistics).

- **Women undergoing hysterectomy**

You will be asked to sign a consent form, which is separate from written consent needed to perform the operation. We will take vaginal swabs and may also perform a trans-vaginal ultrasound at the time of surgery. We may also ask you to give us a sample of urine or 10-20ml of blood. During the surgery we will take a punch biopsy (small pinch of tissue) from the cervix.. This will not interfere with any tests that the pathologists may wish to do on the uterus (womb) once removed. Bleeding after the biopsy is a very rare complication.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or major risks associated with taking part.

What are the possible benefits of taking part?

There will not be any benefit to you from taking part in this study. The information from this research may help us to look after other women with your condition more effectively in the future by devising novel improved management and treatments as mentioned above.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Your personal details will be removed from the information when it is reported and participants will not be able to find out about individual results.

Who is organising the research?

This study is being organised by the staff of the department of Obstetrics & Gynaecology, Imperial College NHS Trust and Imperial College London. This research may be part of a PhD study proposal of one of the investigators.

Who has reviewed the study?

The Research Ethics Committee of Hammersmith and Queen Charlotte's Hospital has reviewed this study.

What if something goes wrong?

Imperial College London holds insurance policies, which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator **Dr Maria Kyrgiou** on 020 8383 3268. The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Office.

Contact for Further Information

If you want more information, before or after you return your form, you can phone **Dr Maria Kyrgiou** on 020 8383 3268.

The local Research Ethics Committee has approved the above statement.

Appendix 4. Patient Consent Forms

Imperial College London

Imperial College London,
Hammersmith Hospital Campus,
Du Cane Road
London, W12 0NN

Participant (Treatment Group) Consent Form v.05 8th June 2014
REC ref No: 13/LO/0126

Risk of preterm labour after treatment for cervical precancer
Principal Investigator- Dr Maria Kyrgiou

Please initial each statement

I have read the Information Sheet for Patients (v.05 – 8th June 2014) ☐

I agree to take part in the study ☐

I have been given the opportunity to ask questions and discuss this study and have received satisfactory answers to all my questions. ☐

I have received enough information about the study. ☐

I agree to have a transvaginal ultrasound. ☐

I agree for cervico-vaginal swabs/smears to be collected and used for research investigations ☐

I agree for urine to be collected and used for research investigations ☐

I agree for blood to be collected and used for research investigations ☐

I agree for extra tissue to be taken and any excess tissue to be used for research purposes ☐

I understand that relevant sections of my medical notes and data collected during the study may be accessed by responsible individuals from the study Sponsor (Imperial College), NHS trust or regulatory authorities. I give permission for these individuals to access my records. I also give permission to access and link information held in national databases (relating to health) and other datasets i.e. NHS data sources such as HES. ☐

I give permission for my samples to be used for future ethically approved research projects ☐

I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care. ☐
The study has been explained to me by:

Dr/Ms/Mr _____

I agree to take part in this study.

Signed..... Date.....

(NAME IN BLOCK CAPITALS).....

Investigator's signature..... Date: .....

(NAME IN BLOCK CAPITALS).....

1 copy for patient, 1 copy for Principal Investigator, 1 copy for hospital notes

Imperial College London

Imperial College London,
Hammersmith Hospital Campus,
Du Cane Road
London, W12 0NN

Participant (Control Group) Consent Form v.06 16th March 2015
REC ref No: 13/LO/0126

Risk of preterm labour after treatment for cervical precancer
Principal Investigator- Dr Maria Kyrgiou

Please initial each statement

I have read the Information Sheet for Patients (v.06 – 16th March 2015) ☐

I agree to take part in the study ☐

I have been given the opportunity to ask questions and discuss this study and have received satisfactory answers to all my questions. ☐

I have received enough information about the study. ☐

I agree to have a transvaginal ultrasound. ☐

I agree for cervico-vaginal swabs/smears to be collected and used for research investigations. ☐

I agree for urine to be collected and used for research investigations. ☐

I agree for blood to be collected and used for research investigations. ☐

I agree for extra tissue to be taken and any excess tissue to be used for research purposes. ☐

I understand that relevant sections of my medical notes and data collected during the study may be accessed by responsible individuals from the study Sponsor (Imperial College), NHS trust or regulatory authorities. I give permission for these individuals to access my records. I also give permission to access and link information held in national databases (relating to health) and other datasets i.e. NHS data sources such as HES. ☐

I give permission for my samples to be used for future ethically approved research projects. ☐

I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting my future medical care. ☐

The study has been explained to me by:

Dr/Ms/Mr _____

I agree to take part in this study.

Signed..... Date.....

(NAME IN BLOCK CAPITALS).....

Investigator's signature..... Date: .....

(NAME IN BLOCK CAPITALS).....

1 copy for patient, 1 copy for Principal Investigator, 1 copy for hospital notes

Appendix 5. Characteristics of included studies for fertility and early pregnancy outcomes meta-analysis

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Weber 1979	Retrospective cohort	Age	CKC	21	20	Interview, mail questionnaire	Pregnancy rates in women wishing to conceive; Conception within given time; Miscarriages (total, 1st and 2nd trimester); Terminations	7
Larsson 1982	Retrospective cohort	Internal* For age, parity, socio-economic status, smoking, surgical interventions, various diseases	CKC	294	341	Hospital records	Miscarriages (total, 1st and 2nd trimester); Ectopic pregnancy; Terminations	9
Buller 1982	Retrospective cohort	Internal	CKC	88	106	Hospital records	Miscarriages (total, 1st and 2nd trimester); Ectopic pregnancy; Molar pregnancy; Terminations	9
Blomfield 1993	Retrospective cohort	Age, parity, ethnicity	LLETZ	40	80	Hospital records	Miscarriages (2nd trimester)	9
Bigrigg 1994	Retrospective cohort	Age, geographic area All controls had negative smears	LLETZ	76-229	66-299**	Telephone interview, mail questionnaire	Total pregnancy rates; Conception within given time; Miscarriages (total); Ectopic pregnancy	6
Cruickshank 1995	Retrospective cohort	Age, parity, height, smoking and partner's social class	LLETZ	149	298	Mail questionnaire (treated) - Maternity Databank (untreated)	Miscarriages (2nd trimester)	8
Sagot 1995	Retrospective cohort	Internal	LC	71	82	Hospital records	Miscarriages (total, 1st and 2nd trimester); Ectopic pregnancy; Molar pregnancy; Terminations	9
Spitzer 1995	Retrospective cohort	Internal for age and parity	LC; LA	433	433	Telephone interview, questionnaire	Total pregnancy rates; Miscarriages (total); Ectopic pregnancy; Terminations	8
Turlington 1996	Retrospective cohort	Untreated women had colposcopy and biopsy but no treatment	LLETZ	54	57	Telephone interview, mail questionnaire	Total pregnancy rates; Pregnancy rates in women wishing to conceive; Miscarriages (total); Terminations	7
Tan 2004	Retrospective cohort	Age, parity	LLETZ	119	119	Hospital records	Miscarriage (total)	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Sjoberg 2007	Retrospective cohort	Age, parity, plurality and regression analysis for smoking, marital status and education	LC; LLETZ	742	742	National registry	Miscarriage (2nd trimester)	9
Albrechtesen 2008	Retrospective cohort	Unmatched - Regression analysis for age and birth order	CKC; LC; LLETZ	15108	2164006	National registry	Miscarriage (2nd trimester)	7
Kalliala 2012	Retrospective cohort	Unmatched - Regression analysis for number of pregnancies and children, age, municipality	LLETZ; CKC; cryo; LC; LA	6179	30436	Hospital records National registries	Total pregnancy rates	8
Frega 2013	Prospective cohort	Unmatched	LLETZ	1329	462	Prospective records	Miscarriage (Gestation not specified)	8
Spracklen 2013	Retrospective case-control	Unmatched - Regression analysis for: age, education, income, race, parity, BMI, smoking, cervical surgery, case status	CKC; LLETZ; Cryo; LA	152	151 - colposcopy only 1021 - untreated	Birth registries, telephone interview	Conception within given time	8

BMI: Body Mass Index; CKC: Cold Knife Conisation; cryo: cryotherapy; LA: Laser Ablation; LC: Laser Conisation; LLETZ: Large Loop Excision of the Transformation Zone
*Self-matching= intra-woman comparison before and after treatment; **Ranges represent different number of cases and controls for every outcome in the same study

Appendix 6. Newcastle-Ottawa scores of included studies for fertility and early pregnancy outcomes meta-analysis

Reference	Selection				Comparability	Outcome		
	Representativeness of exposed cohort	Selection of non exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Follow-up long enough for outcome to occur?	Adequacy of follow up of cohorts
Weber 1979	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	*Study controls for age	*Record linkage	*Yes	* Complete follow-up – retrospective
Larsson 1982	*Truly representative of the average women with CIN in the community	*Internal matching	*Hospital records	*Yes	**Internal matching & study controls for age, parity, socio-economic status and smoking	*Record linkage	*Yes	* Complete follow-up – retrospective
Buller 1982	*Truly representative of the average women with CIN in the community	*Internal matching	*Hospital records	*Yes	**Internal matching	*Record linkage	*Yes	* Complete follow-up – retrospective
Blomfield 1993	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	**Study controls for age, parity, ethnicity	*Record linkage	*Yes	* Complete follow-up – retrospective
Bigrigg 1994	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	**Study controls for age and geographic area. Controls had a negative smear	*Record linkage	*Yes	* Complete follow-up – retrospective
Cruickshank 1995	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	**Study controls for age, parity, height smoking and partners social class	Self-reporting	*Yes	* Complete follow-up – retrospective
Sagot 1995	*Truly representative of the average women with CIN in the community)	*Internal matching	*Hospital records	*Yes	**Internal matching	*Record linkage	*Yes	* Complete follow-up – retrospective
Spitzer 1995	*Truly representative of the average women with CIN in the community	*Internal matching	*Hospital records	*Yes	**Internal matching and study controls for age and parity	*Record linkage	*Yes	* Complete follow-up – retrospective

Turlington 1996	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	Unmatched	Self-reporting	*Yes	* Complete follow-up – retrospective
Tan 2004	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	**Study controls age and parity	*Record linkage	*Yes	* Complete follow-up – retrospective
Sjoberg 2007	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*National registry	*Yes	**Study controls for age, parity, plurality and regression analysis for smoking, marital status and education	*Record linkage	*Yes	* Complete follow-up – retrospective
Albrechtesen 2008	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*National registry	*Yes	**Regression analysis for age and birth order	*Record linkage	*Yes	* Complete follow-up – retrospective
Kalliala 2012	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*National registry and hospital records	*Yes	**Regression analysis for number of pregnancies and children, age, municipality	*Record linkage	*Yes	* Complete follow-up – retrospective
Frega 2013	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	* Hospital records	*Yes	Unmatched	*Record linkage	*Yes	*Subjects lost to follow up <30% unlikely to introduce bias
Spracklen 2013	*Truly representative of the average women with CIN in the community	*Drawn from the same community as the exposed cohort	*Hospital records	*Yes	** Regression analysis for age, education, household income, race, parity, pre-pregnancy BMI, smoking	Self-reporting	*Yes	* Complete follow-up – retrospective

Appendix 7. Characteristics of included studies for obstetric outcomes according to depth of excision meta-analysis

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Jones 1979 (UK)	Retrospective cohort (population-based)	External: matching for age, parity, social class, delivery date, singleton birth	CKC	66	264	Clinical records from Cardiff Cervical Cytology Study - Cardiff Birth Survey (registry)	PTB (<37w); PTB (<37w)(singleton); sPTB (<37w); CS; ID; PrecL (<2h); ProlL (>12h); LBW (<2500g); PM; SB	9
Weber 1979 (Denmark)	Retrospective cohort (hospital-based)	External: matching for age	CKC	48	48	Hospital records; structured interviews	LBW (<2500g)	8
Buller 1982 (USA)	Retrospective cohort (hospital-based)	Internal (pre-treatment pregnancies)	CKC	47	79	Hospital records	PTB (<37w); tPTL; CS	7
Hemmings-son 1982 (Sweden)	Retrospective cohort (hospital-based)	Internal (pre-treatment pregnancies)	CT	115	65	Hospital records	PTB (<36w); pPROM; CS; stenosis; PM	8
Larsson 1982 (Sweden)	Retrospective cohort (population-based)	Internal (pre-treatment pregnancies) matching for age, parity, socioeconomic status, smoking, treatment, diseases	CKC	197	284	South Swedish Regional Tumour Registry, hospital records	PTB (<37w); PTB (<37w)(singleton); PTB (<37w)(multiple); PM; SB	9
Ludviksson 1982 (Sweden)	Retrospective cohort (hospital-based)	External: matching for age, parity, time of delivery	CKC	83	79	Hospital records	PTB (≤37w); PTB (≤33w); PTB (<30w); PPH; MOH	8
Moinian 1982 (Sweden)	Retrospective cohort (hospital-based)	Internal (pre-treatment pregnancies)	CKC	103	720	Hospital records	PTB (<37w); cerclage	8
Anderson 1984 (UK)	Retrospective cohort (hospital-based)	External: matching for age, race, births, miscarriages/TOPs	LA	68	70	Hospital records; postal questionnaires; obstetricians	PTB (<37w); PTB (<37w)(single); CS; ID; ProlL (>12h); LBW (<2500g)	7
Kristensen 1985 (Denmark)	Retrospective cohort (population-based)	External: matching for age, parity	Treatment NOS	85	12792	Hospital records; questionnaires	PTB (<37w); PTB (<37w)(singleton); LBW (<2500g)	9
Kuoppala 1986 (Finland)	Retrospective cohort (hospital-based)	External: matching for age, parity, date of delivery, singleton birth	CKC	62	62	Hospital records	PTB (<37w); CS; ID; IoL; oxytocin; analgesia; cerclage; PM; SB	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Saunders 1986 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity, race, year of delivery, singleton pregnancy	LA	97	97	Hospital records; general practitioners	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); pPROM; CS; ID; LBW (<2500g); PM	6
Gunasekera 1992 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity, race, duration of pregnancy, smoking	LLETZ; LA	140 (LLLETZ=23; LA=117)	140 (LLLETZ=23; LA=117)	Hospital records	PTB (<37w); CS; ID; ProlL(>12h)	9
Blomfield 1993 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity, ethnic group	LLETZ	40	80	Hospital records	PTB (<37w); sPTB (<37w); CS; ID; IoL; oxytocin; epidural; LBW (<2500g); NICU; PM	9
Haffenden 1993 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity	LLETZ	152	152	Hospital records	PTB (<37w); CS; ID; PrecL (<2h); ProlL (>12h); IoL; oxytocin; epidural; LBW (<2500g)	9
Hagen 1993 (Norway)	Retrospective cohort (hospital-based)	External: matching for age, parity; regression for height, marital status, education, smoking, TOP - index pregnancy: hypertension, APH, mode of delivery	LC	56	112	Hospital records	PTB (≤37w); PTB (≤37w)(nulliparous); PTB (≤37w)(parous); PTB (≤37w)(singleton); CS; ID; APH	9
Kristensen 1993 (Denmark)	Retrospective cohort (population-based)	A) External: no matching, no regression B) Internal (self-matching)	Treatment NOS (CKC, laser, electrocaute-ry)	A) 130 B) 62	A) 28124 B) 62	Medical Birth Register; national Register of Hospital Discharges	PTB (<37w); PTB (<37w)(nulliparous); PTB (<37w)(parous); PTB (<37w)(singleton)	7
Braet 1994 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity, smoking	LLETZ	78	78	Hospital records	PTB (<37w); PTB (<37w)(singleton); pPROM; CS; ID; APH; LBW (<2500g); PM	9
Cruickshank 1995 (UK)	Retrospective cohort (hospital-based)	A) External: age, parity, partner's social class, height, smoking B) Internal (pre-treatment pregnancies)	LLETZ	149	A) 298 B) 133	Aberdeen Maternity and Neonatal Databank; postal questionnaires	PTB (<37w); PTB (<28w); PTB (singleton)(<37w); CS; PrecL (<2h); SB	7
Sagot 1995 (France)	Retrospective cohort (hospital-based)	Internal (pre-treatment pregnancies)	LC	53	59	Hospital records	PTB (<37w); tPTL; pPROM; CS; chorioamnionitis; cerclage	7

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Spitzer 1995 (Jamaica)	Retrospective cohort (hospital-based)	Internal (pre-treatment pregnancies) with matching for age, parity	LC; LA	163 (LC=34; LA=129)	112	Hospital/private practice records; questionnaires (by mail, phone or in person)	PTB (<37w)	7
Bekassy 1996 (Sweden)	Retrospective cohort (hospital-based)	A) External: matching for age, parity, time of delivery B) Internal (self-matching)	LC ('miniconisa-tion')	A) 250 B) 148	A) 250 B) 148	National Medical Birth Registry; hospital records	PTB (<37w); PTB (<37w)(nulliparous); PTB (<37w)(parous); PTB (<37w)(single); PTB (<37w)(repeat); CS; ID; ProlL (>12h); stenosis; LBW (<2500g); PM; SB	8
Forsmo 1996 (Norway)	Retrospective cohort (hospital-based)	External: age, parity, place of delivery	LC; LA	71 (LC=51; LA=20)	174	Hospital records, postal questionnaires	LBW (<2500g); LBW (<2000g); LBW (<1500g); PM; SB	8
Turlington 1996 (USA)	Retrospective cohort (hospital-based)	Biopsy but no treatment: regression for age	LLETZ	15	15	Hospital records; telephone interviews/mail-in questionnaires	SB	7
Raio 1997 (Switzeland)	Retrospective cohort (hospital-based)	A) External: matching for age, parity, marital status, social class, smoking, PTB B) Internal (self-matching)	LC	A) 64 B) 26	A) 64 B) 26	Hospital records	PTB (<37w); PTB (<37w)(singleton); PTB (<37w)(D<10mm); PTB (<37w)(D≥10mm); pPROM	9
Andersen 1999 (Denmark)	Retrospective cohort (hospital-based)	External: matching for age, parity	LC	75	150	Hospital records	PTB (≤37w); PTB (≤37w)(D<15mm); PTB (≤37w)(D=15-20mm); PTB (≤37w)(D>20mm); pPROM; CS; PM; SB	9
El-Bastawissi 1999 (USA)	Retrospective cohort (population-based)	A) External: matching for age, country B) HSIL but no treatment Both regression for parity, race, smoking, marital status, TOPs	Excision NOS (CKC, LC, LLETZ); Ablation NOS (LA, CT)	1096	A) 9201 B) 330	Cancer Surveillance System (a population-based cancer registry); Birth Certificates (from the Department of Health in Washington state)	PTB (<37w); PTB (<37w)(singleton); CS; LBW (<2500g)	9
van Rooijen 1999 (Sweden)	Retrospective cohort (hospital-based)	External: matching for age, parity, year of delivery	LA	236	472	Hospital records	PTB (<37w); PTB (<37w)(single); CS; APH; LBW (<2500g); LBW (<2000g); LBW (<1500g); LBW (<1000g)	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Paraskevai-dis 2002 (Greece)	Retrospective cohort (hospital-based)	External: matching for age, parity, smoking, multiple pregnancies, PTBs	LLETZ (for microinva-sion)	28	28	Hospital records	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); sPTB; CS; PrecL (<2h); LBW (<2500g); NICU	9
Sadler 2004 (New Zealand)	Retrospective cohort (hospital-based)	Colposcopy but no treatment: regression for age, ethnicity, socioeconomic status, smoking, obstetric history, transfer to hospital, APH	LC; LLETZ; LA	652	426	Hospital records	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); PTB (<37w)(singleton); PTB (<37w)(D≤10mm); PTB (<37w)(D=11-16mm); PTB (<37w)(D≥17mm); PTB (<32w); sPTB (<37w); pPROM	9
Tan 2004 (UK)	Retrospective cohort (hospital-based)	External: matching for age, parity	LLETZ	119	119	Hospital records	PTB (<37w); CS; ID; ProlL (>12h); IoL; oxytocin; epidural; pethidine	8
Acharya 2005 (Norway)	Retrospective cohort (hospital-based)	A) External: matching for age, parity, date of delivery, smoking, obstetric history B) Internal (pre-treatment pregnancies)	LLETZ	79	A) 158 B) 45	Hospital records	PTB (<37w); tPTL; chorioamnionitis; IoL; LBW (<2500g); PM	9
Samson 2005 (Canada)	Retrospective cohort (hospital-based)	External: matching for age, parity, smoking status, year of delivery	LLETZ	571	571	Registries	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); PTB (<37w)(singleton); PTB (<37w)(multiple); PTB (<34w); PTB (<34w)(multiple); pPROM; CS; IoL; oxytocin; LBW (<2500g); NICU; PM; SB	9
Crane 2006 (Canada)	Retrospective cohort (hospital-based)	External: regression for age, gestation at USS, parity, smoking, APH, sPTB	CKC; LLETZ; CT	132 (CKC=21; LLETZ=75; CT=36)	81	Hospital records	sPTB (<37w); sPTB (<37w)(singleton); sPTB (<34w); CS; IoL; APH; LBW (<2500g); NICU; PM; Apgar (<7)(5min)	8
Klaritsch 2006 (Austria)	Retrospective cohort (hospital-based)	External: no matching, no regression	CKC	76	29711	Hospital records	PTB(<37w); PTB (<37w)(single); PTB (<37w)(singleton); PTB(<34w); pPROM; CS; chorioamnionitis; LBW (<2500g); PM	7
Bruinsma 2007 (Australia)	Retrospective cohort (hospital-based)	A) Colposcopy before pregnancy but no treatment B) Colposcopy during pregnancy but no treatment	CKC; LLETZ; LA; RD	1951	A) 2294 B) 1303	Hospital records and registries	PTB (<37w); PTB (<37w)(singleton); PTB (<32w); PTB (<28w); sPTB; pPROM; CS; ID; LBW (<2500g); PM; SB	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
		Both regression for age, drug use, marital status, medical conditions, TOPs, miscarriages, PTBs, treatment						
Himes 2007 (USA)	Retrospective cohort (hospital-based)	Biopsy but no treatment – no matching, regression	LLETZ	114	962	Hospital records	PTB (<37w); PTB (<37w)(singleton); sPTB; pPROM	8
Jakobsson 2007 (Finland)	Retrospective cohort (population-based)	External: regression for age, parity, smoking	Excision NOS (CKC, LC, LLETZ); Ablation NOS (LA, CT, electrocoa-gulation)	8422 (Excision NOS=4846; Ablation NOS=3576)	1056855	National registers	PTB (<37w); PTB (<28w); LBW (<2500g); PM	9
Sjoborg 2007 (Norway)	Retrospective cohort (population-based)	A) External: matching for age, parity, plurality B) Internal (self-matching) Both regression for smoking, marital status, education	Excision NOS (LC, LLETZ)	A) 742 (LC=609; LLETZ=133) B) 419	A) 742 B) 419	Hospital records	PTB (<37w); PTB (<32w); PTB (<28w); pPROM; LBW (<2500g); LBW (<1500g); LBW (<1000g); PM	8
Albrechtsen 2008 (Norway)	Retrospective cohort (population-based)	A) External B) Internal (pre-treatment pregnancies) Both regression for age, birth order	Excision NOS (CKC, LC, LLETZ)	14882	A) 2155505 B) 56927	National registries	PTB (<37w); PTB (<33w); PTB (<28w)	9
Parikh 2008 (USA)	Retrospective cohort (hospital-based)	External: no matching, no regression	LLETZ	87	18042	Hospital records	PTB (≤34w)	6
Jakobsson 2009 (Finland)	Retrospective cohort (hospital-based)	A) External: no matching B) Internal (self-matching) Both regression for age, parity, or both	LLETZ	A) 624 B) 258	A) 554507 B) 258	National registers and hospital records	PTB (<37w)(nulliparous); PTB (<37w)(parous)	8
Noehr 2009 (singletons) (Denmark)	Retrospective cohort (population-based)	A) External B) Biopsy but no treatment Both regression for age, year of delivery, smoking, marital status	LLETZ; Ablation NOS	10207 (LLETZ=8180; Ablation NOS=2027)	A) 510841 B) 31630	National registries	sPTB (<37w); sPTB (<37w)(D≤12mm); sPTB (<37w)(D=13-15mm); sPTB (<37w)(D=16-19mm); sPTB (<37w)(D≥20mm); sPTB (<37w)(single); sPTB (<37w)(repeat); sPTB	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
							(<37w)(singleton); sPTB (<32w); sPTB (<28w)	
Noehr 2009 (twins) (Denmark)	Retrospective cohort (population-based)	External: regression for age, year of delivery, smoking, marital status, IVF	LLETZ	166	9702	National registries	sPTB (<37w)(multiple); sPTB (<32w)(multiple); sPTB (<28w)(multiple)	9
Shanbhag 2009 (UK)	Retrospective cohort (population-based)	A) External B) CIN3 but no treatment Both regression for age, smoking, socioeconomic status, year of delivery, birth weight, malpresentation, sPTB, pPROM	Excision NOS (CKC, LC, LLETZ); Ablation NOS (LA, CC, diathermy coagulation)	1388 (Excision NOS=1103; Ablation NOS=285)	A) 119216 B) 87	National registries	PTB (<37w); sPTB (<37w); pPROM; CS; LBW (<2500g); PM	8
Fischer 2010 (USA)	Prospective cohort study (hospital-based)	External: matching for age, race, vaginal deliveries, gestational age at USS	Excision NOS (CKC, LLETZ)	85 (CKC=48; LLETZ=68; both=2)	85	Hospital records	PTB (<37w); PTB (<37w)(singleton); PTB (<34w); CS; cerclage	8
Ortoft 2010 (Denmark)	Retrospective cohort (hospital-based)	A) External B) HSIL but no treatment Both regression for age, parity, smoking, education, marital status C) Internal (self-matching)	CKC; NETZ; LLETZ	A/B) 746 [single cone=710 (CKC=67; NETZ=71; LLETZ=572) repeat cones=36] C) 170	A) 72899 B) 383 C) 170	National registries, hospital records, questionnaires	sPTB (<37w); sPTB (<37w)(single); sPTB (<37w)(repeat); sPTB (<37w)(singleton); sPTB (<32w); sPTB (<28w); pPROM (<37w); pPROM (<32w); pPROM (<28w); LBW (<2500g); LBW (<2000g); LBW (<1500g); PM; PM (<37w); PM (<32w); PM (<28w)	9
van de Vijner 2010 (Belgium)	Retrospective cohort (hospital-based)	External: matching for age, parity, year of delivery	LC; LLETZ	55 (LC=5; LLETZ=50)	55	Hospital records and questionnaires	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); PTB (<37w)(singleton); PTB (<37w)(multiple); PTB (<34w); tPTL; pPROM; CS; ID; IoL; oxytocin; LBW (<2500g); NICU; PM; SB	7
Werner 2010 (USA)	Retrospective cohort (hospital-based)	A) External B) Internal (pre-treatment pregnancies) Both regression for age, parity, race	LLETZ	551	A) 240348 B) 842	Hospital records	PTB (<37w); PTB (nulliparous)(<37w); PTB (singleton)(<37w); sPTB (<37w); pPROM; PM; SB	9
Andia 2011 (Spain)	Retrospective, cohort	A) External B) Internal (pre-treatment pregnancies)	LLETZ	189	A) 189 B) 189	Hospital records and registries	PTB (<37w); PTB (<37w)(nulliparous); PTB (<37w)(parous); PTB	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
	(population-based)	Both regression for age, parity, smoking					(<37w)(singleton); PTB (<35w); PTB (<32w); CS; LBW (<2500g); LBW (1500g)	
Armarnik 2011 (Israel)	Retrospective cohort (hospital-based)	External: regression for age, birth order, year of delivery, smoking, cervical cerclage	Excision NOS (CKC, LC, LLETZ, other)	53	104617	Hospital records	PTB (<34w); CS; epidural; cerclage; PM	9
Lima 2011 (Portugal)	Retrospective cohort (hospital-based)	External: no matching, no regression	LC; LLETZ	29 (LC= 11; LLETZ=18)	58	Hospital records	PTB (<37w); PTB (<37w)(D≤10mm); PTB (<37w)(D>10mm); CS; LBW (<2500g); Apgar (<7)(5min)	7
Castanon 2012 (& 2014) (UK)	Retrospective cohort (hospital-based)	A) External (general population) B) Biopsy no treatment C) Internal (pre-treatment pregnancies) D) Internal (self-matching)	Excision NOS (CKC, LC, LLETZ, other)	4776	A) 510660 B) 7263 C) 1173 D) 372	Hospital records and national registries	PTB (<37w); PTB (<37w)(D<10mm); PTB (<37w)(D≥10mm); PTB (<37w)(singleton); PTB (<33w)	8
Poon 2012 (UK)	Prospective cohort (hospital-based)	External: regression for parity, race, smoking, cervical length, PTB, miscarriage, LLETZ	LLETZ	473	25772	Hospital records, private practice records, questionnaires	sPTB (<37w); sPTB (<34w)	8
Reilly 2012 (UK)	Retrospective cohort (population-based)	A) External negative smear B) Colposcopy +/- biopsy Both regression for age, social deprivation, smoking, time to conception, obstetric history	Excision NOS (CKC, LLETZ); Ablation NOS (LA, CC, CT)	2162 (single excision= 1546; single ablation=53; multiple=82)	A) 38983 B) 2534	National registries	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); PTB (<37w)(singleton); PTB (<32w); PTB (<28w); LBW (<2500g)	9
Simoens 2012 (Belgium)	Prospective cohort (hospital-based)	External: matching for hospital; regression for age, parity, ethnicity, smoking, education, HIV	LC; LLETZ; Treatment NOS [only Excision NOS (CKC, LC, LLETZ) or Excision + Ablation NOS (LA, CC, CT)]	97 [Excision=81 (CKC=8; LC=24; LLETZ=53; unknown=4); Ablation=8 (LA=6; CC=1; CT=1); both=8]	194	Hospital records; questionnaires and medical records	PTB (<37w); PTB (<37w)(D≤10mm); PTB (<37w)(D>10mm); PTB (<37w)(singleton); PTB (<32w); sPTB (<37w); sPTB (<32w); CS; LBW (<2500g)	9
Van Hentenryck 2012 (Belgium)	Retrospective cohort (hospital-based)	External: matching for age, parity, smoking, HIV	Excision NOS (CKC, LC, LLETZ)	106	212	Hospital records	PTB (<37w); PTB (<34w); tPTL; pPROM; chorioamnionitis; CS; ID; IoL; LBW (<2500g); NICU	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Frega 2013 (Italy)	Prospective cohort (population-based)	External: matching for parity (nulliparous only), race (white only)	LLETZ	406	379	Hospital records	PTB (<37w); PTB (<37w)(nulliparous); PTB (<37w)(single); PTB (<37w)(singleton)	9
Frey 2013 (USA)	Retrospective cohort (hospital-based)	A) External with smear B) Biopsy but no treatment matching for age, year of treatment; regression for age, parity, race, diabetes, BMI, birth weight, CS	LLETZ	598	A) 588 B) 552	Hospital records and structured phone interviews	PTB (<37w); CS; IoL	8
Heinonen 2013 (Finland)	Retrospective cohort (population-based)	External: regression for age, socioeconomic status, marital status, urbanism, time to conception, PTB	LLETZ	7636	658179	National registers	PTB (<37w); PTB (<37w)(single); PTB (<37w)(repeat); PTB (<37w)(singleton)	9
Guo 2013 (China)	Prospective cohort (hospital-based)	Biopsy +/- CIN but no treatment: matching for smoking (non-smokers only)	CKC; LLETZ	84 (CKC=36; LLETZ=48)	68	Hospital records	PTB (<37w); PTB (<37w)(single); PTB (<34w); pPROM; CS; PrecL (<2h); ProlL (>12h); LBW (<2500g); Apgar (<7)(1min)	8
Wuntakal 2013 (UK)	Retrospective cohort (hospital-based)	A) Biopsy but no treatment B) Internal, (pre-treatment pregnancies) Both regression for parity, ethnicity, deprivation	Excision NOS (CKC, LC, LLETZ)	261	A) 257 B) 181	Hospital records	PTB (<37w)(single); PTB (<37w)(repeat); pPROM; CS; ID; LBW (<2500g)	9
Ciavattini 2014 (Italy)	Retrospective cohort (hospital-based)	External: matching for age, parity, BMI, smoking, hormonal contraception, PTB, cervical incompetence	LLETZ	7	21	Hospital records	sPTB (<36w)(multiple)	8
Ehsanipoor 2014 (USA)	Retrospective cohort (hospital-based)	External: regression for age, parity, race, PTB, smoking, drug use, chorionicity	CKC; LLETZ; Ablation NOS (LA, CT)	110 (CKC=10; LLETZ=36; Ablation NOS=64)	766	Hospital records	PTB (<37w)(multiple); PTB (<34w)(multiple); PTB (<28w)(multiple)	9
Kitson 2014 (UK)	Retrospective cohort (hospital-based)	Biopsy but no treatment: matching for age, parity, smoking	LLETZ	278	278	Hospital records	PTB (<37w); PTB (<37w)(singleton); PTB (<34w); sPTB; pPROM; CS; ID; LBW (<2500g); NICU	9
Sozen 2014 (Turkey)	Retrospective cohort (hospital-based)	External: matching for age, parity, obstetric history	CKC	15	24	Hospital records	PTB (<37w); pPROM; NICU	9

Study	Study Design	Matching factors	Procedure	Treated	Untreated	Source of data	Outcomes	Newcastle-Ottawa score
Martyn 2015 (Ireland)	Retrospective cohort (hospital-based)	Colposcopy but no treatment: matching for age	LLETZ; Excision NOS (CKC, repeat LLETZ)	297 (LLETZ=278; Excision NOS=19)	204	Hospital records and postal questionnaires	PTB (<37w); PTB (<37w)(single)	8
Stout 2015 (USA)	Retrospective cohort (hospital-based)	A) Cytology/biopsy but no treatment: matching for age, hospital, year B) Internal (pre-treatment pregnancies)	LLETZ	598	A) 1129 B) 598	Hospital records and structured phone interviews	sPTB (<37w); sPTB (<37w)(singleton); sPTB (<34w)	9

*Numbers refer to women or pregnancies

APH: antepartum haemorrhage; BMI: body mass index; CC: cold coagulation; CIN: cervical intraepithelial neoplasia; CKC: cold knife conisation; CS: caesarean section; CT: cryotherapy; D: depth; HSIL: high-grade squamous intraepithelial lesion; ID: instrumental deliveries (ventouse/forceps); IoL: induction of labour; LA: laser ablation; LBW: low birthweight; LC: laser conisation; LLETZ: large loop excision of the transformation zone; MOH: massive obstetric haemorrhage; NETZ: needle excision of the transformation zone; NICU: neonatal intensive care unit admission; NOS: not otherwise specified; PM: perinatal mortality; PPH: postpartum haemorrhage; pPROM: preterm premature rupture of membranes; PreL: precipitous labour; ProlL: prolonged labour; PTB: preterm birth; RD: radical diathermy; SB: stillbirth; sPTB: spontaneous preterm birth; (s)PTB (single): (spontaneous) preterm birth (single cone); (s)PTB (repeat): (spontaneous) preterm birth (repeat cones); (s)PTB (singleton): (spontaneous) preterm birth (singleton pregnancies); (s)PTB (multiple): (spontaneous) preterm birth (multiple pregnancies); TOP: termination of pregnancy; tPTL: threatened preterm labour; USS: ultrasound scan.

Appendix 8. Newcastle-Ottawa scores of included studies for obstetric outcomes according to depth of excision meta-analysis

Reference	Selection				Comparability	Outcome		
	Representativeness of exposed cohort	Selection of non exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Follow-up long enough for outcome to occur?	Adequacy of follow up of cohorts
Jones 1979	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity, social class, date of delivery and singleton birth	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Weber 1979	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Structured interview	*Yes	*External: matching for age	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Buller 1982	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Internal (pre-treatment pregnancies)	*Secure record - hospital records	*Yes	*Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	Inadequate: 27% lost to follow-up – no description of those lost
Hemmingsson 1982	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Internal (pre-treatment pregnancies)	*Secure record - hospital records	*Yes	*Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Larsson 1982	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Internal (pre-treatment pregnancies)	*Secure record - registry	*Yes	**Internal (pre-treatment pregnancies) with matching for age, parity, socioeconomic status, smoking, surgical interventions and various diseases	*Record linkage	*Yes – retrospective	*Complete follow-up – retrospective

Ludviksson 1982	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community.	no description of the derivation of the non exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity and time of delivery	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Moinian 1982	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Internal (pre-treatment pregnancies)	*Secure records – hospital records	*Yes	*Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Anderson 1984	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: matching for age, race, births and miscarriages/TOP	Self-report	*Yes - retrospective	Inadequate: 25% lost to follow-up – no description of those lost
Kristensen 1985	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age and parity	*Record linkage (questionnaires for a minority that moved away)	*Yes - retrospective	*Complete follow up - retrospective
Kuoppala 1986	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity and date of delivery	*Record-linkage	*Yes - retrospective	*Complete follow up - retrospective
Saunders 1986	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	Hospital case notes and contact with local general practitioners	*Yes	**External: matching for age, parity, race, year of delivery and singleton pregnancy	Hospital case notes and contact with local general practitioners	*Yes - retrospective	No description
Gunasekera 1992	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record- hospital records	*Yes	**External: matching for age, parity, race, duration of pregnancy and smoking habit	*Record linkage	*Yes- retrospective	*Complete follow up - retrospective

Blomfield 1993	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: matching for age, parity and ethnicity	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Haffenden 1993	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age and parity	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Hagen 1993	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community.	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age and parity; regression analysis for maternal height, marital status, level of education, smoking, previous TOP, and, in the index pregnancy, occurrence of gestational hypertension or antepartum haemorrhage and the mode of delivery	*Record linkage	*Yes - retrospective	*Subjects lost to follow up (1.7%) unlikely to introduce bias
Kristensen 1993	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (self-matching)	*Secure record - registry	*Yes	A) External: no matching, no regression analysis B) Internal (self-matching)	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Braet 1994	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity and smoking	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Cruickshank 1995	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (pre-treatment pregnancies)	*Secure record – registry	*Yes	**A) External: matching for maternal age, parity, husband's or partner's social class, height and daily cigarette consumption B) Internal (pre-treatment pregnancies)	Record linkage but also self-report	*Yes - retrospective	Inadequate: 34.7% did not respond to the questionnaire – no description of those lost

Sagot 1995	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community.	*Internal (pre-treatment pregnancies)	*Secure record - hospital records	*Yes	*Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	Inadequate: 21.6% could not be recontacted – no description of those lost
Spitzer 1995	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Internal (pre-treatment pregnancies)	*Secure record – hospital/private practice records	*Yes	**Internal (pre-treatment pregnancies) with matching for age and parity	Self-report	*Yes - retrospective	Inadequate: 47.9% lost to follow-up – no description of those lost
Bekassy 1996	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	A) External: drawn from a different source B) Internal (self-matching)	*Secure record - hospital records	*Yes	**A) External: matching for age, parity and time of delivery B) Internal (self-matching)	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Forsmo 1996	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*drawn from a same area & period but may be other institutions	*Secure record - hospital records	*Yes	**External: matching for age, parity and place of delivery	Self-report & record linkage for some outcomes	*Yes - retrospective	*Subjects lost to follow-up (3.4%) unlikely to introduce bias
Turlington 1996	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**Women with colposcopically directed biopsy: regression analysis for age	Self-report	*Yes - retrospective	Inadequate: 29.7% did not respond - no description of those lost
Raio 1997	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (self-matching)	*Secure record - hospital records	*Yes	**A) External: matching for age, parity, marital status, social class, smoking habits and previous PTB B) Internal (self-matching)	*Record linkage	*Yes - retrospective	*Subjects lost to follow-up (11.4%) unlikely to introduce bias
Andersen 1999	*Somewhat representative of the average pregnant woman with a previous history of	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age and parity	*Record-linkage	*Yes - retrospective	*Complete follow up - retrospective

	treatment for CIN in the community							
El-Bastawissi 1999	*Truly representative of the average pregnant woman with a previous history of treatment for CIS in the community	*Drawn from the same community as the exposed cohort	*Secure record – population-based cancer registry and birth certificates	*Yes	**A) External: matching for age and country of origin B) Women with untreated HSIL: no matching Both had regression analysis for parity, race, maternal smoking, marital status and history of TOPs	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
van Rooijen 1999	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same source as the treated group	*Secure record - hospital records	*yes	**External: matching for age, parity and year of delivery	*Record linkage	*Yes - retrospective	*Subjects lost to follow-up (16.5%) unlikely to introduce bias
Paraskevaidis 2002	*Somewhat representative of the average pregnant woman with a previous history of treatment for IA1 cervical carcinoma in the community	*drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity, smoking, multiple pregnancies and history of previous PTBs	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Sadler 2004	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**Women with colposcopy: regression analysis for age, ethnicity, socioeconomic status, smoking in pregnancy, previous obstetric history, transfer to the National Women's Hospital and antepartum hemorrhage	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Tan 2004	*Somewhat representative of the average woman with CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age and parity	*Record linkage	*Yes - retrospective	Inadequate: in 29.7% incomplete retrieval of data
Acharya 2005	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (pre-treatment pregnancies)	*Secure record – hospital records	*Yes	**A) External: matching for age, parity, date of delivery, smoking and previous obstetric history B) Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective

Samson 2005	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – official databases	*Yes	**External: matching for age, parity, smoking status, year of delivery	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Crane 2006	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	no description	*Yes	**External: regression analysis for maternal age, gestational age at the time of transvaginal ultrasonography, parity, smoking, antepartum bleeding after 20 weeks of gestation and previous sPTB	*Record-linkage	*Yes - retrospective	*Complete follow-up – retrospective
Klaritsch 2006	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	External: no matching, no regression analysis	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Bruinsma 2007	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**Women with colposcopy but no treatment: regression analysis for for age, illicit drug use during pregnancy, delivery at the RWH, marital status, maternal medical condition, previous TOP, previous miscarriage, previous PTB and previous treatment	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Himes 2007	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	*Women with colposcopic biopsy but no treatment – no matching, no regression analysis	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Jakobsson 2007	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – national registers	*Yes	**External: regression analysis for age, parity and smoking	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Sjoborg 2007	*Somewhat representative of the average pregnant woman with a previous history of	*A) External: drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**A) External: matching for age, parity and plurality B) Internal (self-matching) Both had regression analysis for smoking, marital status and education	*Record linkage	*Yes - retrospective	Inadequate: 69% of the women did not respond or did not give their

	treatment for CIN in the community	B) Internal (self-matching)						consent – no description of those lost
Albrechtesen 2008	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (pre-treatment pregnancies)	*Secure record - national registries	*Yes	**A) External B) Internal (pre-treatment pregnancies) Both had regression analysis for age and birth order	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Parikh 2008	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	No description	*Yes	External: No matching, no regression analysis	*Record linkage	*Yes - retrospective	*Subjects lost to follow-up (10.3%) unlikely to introduce bias
Jakobsson 2009	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	A) External: treated group drawn from hospital while controls from population-based registry B) Internal (self-matching)	*Secure record – national registers and hospital records	*Yes	**A) External: no matching B) Internal (self-matching) Both had regression analysis for age, parity, or both	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Noehr 2009 (singletons)	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – national registries	*Yes	**A) External B) Women with biopsy but no treatment Both had regression analysis for age, year of delivery, smoking during pregnancy and marital status during pregnancy	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Noehr 2009 (twins)	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – national registries	*Yes	**External: regression analysis for age, year of delivery, smoking during pregnancy, marital status during pregnancy and IVF	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Shanbhag 2009	*Somewhat representative of the average pregnant woman with a previous history of	*Drawn from the same community as the exposed cohort	*Secure record – national registries	*Yes	**A) External B) Women with untreated CIN 3 Both had regression analysis for maternal age at delivery, smoking,	*Record linkage	*Yes - retrospective	Inadequate: for 69% of the treated population the

	treatment for CIN3 in the community				socioeconomic status, year of delivery, birth weight, malpresentation, sPTB and pPROM			type of treatment was not known – no description of those lost
Fischer 2010	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	No description	*Yes	**External: regression analysis for age, race, the number of prior vaginal deliveries at ≥20 weeks and gestational age at the time of cervical sonography	*Record linkage	*Yes	*Complete follow-up
Ortoft 2010	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External B) Women with untreated HSIL Both were drawn from the same community as the exposed cohort C) Internal (self-matching)	*Secure record – national registries	*Yes	** A) External B) Women with untreated HSIL Both had regression analysis for age, parity, smoking status, educational level and marital status C) Internal (self-matching)	*Record linkage (but questionnaires for the outcomes of previous pregnancies when internal matching (self-matching) was used)	*Yes - retrospective	*Complete follow-up
van de Vijner 2010	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: matching for age, parity and year of delivery	Self-report	*Yes - retrospective	No statement
Werner 2010	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External: drawn from the same community as the exposed cohort B) Internal (pre-treatment pregnancies)	*Secure record – hospital records	*Yes	**A) External B) Internal (pre-treatment pregnancies) Both had regression analysis for age, parity and race	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Andia 2011	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	A) External: drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**A) External B) Internal (pre-treatment pregnancies) Both had regression analysis for age, parity and smoking	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective

		B) Internal (pre-treatment pregnancies)						
Armarnik 2011	*Somewhat representative of the average pregnant women with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: regression analysis for age, birth order, year of delivery, smoking and cervical incompetence with cerclage	*Record linkage	*Yes - retrospective	*Subjects lost to follow-up (7%) unlikely to introduce bias
Lima 2011	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	No matching, no regression analysis	*Record linkage	*Yes - retrospective	*Complete follow-up – retrospective
Castanon 2012 (& 2014)	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) External (general population) B) Women with punch biopsy C) Internal (pre-treatment pregnancies) D) Internal matching (self-matching)	*Secure record – hospital records	*Yes	**A) General population B) Women with punch biopsy C/D) Internal controls Regression analysis for age parity and study site for a variant of the groups that we used	*Record linkage	*Yes - retrospective	Inadequate: 29.9% lost to follow-up because of unknown gestational age – no description of those lost
Poon 2012	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	Written self-report (questionnaires)	*Yes	**External: regression analysis for parity, race, smoking, cervical length, previous delivery at term, previous PTB, previous miscarriage and previous LLETZ (for the prediction of sPTB)	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Reilly 2012	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – national registries	*Yes	**A) External B) Women with colposcopy +/- punch biopsy Both had regression analysis for maternal age at birth, social deprivation, smoking status, time interval between screening/colposcopy/treatment and conception, any history of a previous	*Record linkage	*Yes - retrospective	*Subjects lost to follow-up (10.6%) unlikely to introduce bias

					adverse pregnancy outcome (and gestational age for LBW outcome)			
Simoens 2012	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – questionnaires in combination with checking of medical files	*Yes	**External: matching for admittance in the same maternity ward; regression analysis for age, parity, ethnicity, smoking, education, HIV status	*Record linkage	*Yes	*Complete follow-up
Van Hentenryck 2012	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: matching for age at delivery, parity, smoking, history of gestation and HIV status	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Frega 2013	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: women of the same parity (only nulliparous) and race (only white)	*Record linkage	*Yes	*Subjects lost to follow up (4.1%) unlikely to introduce bias
Frey 2013	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**A) External B) Women with punch biopsy Both had matching for age and year of treatment, and regression analysis for age, parity, race, maternal diabetes, maternal BMI, neonate birth weight and prior CS	*Record linkage (structured phone interviews and then confirmation from medical files)	*Yes - retrospective	No statement
Heinonen 2013	*Truly representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: regression analysis for maternal age, socioeconomic status, marital status, urbanism, time since LLETZ and previous PTBs	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Guo 2013	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**Women with colposcopic biopsy +/- CIN: all were non-smokers	*Record linkage	*Yes	No statement
Wuntakal 2013	*Somewhat representative of the average pregnant woman with a previous history of	*A) Women with biopsy: drawn from the same community as	*Secure record – hospital records	*Yes	**A) Women with biopsy B) Internal (pre-treatment pregnancies)	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective

	treatment for CIN in the community	the exposed cohort B) Internal (pre-treatment pregnancies)			Both had regression analysis for parity, ethnicity and deprivation			
Ciavattini 2014	Selected group of users (twin deliveries after assisted reproduction techniques)	*Drawn from the same community as the exposed cohort	*Secure record - hospital records	*Yes	**External: matching for age, parity, BMI, tabagism, previous hormonal contraception, previous PTB and cervical incompetence at 1st trimester	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Ehsanipoor 2014	*Somewhat representative of the average pregnant woman (with a twin pregnancy) with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: regression analysis for age, parity, race, history of PTB, history of tobacco use, history of drug use and chorionicity	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Kitson 2014	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**Women with punch biopsy: matching for age, parity and smoking	*Record linkage	*Yes - retrospective	*Complete follow-up - retrospective
Sozen 2014	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record – hospital records	*Yes	**External: matching for age, parity and obstetric history	*Record linkage	*Yes - retrospective	*Complete follow up - retrospective
Martyn 2015	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*Drawn from the same community as the exposed cohort	*Secure record - questionnaires which were then confirmed from hospital records	*Yes	**Women with colposcopy: matching for age	Self-report	*Yes - retrospective	*Complete follow up - retrospective
Stout 2015	*Somewhat representative of the average pregnant woman with a previous history of treatment for CIN in the community	*A) Women with cervical cytology/punch biopsy: drawn from the same community as	*Secure record – hospital records	*Yes	**A) Women with cervical cytology/punch biopsy: matching for age, hospital site and calendar year of cervical procedure B) Internal (pre-treatment pregnancies)	*Structured phone interviews which were then confirmed from medical files	**Yes - retrospective	*Subjects lost to follow up (<6%) unlikely to introduce bias

		the exposed cohort B) Internal (pre-treatment pregnancies)						