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Predictors of HSIL Treatment Failure

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Predictors of HSIL Treatment Failure

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Résumé

Objectif Les traitements répétés des lésions précancéreuses du col utérin (HSIL), nécessaires en cas d'échecs de traitement, sont associés à des issues obstétriques négatives, telle qu'une augmentation de la mortalité néonatale. Nous avons investigué l'association entre un grand nombre de facteurs de risque potentiels pour l'échec de traitement des HSIL dans le but d'identifier des prédicteurs potentiellement modifiables de l'échec de traitement.

Méthodes La population source était constituée de 1 548 femmes canadiennes qui ont subi un premier traitement pour HSIL. L'échec de traitement a été défini comme étant un diagnostic histologique de HSIL ou cancer au cours des deux années suivant le traitement. Nous avons mené une étude cas-témoins nichée incluant les 101 cas d'échec de traitement ainsi que les témoins appariés 1 :1 par centre de traitement et par date d'échec. Nous avons calculé des rapports de cotes (OR) et intervalles de confiance (CI) à 95% à l'aide de régressions logistiques conditionnelles, pour les associations entre l'échec de traitement et l'âge, le nombre d'accouchements, le statut tabagique, le nombre de partenaires sexuels, l'utilisation du condom, la méthode de contraception, les marges, le nombre de passages, le diagnostic sur le spécimen de traitement, le génotype du VPH, et le nombre de types. Nous avons aussi estimé l'association entre la charge virale et les variants du VPH16 et du VPH18 et l'échec de traitement.

Résultats Les marges positives vs négatives (OR ajusté=4.05, 95% CI 1.57-10.48), la positivité pour le VPH16/18 vs autres types (OR ajusté=2.69, 95% CI 1.32-5.49), et avoir un variant similaire au prototype du VPH16 vs le prototype (OR ajusté=2.49, 95% CI 1.07-5.83) étaient des prédicteurs de l'échec de traitement des HSIL. Être plus âgé, avoir des lésions plus sévères, avoir une infection monotype, et avoir une variation à la position 7521 chez celles avec le VPH16 pourraient augmenter le risque d'échec de traitement, mais les associations n'étaient pas statistiquement significatives. Les estimations pour les autres facteurs étaient proches de la valeur nulle. Nous n'avons pas observé de modification d'effet du génotype sur le risque de l'échec de traitement par le tabagisme, ni par les marges.

Conclusion Seules les marges positives, la positivité pour le VPH16/18 et avoir un variant similaire au prototype étaient des prédicteurs d'un échec de traitement au cours des deux années

suivant le traitement. Malgré l'aspect non-modifiable des prédicteurs identifiés, ils sont informatifs et pourront éclairer la prise en charge et le suivi clinique.

Mots clés lésions malpighiennes intra-épithéliales du col de l'utérus, néoplasies intra-épithéliales du col de l'utérus, cancer du col de l'utérus, virus du papillome humain, conisation cervicale, LEEP, échec de traitement, facteurs de risque, épidémiologie

Abstract

Objective Repeated treatments for high-grade squamous intraepithelial lesions (HSIL), which are necessary in the case of treatment failure, are associated with negative obstetric outcomes, such as an increased risk of neonatal death. We investigated the association between a large number of potential risk factors and HSIL treatment failure in an effort to identify potentially modifiable predictors of treatment failure.

Methods The source population included 1,548 Canadian women who received a first treatment for HSIL. Treatment failure was defined as the histological diagnosis of HSIL or cancer within the two years following treatment. We conducted a nested case-control study that included all 101 cases of treatment failure and controls that were matched 1:1 on treatment center and date of failure. We used conditional logistic regression to calculate the odds ratios (OR) and 95% confidence intervals (CI) between treatment failure and age, parity, smoking status, number of sexual partners, condom use, method of contraception, margins, number of passes, diagnosis on the treatment specimen, HPV genotype and number of types. We also estimated the association between HPV16 and HPV18 viral loads and variants and HSIL treatment failure.

Results Having positive vs. negative margins (adjusted OR=4.05, 95% CI 1.57-10.48), being positive for HPV16 and/or HPV18 vs. any other type (adjusted OR=2.69, 95% CI 1.32-5.49), and having a prototype-like variant of HPV16 vs. the prototype (adjusted OR=2.49, 95% CI 1.07-5.83) were predictors of HSIL treatment failure. Older age, more severe lesions, single-type infections and a variation at the 7521 position of the HPV16 genetic sequence may lead to a higher risk of treatment failure but were not statistically significant. Estimates for all other factors were near the null value. The effect of genotype on the risk of treatment failure was not modified by smoking status, nor by margin status.

Conclusion Only positive margins, HPV16/18 positivity, and having a prototype-like variant of HPV16 were predictors for HSIL treatment failure within two years of treatment. Despite being non-modifiable, the identified predictors are clinically significant in regards to management and follow-up of patients.

Keywords squamous intraepithelial lesions of the cervix, cervical intraepithelial neoplasia, cervical cancer, *Human Papillomavirus*, cervical conization, LEEP, treatment failure, risk factors, epidemiology

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List of abbreviations

AGC: Atypical glandular cells

AIS: Adenocarcinoma in situ

ASC-H: Atypical Squamous Cells that cannot exclude HSIL

ASCCP: American Society for Colposcopy and Cervical Pathology

ASCUS: Atypical Squamous Cells of Undetermined Significance

CHUM: Centre Hospitalier de l'Université de Montréal

CIN: Cervical Intraepithelial Neoplasia

CIS: Carcinoma in situ

CKC: Cold Knife Conisation

CoHIPP: Colposcopy vs. HPV testing to identify persistent precancers post treatment

CI: Confidence Interval

DAG: Directed Acyclic Graph

DMSP: Département de Médecine Sociale et Préventive

DNA: Deoxyribonucleic Acid

ESPUM: École de Santé Publique de l'Université de Montréal

FESP: Faculté des Études Supérieures et Postdoctorales

HC2: Hybrid Capture 2

HPV: Human Papillomavirus

hr-HPV: high-risk Human Papillomavirus

HSIL: High-grade Squamous Intraepithelial Lesion

IARC: International Agency for Research on Cancer

LCR: Long Control Region

LEEP: Loop Electrosurgical Excision Procedure

LLETZ: Large Loop Excision of the Transformation Zone

lr-HPV: low-risk Human Papillomavirus

LSIL: Low-grade Squamous Intraepithelial Lesion

OR: Odds Ratio

pPROM: preterm Premature Rupture Of the Membranes

pRb: Retinoblastoma protein

p53: Tumor suppressor protein

qPCR: quantitative (real time) Polymerase Chain Reaction

RCT: Randomized Controlled Trial

RR: Relative Risk

RLU: Relative Light Units

ROC: Receiver Operating Characteristic (curve)

SD: Standard Deviation

SIL: Squamous Intraepithelial Lesion

SNP: Single Nucleotide Polymorphism

STI: Sexually Transmitted Infection

SWETZ: Straight-Wire Excision of the Transformation Zone

TZ: Transformation Zone

To my parents,
Who taught me perseverance and determination,
And who believed in me even when I didn't believe in myself.
Thank you for your endless support,
And for all of the meal deliveries.

Thanks

I would first like to express my deep gratitude to my research directors, Dr. Marie-Hélène Mayrand and Dr. Anita Koushik. Thank you for your confidence in me, for your guidance and for your support throughout this project. Thank you for everything you have taught me about scientific research, which extends well beyond the content of this thesis.

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Finally, I must thank my parents and friends, who were an essential source of motivation throughout this research project.

Chapter 1 - Introduction

1.1 Context and rationale

Cervical cancer is the fourth most frequent and deadly cancer affecting women globally [1]. The disease poses an enormous threat to women's health, especially in countries that have not implemented successful methods of prevention. Screening at regular intervals, usually performed by Papanicolaou smear (Pap test), and anti-*Human Papillomavirus* (HPV) vaccination are proven to be effective methods at reducing the incidence of cervical cancers and precancers [2-10]. Cervical cancer has a long pre-invasive phase and lends itself well to screening as a secondary method of prevention [11]. In Canada, cervical cancer screening is widely available and has been responsible for a dramatic decrease in the incidence of cervical cancer in the last 50 years [3, 4, 6, 9, 10]. Despite these advancements, it is estimated that 1,350 women will be diagnosed with cervical cancer and 410 women will die of it in Canada in 2020 [12].

The necessary cause for cervical cancer and its precursors is persistent infection with a high-risk type of HPV (hr-HPV) [13-15]. HPV is the most prevalent sexually transmitted infection (STI) in the world [16]. However, not all HPV infections are created equal. Approximately 90% of HPV infections will clear without any intervention [16, 17], while others have the capacity to evade immune responses and persist within their host [18-22]. Persistent infections with hr-HPV can lead to oncogenesis. Precancers called High-Grade Squamous Intraepithelial Lesions (HSIL) can be diagnosed on cervical biopsies. If HSIL is diagnosed, a patient must be treated in order to prevent progression to cervical cancer [3].

Treatment for HSIL is successful in most cases. However, approximately 10-15% of those treated will have recurrent disease, called treatment failure, within two years of treatment and will need to be retreated [23]. Treatment, and especially repeated treatments have been associated with negative obstetric outcomes in subsequent pregnancies [24]. On average, cervical precancers are diagnosed at a younger age than other female reproductive cancers, occurring during a woman's childbearing years [24].

It is therefore of great importance to understand why some women fail treatment in an effort to reduce these negative outcomes, and to reduce the overall harms associated with screening and treating HSIL.

Previous studies that have investigated risk factors for HSIL treatment failure have mostly been very limited in sample size and in number of cases of treatment failure [25-54]. They have usually relied exclusively on univariate analysis or focused only on two or three potential predictors. Results have been inconsistent, but suggest that older age, positive treatment margins and high viral load may be predictors of treatment failure.

1.2 Specifications

In this thesis, we refer to the most recent terminology for the classification of cervical precancers. In the prior system, cervical intraepithelial neoplasia (CIN) were divided into 3 categories: CIN1, CIN2 and CIN3. The current system requires specimens that previously would have been characterized as CIN2 to undergo additional examination. CIN2 that test positive for the p16 biomarker, a marker of abnormal cell proliferation, and CIN3 are now classified as HSIL. CIN1 and CIN2 that are p16-negative are classified as low-grade SIL (LSIL) [55]. When referring to other studies that use the old terminology, we have conserved the use of CIN classification for consistency.

1.3 Thesis organisation

This thesis was written according to the guidelines presented in the *Guide de présentation des mémoires et des thèses de la Faculté des Études Supérieures et Postdoctorales (FESP)* for submission to the *Département de Médecine Sociale et Préventive (DMSP)* of the *École de Santé Publique de l'Université de Montréal (ESPUM)*. This thesis is written by article. Chapter 2 includes a detailed background and literature review. Chapter 3 presents our study objectives. Chapter 4 describes the methods used to investigate potential predictors of HSIL treatment failure. Chapter 5 consists of an article manuscript prepared for publication in a peer-reviewed journal. Chapter 6 presents additional results obtained from our laboratory analyses. Finally, Chapter 7 is a discussion of our results.

1.4 Student's contribution

Under the supervision of Dr. Marie-Hélène Mayrand and Dr. Anita Koushik, Sarah Botting-Provost conducted a literature review on exposure variables that were potentially associated with HSIL treatment failure, determined the objectives of this master's project, cleaned the data, performed statistical analyses and interpreted their results. In collaboration with Julie Guenoun, the student prepared purified HPV DNA for sequencing and analysed HPV variant sequences. Sequencing results were verified by Dr. François Coutlée. The student also analysed viral load results provided by Emilie Comète. Finally, the student wrote the first drafts of the article manuscript and the entirety of this thesis.

Chapter 2 – Background and Literature Review

2.1 *Human Papillomavirus*

HPV is the most common STI worldwide [16], transmitted through skin-to-skin or skin-to-mucosa contact. In North America, crude prevalence of HPV DNA in the general female population is 13.8% [56]. HPV belongs to the *Papillomaviridae* family [56], which includes viruses capable of infecting a range of animal and human hosts [16]. HPV taxonomy is based largely on genomic sequence, as well as biological function and pathological effect [57]. Over 130 genotypes of HPV have been isolated from humans [11, 57].

HPV is a circular double-stranded DNA virus that infects epithelial cells at various sites in the human body [16, 56-58]. Along with a noncoding long control region (LCR), the viral genome encodes 8 proteins: capsid proteins L1 and L2, and replication, transcription and transformation proteins E1, E2, E4, E5, E6, and E7 [59]. Initial infection of basal epithelial cells likely occurs through micro-abrasions to the cell's surface, with internalisation of the virus happening during the wound healing process [16, 56]. Once transported into the host cell's nucleus, HPV establishes itself as a low copy-number nuclear plasmid [56]. Persistence of the virus allows for viral DNA to be passed to daughter cells through cell division [16, 56]. Viral replication relies on suprabasal, differentiating cells, where the cell's DNA replication mechanisms are hijacked by the virus [56]. HPV DNA amplification occurs and viral gene products E6 and E7 bind to tumour suppressor protein p53 and cell-cycle regulator retinoblastoma (pRb) respectively, promoting cellular proliferation, prolonging the cell-cycle, and preventing apoptosis [11, 16, 56, 60]. Infected cells become factories for viral replication, producing hundreds or thousands of HPV genome copies each [16]. Viral assembly takes place at the surface of the epithelium, which expresses the capsid proteins L1 and L2. Both episomal and integrated versions of HPV DNA are often found within the same cell [16].

The majority of HPV infections are transient and will clear spontaneously [16]. In fact, more than 90% of cases of HPV infection will regress within 6 to 18 months due to cell-

mediated immunity [16, 17]. However, some types of HPV possess an enhanced capacity to evade the host's immune responses. Gene products expressed during early stages of infection and replication, like E6 and E7, are involved in preventing natural clearance by the host [18-22]. The evasion mechanisms result in decreased expression of viral antigens at the cell's surface, inhibition of innate immune responses such as those by toll-like receptors, prevented activation of cytotoxic T lymphocytes, inhibition of interferon production, down-regulation of pro-inflammatory cytokines with up-regulation of anti-inflammatory cytokines, and decreased concentration of antimicrobial peptides in the cervical-vaginal tract [18-22]. Deregulation of the cell cycle allows for an accumulation of genomic mutations and effective immortalization of cells [61]. In the genital area, malignant transformation can occur, most commonly in cells of the cervix in what is called the transformation zone (TZ). The resulting transformed cells are called CIN or SIL, which can in turn progress to invasive disease [62].

HPV has been identified as a human carcinogen, associated with the development of cancer at multiple sites, including the cervix, vulva, anus, vagina, penis and oropharynx [11, 63]. Of 14 million incident cases of cancer that developed in 2012, 2.2 million were caused by infections, including *Helicobacter pylori*, HPV, hepatitis B and C, and Epstein-Barr virus. In the 2012 analysis, HPV was deemed responsible for 29.5% of all cancers attributable to infections [63].

2.1.1 Causal association between HPV and cervical cancer

HPV was the first-ever identified necessary cause for a human cancer [13, 14]. Theoretically, there are no cases of epithelial cervical cancer without the presence of persistent oncogenic HPV DNA. Technology developed in the 1980's allowed for etiological confirmation through the identification of HPV DNA in cellular specimens [14]. The original discovery was made by Harald zur Hausen and his team, who successfully identified HPV16, and then HPV18, in tissue from cervical cancer specimens in 1983 and 1984 respectively [64]. Zur Hausen was awarded the Nobel Prize in Physiology or Medicine in 2008 for this discovery [16]. The causal relationship between HPV and cervical cancer has since been confirmed through molecular biology,

clinical and epidemiological evidence [13, 14, 16]. In a meta-analysis by Koshiol et al., which included 41 studies and over 22,500 women, 92% of studies resulted in a relative risk of over 3.0, with an overall range of 1.3 to 813.0, for the association between persistent carcinogenic HPV infection, defined as HPV positivity at two or more visits, and cervical cancer and its high-grade precursors [65].

Among the wide range of HPV genotypes, different levels of oncogenic potential can be observed. According to the International Agency for Research on Cancer (IARC) Monograph from 2018, 12 types have been confirmed as high-risk of causing malignant transformation, or oncogenic: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 [66]. These hr-HPV types are classified as group 1 human carcinogens. Low-risk types can also produce disease, but often benign, like condylomas, and include: HPV6, 11, 40, 42, 43, 44, 53, 54, 61, 66, 68, 72, 73 and 82 [11, 66]. While the vast majority of infections clear spontaneously, the persistence of hr-HPV infection can lead to carcinogenesis [16]. In fact, the necessary steps for cervical carcinogenesis include initial infection, persistence, progression to precancerous lesions, and invasion [58]. Certain types of HPV have been shown to be more likely to persist, and more capable of causing malignant transformation, notably HPV16 and HPV18 [11].

The time from initial infection with HPV to development of cervical precancer is highly variable. The delay is often around 10 years, which is illustrated by the fact that the highest rate of incident infection occurs in the late teens or early twenties, but the most frequent age of diagnosis of cervical precancers is around 30 years of age [58]. In turn, progression to invasive cancer can take decades. However, progression can also occur much faster, especially for HPV16 infections. Since only the minority of HPV infections progress to cervical cancer, even amongst those with high-risk types, cofactors must be involved in the transformation process [56]. Indeed, a variety of patient, behavioural, clinical and viral cofactors have been studied in relation to the progression of HPV infection to cervical cancer. Generally, these cofactors aid in the transformation process by promoting immunosuppression or by up-regulating hr-HPV viral expression [11]. Infection with multiple types of HPV or co-infection with Human Immunodeficiency

Virus and/or other disease, along with genetic predisposition, age, smoking, parity, oral contraceptive use and hormone replacement therapy, viral load, virus type and variant, lack of screening, among others, have been proposed or confirmed, as co-factors for the development of cervical cancer in the presence of hr-HPV infection [11, 26, 56, 67].

2.2 Descriptive epidemiology of cervical cancer

The prevalence of HPV infection peaks in young women following sexual initiation, up to around 25 years of age, and declines at older ages in most populations [56, 58]. This trend can be explained by the general transience of HPV infection, which will most often clear naturally within two years of initial infection [58]. There is no evidence that duration of infection with incident HPV infection is associated with age [67, 68]. However, prevalent HPV infections in women over the age of 30 tend to be more likely to progress to cervical cancer because these infections are more likely to be persistent, as opposed to newly acquired [67]. In fact, rates of incident infection decrease significantly with age in most populations, and incident infections have a low risk of persistence [69]. The increased capacity of hr-HPV types to persist in the host, compared to low-risk HPV (lr-HPV) types, likely explains why these types constitute between 50% and 80% of infections in women over the age of 30 [70]. In addition, persistence could explain why the most prevalent type of HPV worldwide is HPV16, even in women without cervical pathology [56]. According to a meta-analysis conducted by Guan, Howell-Jones, Li *et al*. in 2012, the prevalence of HPV16 increased as the grade of lesion increased, reflecting the higher oncogenic potential of HPV16 [71]. They found that the most common types of HPV in invasive cervical cancer worldwide were HPV16 (57%), HPV18 (16%), HPV58 (5%), HPV33 (5%), HPV45 (5%), HPV31 (4%), HPV52 (3%), and HPV35 (2%) [71].

2.2.1 Global burden of cervical cancer

Globally, HPV poses a major threat to women's health, especially in countries that have not implemented effective prevention of cancer through vaccination or screening [11, 13, 56]. Most sexually active people will acquire at least one genotype of HPV in their lifetime [56, 58], with a lifetime cumulative risk of over 80% [70]. In 2018, IARC

estimated that approximately 570,000 new cases of cervical cancer developed, or 15.1 new cases for every 100,000 women, and over 311,000 deaths resulted from cervical cancer, or 10.1 deaths for every 100,000 women. The highest prevalence of HPV can be found in Africa and South America, with the lowest in Europe [58], while the highest incidence of cervical cancer is in Africa, Asia and South America. Among cancers affecting women, cervical cancer ranks fourth globally in terms of incidence and mortality, after breast, lung, and colorectal cancers [1]. In May 2018, the World Health Organization launched a call to action to end global suffering caused by cervical cancer [72].

2.2.2 Canadian burden of cervical cancer

Between 2010 and 2015, the incident rate of cervical cancer in Canada decreased by approximately 3.3% per year [4]. The Canadian Cancer Society estimated that 1,350 Canadian women would be diagnosed with cervical cancer in 2020, with 410 women dying from the disease [4]. Women in their early forties are at the highest risk of cervical cancer diagnosis, which is younger than what is observed for other female reproductive cancers [9]. In fact, almost 30% of cases of cervical cancer occur in women under 40 years of age [9].

2.3 Cervical cancer prevention

2.3.1 Primary prevention: Vaccination

HPV16 and HPV18 are the most common genotypes found in invasive cervical cancer. According to a meta-analysis, HPV16 and HPV18 account for 70% of invasive cervical cancers globally [73]. Therefore, development of effective prophylactic vaccines against these genotypes has the potential to prevent approximately 70% of cervical cancers [16]. HPV vaccination should play an important role in preventing deaths caused by cervical cancer, especially in unscreened or under-screened populations, as well as being cost-effective by reducing costs associated with screening and treatment in settings where screening is implemented. The long-term reduction in deaths from cervical cancer that will be attributable to anti-HPV vaccination has not yet been characterised. Based on age

of vaccination and the natural history of the disease, it has not been long enough since the implementation of vaccination programs to measure the full effect.

Currently, three prophylactic vaccines have been approved: a bivalent vaccine (Cervarix®, GlaxoSmithKline), a quadrivalent vaccine (Gardasil/Silgard®, Merck) [16], and more recently a nonavalent vaccine (Gardasil9®, Merck). In 2006, Gardasil® was approved for use in females aged 9 to 26 in Canada, followed by Cervarix® in 2010 and by Gardasil9® in 2015[74, 75]. Since, HPV vaccines have been authorized for use in women up to 45 years of age, and in males aged 9 through 26 years [74, 75]. All three vaccines use virus-like particles to induce production of type specific antibodies. The bivalent vaccine targets HPV16 and 18, the quadrivalent vaccine also targets HPV16 and HPV18, adding HPV6 and 11 (responsible for most genital warts). The nonavalent vaccine targets the types targeted by the quadrivalent vaccine, along with another five oncogenic types: 31, 33, 45, 52, and 58 [7]. These vaccines contain L1 capsid proteins that self-assemble into virus-like particles capable of inducing strong neutralising antibody responses, and preventing HPV from accessing the basal layer of the epithelium [76].

Since the approval of the first HPV vaccine in 2006, prophylactic vaccination programs have been implemented in over 100 countries worldwide and constitute a major public health initiative to limit the incidence of cervical cancer [7, 77]. Levels of coverage and specific methods of implementation vary between countries [7]. Given the vaccine's optimal efficacy in those who have never been infected with HPV, vaccine programs have targeted pre-adolescent and adolescent females, and most recently pre-adolescent and adolescent males in some developed countries. In a randomized placebo-controlled trial of the quadrivalent vaccine, vaccine efficacy was shown to be 98% (95% confidence interval (CI) 86-100%) in HPV-naïve patients [2]. Both bivalent and quadrivalent vaccines have been clinically shown to prevent precancerous lesions associated with HPV16 and 18 [2, 8, 78]. In a meta-analysis of articles comparing the frequency of different HPV-related outcomes during pre- and post-vaccination periods in 14 high-income countries, it was found that after 5-8 years of vaccination, there was a significant

decrease in the prevalence of HPV16 and HPV18 in girls aged 13-19 years (83% decrease, RR=0.17, 95% CI 0.11-0.25) and in women aged 20-24 (66% decrease, relative risk (RR)=0.34, 95% CI 0.23-0.49) [5]. A study in Australia, which implemented a National HPV Vaccination Programme with the quadrivalent vaccine for girls in 2007 and for boys in 2013, found that the rate of high-grade cervical abnormalities in screened women under 20 years of age in 2014 was less than half the rate screened in 2007 [79]. The bivalent vaccine, in the PATRICIA trial [80], and the quadrivalent vaccine have also been shown to induce cross-protective immune responses against non-vaccine types of HPV [78]. Even with cross-protective capacities, the vaccines do not protect against all types of HPV. Effective vaccination programs do not eliminate the need for screening but could modify recommendations for screening programs in the future [81].

Effectiveness and impact of HPV vaccination depends on coverage within a population, on age of birth cohorts targeted by vaccination programs, and on presence of catch-up programs to immunise older generations [7]. In Canada, all provinces and territories currently provide school-based publicly funded HPV vaccination [82]. All provincial and territorial governments implemented vaccination programs for eligible girls between 2007 and 2010 [83]. Since carcinogenesis by HPV often requires decades, a decrease in cervical cancer incidence due to the vaccine will only be observable on the longer term [7]. In 2017, overall uptake of the vaccine in school-aged girls was calculated to be 67% [83]. The Government of Canada has set a target of 90% coverage by 2025 [84]. Exact implementation of the school-based vaccination programs and coverage of those programs varies between provinces and territories. In 2017, coverage of anti-HPV vaccination varied from 55.0% in the Northwest Territories to 92.0% in Newfoundland and Labrador (data was not available for Nunavut) [83].

2.3.2 Secondary prevention: Screening for cervical pre-cancers and cancers

There has been a dramatic decrease in the incidence of cervical cancer in the last 50 years, due, in large part, to screening [3, 4, 6, 9, 10]. Screening is an important method of cancer prevention in many countries, including Canada [10]. The goal of screening is to identify precancerous lesions or cancers at an early stage in order to allow for prompt

diagnosis and treatment [11]. Identifying pre-cancerous lesions can reduce the incidence of cancer. Cervical cancer is an ideal candidate for using screening as a secondary method of prevention, largely due to its long pre-invasive phase that can be identified on histopathology [11].

Systematic screening in Canada relies on the Pap test for the identification of precancerous lesions [82]. Pap tests consist of swabbing the cervix in order to collect a sample that will then undergo cytological examination. In cytology, the sample is examined under a microscope to identify morphological abnormalities that could be indicative of cervical precancers. According to the 2001 Bethesda cytological terminology, lesions are categorised as: atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells that cannot exclude HSIL (ASC-H), LSIL, HSIL, atypical glandular cells (AGC), adenocarcinoma in situ (AIS), and squamous cell carcinoma in situ (CIS)[85]. Recent guidelines by the Canadian Task Force on Preventive Health (2013) recommend routine Pap screening of asymptomatic women every three years starting at 25 years and ending at 69 years of age [3]. Cervical cytology results indicate which women may benefit from colposcopy, an examination of the cervix with a magnifying lens. During colposcopy, biopsies of suspected lesions are obtained and their histologic analysis is required for diagnosis of cervical pre-cancers or cancers [3, 86]. Different grades of lesions can be identified on histopathology, and confirmation of HSIL or worse requires treatment because over 30% of HSIL will progress to cervical cancer [24, 87-89].

Pap tests have a relatively low sensitivity and therefore need to be repeated at regular intervals in order to effectively detect cervical precancers [86]. According to a randomised controlled trial (RCT) that included over 10,000 participants, a single Pap test had a sensitivity of only 55.4% (95% CI 33.6-77.2) for the detection of CIN2 and CIN3 [90]. Hr-HPV DNA testing has an increased sensitivity, but a decreased specificity compared to Pap [86]. The same RCT reported a sensitivity of 94.6% (95% CI 84.2-100) for HPV test. They also reported a specificity of 96.8% (95% CI 96.3-97.3) for Pap test and 94.1% (95%CI 93.4-94.8) for HPV test. Infection with hr-HPV does not necessarily

equate underlying disease [17], but could instead reflect an HPV infection that will clear naturally. It is therefore important not to overly rely on HPV testing because of the risk of over-treatment of those who test positive for hr-HPV DNA, but who would not show or develop any cytological abnormalities [3, 86]. Co-testing (cytology and hr-HPV test) has been shown to increase HSIL detection with a sensitivity of 100% and a specificity of 92.5% [90].

Despite the need for caution when managing positive HPV DNA results, HPV testing could improve risk stratification when triaging women with abnormal cytology [91], ensuring that those at high-risk of progression to cervical cancer are referred for colposcopy [17]. In addition, HPV testing could improve surveillance of patients after receiving treatment for cervical pre-cancers or cancers [86, 91].

2.4 Treatment of cervical pre-cancers and its complications

2.4.1 HSIL treatment

Ablative or excisional techniques are used to remove HSIL. These techniques leave the uterus intact and do not prevent future pregnancies [24]. Excisional treatment techniques include Large Loop Excision of the Transformation Zone (LLETZ), also known as Loop Electrosurgical Excision Procedure (LEEP), Straight-Wire Excision of the Transformation Zone (SWETZ), and laser cone biopsy [86, 92, 93]. Cold-Knife Conisation (CKC) is another excisional treatment option, but often excises an unnecessarily large portion of the cervix, and carries a higher risk of complications like haemorrhage, cervical stenosis, cervical incompetence, morbidity and long-term complications in pregnancy [86]. Ablative methods of treating HSIL, like cryotherapy and laser ablation, take longer to perform and are less versatile than excisional treatment in terms of size and location of the transformation zone. An advantage of excisional techniques compared to ablative treatment is a resulting specimen that can be histologically analysed [24].Hysterectomy is not recommended as a first line treatment for HSIL [86]. In countries with adequate healthcare resources, LEEP/LLETZ is the preferred procedure for treating cervical pre-cancers or micro-invasive cancers [86].

Colposcopy with histopathology, followed by treatment with LEEP is the "gold-standard" for management and treatment of women with confirmed HSIL [86].

2.4.2 HSIL treatment complications

LSIL have a high likelihood of natural regression, and only histologically confirmed HSIL or worse, should be treated. Over-treating should be avoided since treatment has been associated with negative obstetric outcomes [86, 92, 93]. The goal of treating HSIL is to eradicate the lesion while minimising morbidity [94]. The risk of negative obstetric outcomes is of particular concern because pre-cancerous lesions are most often detected in women during their reproductive years [24].

A recent Cochrane Database Systematic Review on obstetric outcomes after treatment for intraepithelial or early invasive cervical lesions determined that the risk of overall preterm birth (earlier than 37 weeks), severe preterm birth (less than 32-34 weeks), extreme preterm birth (earlier than 28-30 weeks), and spontaneous preterm birth was increased after local cervical treatment [24]. Multiple treatments increased the risk of preterm birth even more [24]. Specifically, for LEEP/LLETZ, the relative risk of prematurity after two treatments was 2.81 (95% CI 2.33-3.39) when compared with no treatment [24]. Deeper excisions also play a role in increasing risk of preterm delivery [24]. Cervical treatment increased the risk of certain maternal outcomes like preterm premature rupture of the membranes (pPROM) earlier than 37 weeks, admission for threatened preterm birth, chorioamniotitis, and postpartum haemorrhage [24]. Finally, cervical treatment was associated with an increased risk of negative neonatal outcomes, specifically low birth weight, neonatal admission to intensive care, and perinatal mortality [24]. The authors note, however, that the increased risk of negative outcomes after small excisions compared with simply having the disease is likely to be small [24]. Overall, limiting the harms associated with treating HSIL, and the risks when multiple treatments are required is of great importance.

2.4.3 HSIL treatment failures

IARC's Technical Publication cited success rates for excisional methods at 90-94% for CKC, 91-98% for LEEP/LLETZ, and 93-96% for laser cone biopsy [86]. While it would be easy to choose the best treatment if one technique produced significantly less failure and less morbidity than others, a Cochrane Database review concluded that there is no obvious choice of technique to limit treatment failures or morbidity [23]. Follow-up remains extremely important for the identification of women who will have persistent/recurrent disease, because women who have been treated for precursors of cervical cancer have approximately 5 times the risk of cervical cancer compared to the general population [95]. A meta-analysis found that the risk of high-grade CIN after treatment was even greater for those who had positive margins or incomplete excision [95, 96]. Follow-up after treatment for HSIL usually consists of Pap smears and colposcopy at regular intervals for 1 to 2 years after treatment [86]. Currently, the American Society for Colposcopy and Cervical Pathology (ASCCP) recommends that follow-up after HSIL treatments include co-testing, which is cytology and hr-HPV testing in sequence, at 12- and 24-months post-treatment [97]. Studies have found that hr-HPV testing should be included in post-treatment follow-up to improve sensitivity of Pap tests alone [98]. If all tests are negative, the ASCCP recommends retesting in 3 years. Routine screening can then resume, but should continue for 20 years due to increased risk compared to the general population [95, 97]. However, if any test is positive, colposcopy with endocervical sampling should be performed [97]. Histological diagnosis of HSIL or worse during follow-up requires repeat excisional treatment or hysterectomy in cases where excision is not possible [97].

Despite efforts to best identify and treat women diagnosed with cervical precancers, most studies identified in a 2010 Cochrane systematic review showed that between 10% and 15% of patients will have recurrent or residual disease, or treatment failure [23]. Treatment failure is defined as identification of HSIL or higher on histology within two years of treatment. This includes both persistent and recurrent disease due to difficulties in distinguishing lesions remaining after incomplete excision from new lesions [99]. Identification of risk factors associated with treatment failure is complicated by this and

by the differing definitions of treatment failure found in the literature. A two-year follow-up period is used to identify treatment failure since most recurrent disease occurs within this period, according to a long-term follow-up of the British Columbia Cohort Study [100].

We conducted a literature review using the MEDLINE (PubMed) online database in the Fall of 2018 and then again in January 2020. All articles that studied the outcome of interest, HSIL treatment failure, were identified. We excluded articles that focused on immunosuppressed study populations, as they exhibit different characteristics (progression and persistence of disease, response to treatment) than the general population. We have categorised the potential predictors found in the literature into 4 categories: patient, behavioural, clinical and viral.

2.5 Patient risk factors

2.5.1 Age

A total of 16 studies that investigated the association between age and treatment failure were identified in the literature review. Some identified older age as a risk factor for treatment failure [32, 39, 48, 50-53]. Flannelly et al. found that the highest risk of recurrence was found in women 50 years or older [32]. In a retrospective cohort study, Lu et al. identified being 50 years or older as the only preoperative predictor of CIN3 persistence or recurrence (odds ratio (OR)=3.070, 95% CI 1.421-6.630) [39]. Wu et al. also found that, in their study, the risk of residual/recurrent CIN after treatment increased with age in univariable analysis. Though the authors did not provide an estimate of the strength of the association, the observed association between age and disease recurrence was statistically significant [51]. In turn, Verguts et al. analysed age as a continuous variable and found that as age increases, the risk of recurrent disease after treatment significantly increases [50]. In contrast, other studies have demonstrated that age does not predict recurrent disease [26, 28, 31, 34, 36, 37, 41, 43, 101]. Most of the studies that investigated age were limited to univariable analysis and did not adjust for important potential confounders such as margins. Ghaem-Maghami et al. suggested that the significance of age in other studies is due to the location of the upper limit of the lesion in

older women [34]. In older women, the lesion is more likely to extend into the endocervix. The upper limit of the lesion is not visible to the clinician in such cases, and the endocervical margin is more likely to be positive. Since positive margins could be considered to be associated with age and with HSIL treatment failure, margin status could confound the association between age and HSIL treatment failure.

2.5.2 Pregnancy and Parity

We identified two studies that investigated parity and HSIL treatment failure. Serati et al. found, upon multivariable analysis, that one or more vaginal deliveries was not significantly associated with disease recurrence [45]. Zivadinovic et al. also found that parity was not a significant independent predictor of CIN recurrence after treatment [53]. Findings from both studies may have been limited by small sample sizes and number of cases of treatment failure, with n=282 (64 cases) and n=65 (35 cases) respectively.

2.5.3 Menopause

There have been two prior studies that investigated the association between menopausal status and treatment failure. There was no significant difference in menopausal status between the women with or without recurrent disease in the studies by Verguts et al. and Kong et al. [50, 99]. It should be noted that since menopause usually occurs around 50 years of age, it may be impossible to distinguish the impact of age and menopause.

2.6 Behavioural risk factors

2.6.1 Number of sexual partners

Despite the relationship between sexual history and HPV acquisition, we did not identify any studies that investigated the association between number of sexual partners and treatment failure. Recent sexual history could provide information on a patient's risk of acquiring a new HPV after their treatment. However, we believe this risk is low since most women who are treated for HSIL are over the age of 25, who are at a lower risk of acquiring an HPV infection than teens and younger women [56, 58].

2.6.2 Smoking

Smoking has been associated with an increased risk of progression of HPV infection to HSIL and cervical cancer [102]. It is possible that this risk factor is also associated with treatment failure. We found a total of 3 studies that estimated the association between treatment failure and smoking. In a British study by Acladious et al., current smokers had 3.17 (95% CI 1.68-5.98) the odds of treatment failure of never smokers [25]. This study defined treatment failure as histological confirmation of any grade of lesion within two years of treatment [25]. In another study by Zivadinovic et al., smoking in conjunction with HPV positivity was associated with significantly higher grade of recurrent disease (CIN3 and microinvasive carcinoma compared to CIN1-2) [53]. In the small study by Verguts et al., however, a link between current smoking status and recurrence could not be examined as there were 6 cases of treatment failure, of whom none smoked more than a pack per day [50]

2.6.3 Hormonal contraceptive use

Although oral contraceptive use has been associated with an increased risk of progression to high grade lesions [102] and with cervical cancer [103, 104], only one study has investigated the association between the use of oral contraceptives and recurrent disease after treatment. Frega et al. found that there was no statistically significant difference in recurrence rates between current users of oral contraceptives and never users [33].

2.7 Clinical risk factors

2.7.1 Margins on conisation

Many studies have shown that positive/involved excision margins are associated with an increased risk of residual disease [26, 27, 32, 34, 38-40, 43, 49, 87, 96, 99, 101, 105]. This is due to remaining lesion that was not successfully excised, which can depend on size, grade and depth of the lesion [34]. In the study by Lu et al., those with positive margins had 2.972 (95% CI 1.401-6.281) times the odds of persistent or recurrent disease [39]. In univariate analysis, Ryu et al. found that margins were the only significant predictor of residual/recurrent disease (OR=39.079, 95% CI 4.399-347.184) [43]. Their results were limited by the small number of cases of recurrence within their study

population (12 cases (6.6%) in 183 participants). Using multivariate analysis, Ghaem-Maghami et al. identified “completeness of excision” as a risk factor for disease after treatment, with incomplete excisions/positive margins significantly associated with high-grade post-treatment disease [34]. Lubrano et al. also identified positive resection margins as a significant predictor for recurrence (OR=2.7) [40]. The evidence for positive margins is by far the most conclusive of all of the potential predictors of treatment failure.

2.7.2 Number of passes

In the case of deep cervical lesions, surgeons will sometimes opt to perform a second or multiple passes with a small loop instead of performing one deep and large excision with a large loop. Performing multiple passes can lead to the identification of positive margins on the LEEP specimen, even if the entire lesion was successfully removed and the overall margins were negative. The use of the second-pass technique (also called “apical excision,” “top-hat excision”) has been shown to significantly reduce endocervical margin positivity (OR=0.36, 95% CI 0.21-0.63)[106]. In the same study, the second-pass technique was associated with a decrease in treatment failure, though not statistically significant (OR=0.62, 95% CI 0.29-1.32). Though the aforementioned ORs are the results of multivariate analyses, the authors did not provide information on adjustment variables.

2.7.3 Grade of lesion on conisation

Higher grade of lesion upon treatment is also associated with treatment failure [34, 46, 107]. True HSIL (positive for the p16 biomarker) likely carries a higher risk of treatment failure than LSIL. In a study by Ghaem-Maghami et al., multivariable analysis showed that grade of disease was significantly associated with recurrent CIN2+ [34]. A study by Ryu et al. showed that there did not seem to be a difference in the recurrence of disease evaluated as CIN2 or CIN3 upon conisation, but that these were more likely to cause recurrence than lesions evaluated as CIN1 [43]. There are inconsistencies on the significance of this variable as a predictor for recurrent disease, with Orbo et al., Kang et al., Bae et al. and Torné et al. finding no significant association [28, 41, 49, 101]. The inconsistencies could be explained by the fact that most studies relied on the older CIN

classification, without identification of the p16 biomarker. Without the p16 biomarker, CIN2 should be treated as LSIL and not as true HSIL. The p16 biomarker can also be used to differentiate between true HSIL and benign mimics [108]. Therefore, in studies that did not confirm high-grade lesions by identifying the p16 biomarker, the effect of having a higher grade of lesion could have been dampened by the misclassification of some low grade of lesions.

2.7.4 Depth of excision

Ghaem-Maghami et al. identified the depth of excision as a predictor for high-grade lesions after treatment [34]. When analysed as a continuous variable in multivariable analysis, it was just barely significant, but depth became much more significant when only lesions whose upper limit was located in the endocervical canal were included. They concluded that depth of treatment was an important factor in guaranteeing complete excision of the lesion, and therefore in reducing recurrent disease, but depth must be based on location of the lesion. This conclusion was limited by the fact that the treatment policy recommended different depths of excision for different locations of lesions. In addition, the investigators did not provide estimates of the strength of the association between depth of excision and the presence of high-grade lesions post-treatment. On the other hand, in articles by Lu et al. and by Fan et al., no significant difference in depth of cone biopsy was observed between those with recurrent disease and those with no persistent/recurrent disease (OR=1.73, 95% CI 0.761-3.932 for depths of ≤10mm and OR=1.370, 95% CI 0.560-3.335 for 11-15mm in reference to ≥16mm in multivariate analysis) [31, 39].

2.8 Viral risk factors

2.8.1 Genotype

It is possible that the HPV genotypes that are most likely to persist and to cause malignant transformation are also most likely to cause treatment failure. Genotype was sometimes identified on the treatment specimen, and other times was determined over the course of follow-up (persistent HPV type). A total of 6 studies investigated the association of persistence of certain genotypes after treatment and treatment failure. Fan

et al. found that persistence of HPV infection post-conization was an independent predictor of residual/recurrent CIN when compared to participants whose HPV infection did not persist after treatment [31]. Kang et al.'s retrospective cohort of 672 women found that persistent HPV16 and HPV18 after LEEP led to a significantly higher rate of recurrent CIN2-3 in univariable analysis in comparison to those with other persistent HPV types. In their study, HPV16 contributed 54.1% (20/37) and HPV18 contributed 16.2% (6/37) of recurrent disease [101]. Lindroth et al. identified recurrent HSIL in 15% (11/71) of the women with persistent hr-HPV (hr-HPV before and after treatment) in their study compared to 0.27% (1/375) of those without persistent hr-HPV [109]. They reported that persistence of type-specific hr-HPV was significantly associated with recurrent or residual HSIL (OR=58.1, 95% CI 7.4-457). The OR was likely impacted by an analysis with a category containing only one participant, making for a wide CI and resulting in a very weak inference. Bae et al. also found that persistence of the same type of HPV after treatment was significantly predictive of treatment failure compared to those who did not have persistence of the same type [28]. Bruno et al. highlighted the importance of HPV testing post-treatment in order to most effectively identify and manage cases of recurrent CIN2+ after treatment. In their study, women with persistent HPV16 infection had 11.33 times the risk of recurrent disease compared to those with other types of HPV post-treatment and the association was statistically significant [29]. In addition, 100% of women in their study who had HPV16 and positive margins developed recurrent high-grade disease. However, when comparing infection with HPV16 and/or 18 to infection with other HPV types, genotype was not a predictor of recurrent disease in Ryu et al.'s study (OR=0.590, 95% CI 0.124-2.860) [43]. When it comes to persistence of HPV, those with persistent/recurrent disease will all necessarily have some type of HPV infection. The measure of persistence can be considered less of a potential predictor and more of a measure of treatment failure itself.

We identified only 2 studies that estimated the association between pre-treatment HPV type and treatment failure. We considered this a more useful perspective for identifying predictors of treatment failure since it would allow for risk stratification upon treatment and before treatment failure. In a study limited to LEEP with negative margins, Wu et al.

identified a significant association between single-type pre-conisation infections with HPV types 16, 18, 33 and 45 and biopsy proven residual/recurrent disease and no significant association with HPV31, 52, 53 and 58 [51]. However, it is unclear what their comparison group was for this analysis. In their study, infection with multiple hr-HPV types compared to a single hr-HPV type was significantly associated with the highest rate of recurrence [51]. Gök et al. found that pre-LEEP HPV16 specifically was significantly associated with post-treatment CIN3 compared to other hr-HPV types [54].

2.8.2 Variant

Genetic variants occur within HPV genotypes, characterised by less that 2% nucleotide difference in the L1 gene and less than 5% difference in less conserved regions of the viral genome like the LCR [59]. Variants can be classified by phylogeny and by lineage and can be grouped as prototype (the first ever identified sequence of each type), prototype-like (only one point-mutation compared to the prototype), or non-prototype-like (more than one mutation compared to the prototype). Genomic mutations are more likely to persist through time and replication cycles if they confer a pathogenic advantage to the virus. Variants of HPV types could therefore have different levels of pathogenicity due to their genetic diversification [59]. In fact, studies have shown that some variants have an increased capacity to persist and to progress to cervical neoplasia when compared with other variants of the same type. Some case-control studies have shown that non-European variants of HPV16 have increased pathogenicity or ability to persist when compared with the genotype's European variants [110-113]. The study by Xi et al. found that those who had non-prototype-like variants of HPV16 had 6.5 (95% CI 1.6-27.2) times the risk of developing CIN2-3 of those with prototype-like variants [113]. A Korean study showed that a common variant of the HPV16 E7 protein was associated with the majority of cervical carcinomas [47]. Another Korean study found that distribution of HPV variants within the population could be associated with the incidence of cervical neoplasia [42]. Since variants are associated with persistence and progression of the infection, it is of interest to determine whether they are also linked to treatment failures. No studies were identified that examined the association between genetic variants of HPV and HSIL treatment failure. Small sample size did not allow for analysis

of the association between variants and HSIL treatment failure in the studies we identified.

2.8.3 Viral load

We reviewed 10 studies that analysed the association between HPV viral load and treatment failure [26, 28, 35, 37, 43, 47, 99, 101, 114, 115]. All 10 studies had a rate of treatment failure comparable with the literature [23], ranging from 5.5% to 31%. All used semi-quantitative Hybrid Capture 2 (HC2) with relative light units (RLU), to quantify viral load. All studies analysed the variable as dichotomous, except Gosvig et al. who used a continuous variable for infections with a single type of HPV [35]. For those who used a binary variable, choice of cut-off used to distinguish low from high viral load was data-driven with the exception of Kong et al. who plotted all RLU values and performed receiver operating characteristic (ROC) curve analysis to maximise sensitivity (88.2%) and specificity (98.3%) [99]. Bae et al., Ryu et al. and Park et al. defined high viral load as ≥100RLU [28, 43, 115], whereas Kong et al. used ≥1.16RLU [99], Song et al. and Mo et al. used ≥500RLU [47, 114], Jeong et al. used ≥1000 RLU for pre-treatment load and both ≥1000RLU and ≥100 RLU for post-treatment load [37], Alonso et al. used ≥1000 RLU [26], and Kang et al. used many different thresholds ≥1, ≥10, ≥100, ≥1000 RLU[101]. The inconsistencies in analysis of the variable greatly reduced comparability of results.

Most studies used exclusively viral load pre-treatment as a possible predictor of treatment failure [35, 37, 43, 47, 101, 114, 115]. Studies by Bae et al., Alonso et al., Jeong et al. and Kong et al. analysed both pre- and post-treatment viral load [26, 28, 37, 99]. However, similar to genotype, post-treatment viral load could be considered more of an indicator or measurement of treatment failure rather than a predictor of the outcome. In addition, treatment failure was defined in many different ways. Seven of the ten articles required histological confirmation of low or high-grade lesions [26, 28, 37, 43, 99, 101, 115], of which, Park et al. required histological confirmation in combination with identification of high-risk HPV by HC2. Among the three articles without histological confirmation of treatment failure, Gosvig et al. required type-specific persistence of HPV

to identify treatment failure [35], Song et al. required only persistence of any HPV DNA during follow-up [47], and Mo et al. defined treatment failure as presence of high-risk HPV DNA or positive diagnosis on cytology [114].

There was no consensus on significance of viral load as a risk factor for treatment failure. Bae et al. concluded that pre-treatment viral load as measured by HC2 was not associated with persistent/recurrent disease, but a high viral load at 6 months post-treatment was significantly associated with treatment failure (RR = 5.88) [28]. In this study, persistence of the same HPV type after treatment was also a significant predictor of treatment failure [28]. Gosvig et al. also found that high viral load at 4-6 months post-treatment was predictive of HPV persistence (OR=1.36, 95% CI 1.13-1.63) [35]. Alonso et al. concluded by univariable analysis that high viral load both pre- (OR=1.226) and post-treatment (OR=1.582) was significantly associated with recurrence of disease, while multivariable analysis only showed significance for post-treatment high viral load (OR=1.44) [26]. Park et al. concluded, by multivariable analysis, that pre-conisation high viral load was a significant risk factor for both viral persistence after treatment and recurrence of histological abnormalities (OR=5.748) [115]. Song et al.'s study concluded that high viral load pre-treatment is the only significant risk factor for persistence of HPV infection in those with negative margins [47]. Mo et al. observed a linear relationship between pre-treatment viral loads >500 RLU and presence of disease on HC2 and/or cytology after treatment, with no relationship between post-treatment viral load and recurrence [114]. Jeong et al. observed significantly higher pre-treatment viral loads (continuous analysis, no estimate of the strength of the association) and post-treatment loads (OR=9.3, 95%CI 2.2-38.2 for ≥100RLU compared to <100RLU) in recurrent cases when compared with no recurrence [37]. Studies by Ryu et al., Kong et al. and Kang et al. all concluded that viral load was not significantly associated with treatment failure, however it was defined in the respective studies [43, 99, 101].

2.9 Summary of limitations and expected impact of study

There was no consensus on a standard definition for treatment failure [56]. This was, in part, due to difficulties in distinguishing between persistent and recurrent disease [99].

Some studies focused solely on the presence of any HPV DNA detected by HC2 [47], others focused on persistence of the same genotype [35], some required the presence of cytological abnormalities [114], while others used histological confirmation of varying grades of lesions [26, 28, 37, 43, 99, 101, 115] to confirm treatment failure. Analysis specifically of predictors of HSIL or worse within two years of the first treatment was therefore limited.

Many studies were limited to a small sample size and a very small number of cases of treatment failure. Of the 30 studies we found that estimated the association between different risk factors and treatment failure, 17 had less than 50 cases of treatment failure or persistent/recurrent disease and 23 had less than 100 cases. There was often not enough power to draw solid conclusions from the associations identified, only suggestions. Estimated associations were extremely inconsistent from one study to the next, except for margins. In addition, we did not find any literature that investigated the association between HPV variant and HSIL treatment failure.

Most studies investigated only two or three risk factors for HSIL treatment failure. Factors that could be related to treatment failure are often correlated with each other [34] and this must be taken into consideration in the analysis of predictive variables. Some studies did not consider confounding variables in their analysis, or at least did not provide details of confounders considered [26, 37, 43, 47, 94, 99, 101, 114]. This could have created biased results regarding strength of association between predictors and treatment failure.

Our study is expected to provide additional and original information on the identification of predictors of treatment failure, with the goal of reducing overall harms linked to cervical cancer screening and treatment. The Cochrane Colposcopy and Cytopathology Collaborative Group described the lack of consensus and varying recommendations on surveillance procedure after treatment for HSIL [116]. Identifying predictors of HSIL treatment failure could help clarify and standardise follow-up procedures. This could decrease the number of women requiring re-treatment and lead to a reduction of poor

obstetric outcomes associated with multiple treatments [24]. Specifically, the identification of risk factors which we consider to be modifiable would help clinicians to provide informed and specific recommendations upon treatment. Of the risk factors studied here, we considered smoking, condom use and method of contraception to be modifiable by the patient. The analysis of clinical risk factors such as margins and number of passes could influence treatment protocols and may be considered to be modifiable by the clinician. The identification of non-modifiable predictors, including viral predictors, could allow for better risk-stratification at the time of treatment. In turn, this could allow for a simplified post-treatment monitoring process for women at an extremely low risk of treatment failure [87]. Analysis of viral risk factors, especially genotype, could have implications for our understanding of biomolecular pathways of viral carcinogenesis and for planification of vaccination programs and for follow-up tests.

Chapter 3 – Objectives

1. To identify predictors of HSIL treatment failure.
2. To estimate the effects of HPV16 and HPV18 viral load and variants on the risk of HSIL treatment failure.

Chapter 4 – Methods

4.1 Source Population and Study Design

4.1.1 Overview of the CoHIPP Study

This project used questionnaire data and clinical specimens collected in the Colposcopy vs. HPV testing to identify persistent precancers post treatment (CoHIPP) study, a RCT (ClinicalTrials.gov Identifier: NCT01051895) designed to compare the sensitivity of routine follow-up to HPV-testing with HC2 for the identification of treatment failure following excisional treatment for HSIL. Over a three-year recruitment period from January 2010 to March 2013, 2,167 women from 13 treatment centers across Canada joined the CoHIPP cohort. In order to be eligible for CoHIPP, women had to be 18 years of age or older (19 or older in British Columbia, Nova Scotia and Newfoundland), had to understand English or French, had to fully comprehend the risks associated with the study and alternative treatment options, had to have an HSIL diagnosis that was confirmed by histopathology of the excised tissue, and had to have voluntarily consented by signing the Informed Consent Form. Women were not eligible for CoHIPP if they had been previously treated for a cervical pre-cancer or cancer, were immunosuppressed or had an immunodeficiency, had a planned hysterectomy, had received immunosuppressive therapy in the three months prior to enrollment, had received corticosteroid treatment in the two weeks prior to enrollment, had received two or more courses of corticosteroids orally or parenterally lasting at least one week in duration in the year prior to enrollment, or had a strong probability of loss to follow-up. Of the women who were approached, 85% were eligible and consented to participate. The CoHIPP cohort should be broadly similar to the population of Canadian women who underwent treatment for HSIL from 2010 to 2013 thanks to the high rate of participation amongst women treated for HSIL across Canada.

Participants all underwent excisional treatment for HSIL, 98% of which was performed using LEEP. They were then followed for two years post-treatment, with 2 visits at six-month intervals and a last visit 12 months after, for a total of 3 visits post treatment. **Figure 1** is a schema of the follow-up procedure during CoHIPP.

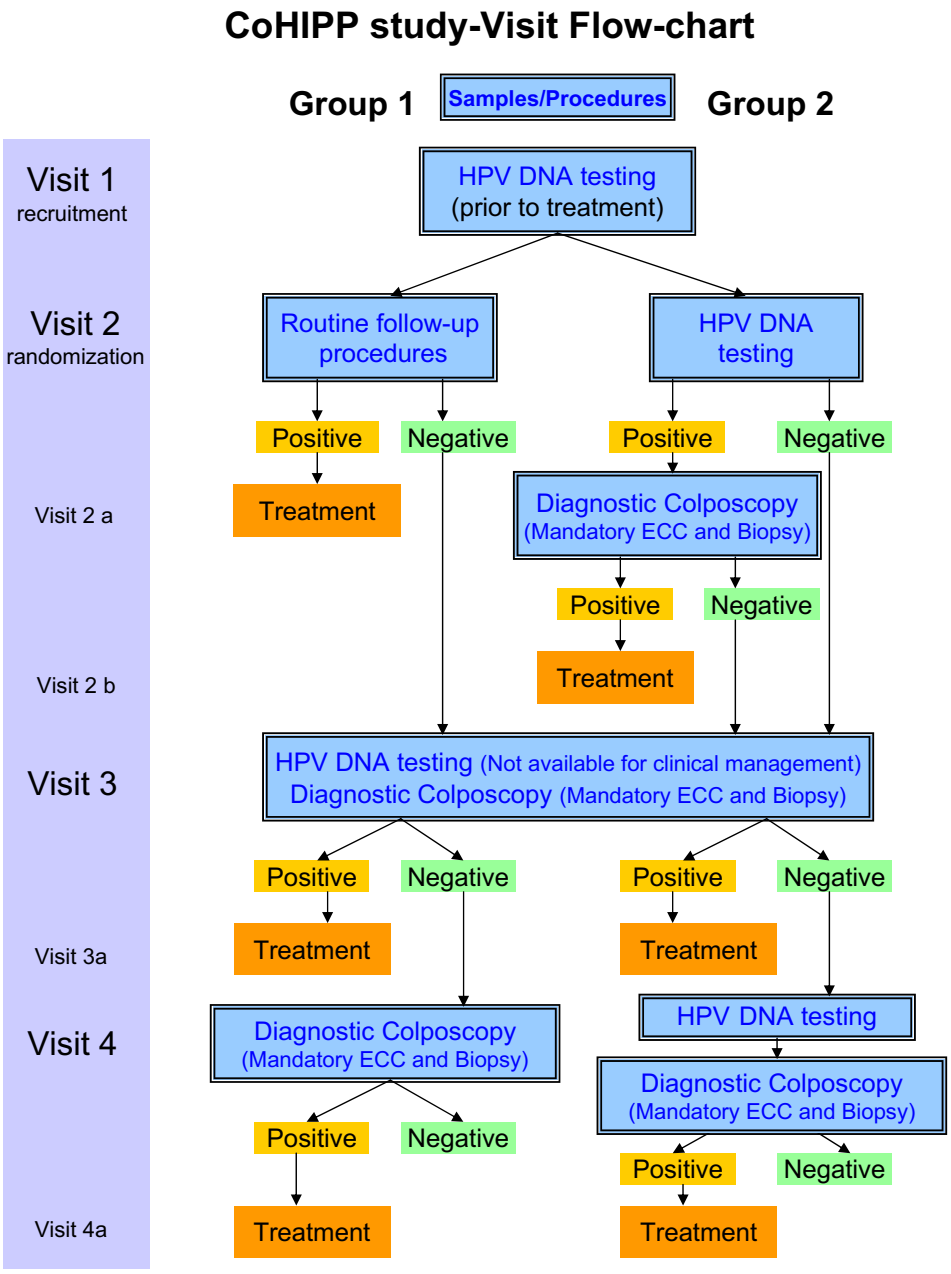

Figure 4.1. Flowchart CoHIPP follow-up protocol. After excisional treatment for HSIL, CoHIPP participants were randomised to either routine follow-up or to HPV DNA testing with Hybrid Capture 2 at six-month intervals. Those who had a positive diagnosis of HSIL or worse (labeled as "positive" in the flow chart) at any point during follow-up were identified as cases of treatment failure. (ECC: Endocervical curettage)

Self-administered questionnaires in the presence of a nurse were used to collect data on the patient, and on their behavioural and gynecological history at follow-up visits 2 through 4. The medical staff provided data on the clinical procedure. Immediately prior to treatment, cervical specimens were banked for laboratory testing. The cells were obtained using a cytobrush and were preserved in PreservCyt (Hologic Inc), a liquid medium used to preserve cells for cytologic diagnosis. Specimens were kept at 4° Celsius when possible. Remaining specimens after HC2 were centrifuged and resuspended in TE buffer solution before freezing. Specimens were placed back at 4° degrees Celsius for this project.

4.1.2 Ethical considerations

The CoHIPP study received approval from Le comité d'éthique de la recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) and all other participating treatment centers across Canada. Participants were informed of possible risks associated with the study, as well as alternative treatment options. All participants in CoHIPP provided free and informed consent. In addition, participants consented separately for their data and specimens to be used for additional studies on HPV and cervical precancers. All patient information accessed in order to proceed with this project was anonymous.

4.1.3 Study Design of this Project

The study design used to assess the association between HSIL treatment failure and potential patient, behavioural, clinical and viral risk factors was a nested case-control within the CoHIPP cohort. Assessment and analysis of the exposures of the entire cohort would be expensive in terms of time and money. A nested case-control design allowed for efficient use of resources, especially for the analysis of biological specimens since laboratory tests can be very costly [117, 118]. Additional viral load quantification and variant sequencing only had to be performed on those selected for the case-control study.

4.1.4 Cases and Controls

Cases were defined as all participants within the CoHIPP cohort who had biopsy proven HSIL or worse within the two-year follow-up post-treatment. The date of the biopsy-

proven treatment failure was considered as the "index" date or time 0 in analysis. Over the course of the study, 101 cases were identified.

Controls were defined as those within the CoHIPP cohort who did not experience treatment failure and who had not been censored at the time of matching, the "index" date. In order to control for the time between treatment and recurrent HSIL diagnosis, incidence density sampling was used. A control could have therefore still become a case after the date that they were "sampled" as a control. The same participant could have also been selected as a control for more than one case. Incidence-density or "risk-set" sampling allowed for the controls to represent the distribution of the exposure over the source-population's person-time at risk. This reduced bias in the estimation of the relative risk by controlling for confounding caused by differing follow-up times [119]. Controls were individually matched to cases 1:1 by treatment center and by visit, which had to occur within three months of identification of the case. Of the 101 matched controls in our study, one control later became a case and nine controls were matched to two different cases.

4.2 Data Collection

Most of our data came from information already collected and available through the CoHIPP study. We performed additional laboratory analyses in order to investigate our second objective.

4.2.1 Viral load

Viral load was measured by quantitative real time PCR with a Light Cycler PCR and detection system (Roche Molecular Systems) for those who had HPV16 or HPV18 detected in the genotyping by Linear Array. Each sample underwent the amplification reaction three times, first as a control to ensure that the reaction was not affected by inhibitors, and then twice in order to calculate average measurements for the quantity of cellular β-globin and number of copies of HPV DNA. A ratio between the number of β-globin and the number of copies of HPV, and a logarithmic conversion are used to

determine the number of HPV copies per cell. The entire process was performed by a highly trained laboratory technician.

4.2.2 Variant sequencing

Specimens that had either HPV16 or HPV18 were purified by MasterPure. We first confirmed that the samples were positive for HPV16 or HPV18 by PCR with GeneAmp PCR System 9700 with hybridisation to the appropriate primer for each genotype. In order to identify different variants of HPV, the LCR region had to be isolated for sequencing. Purification of the LCR regions was done using QIAquick® PCR Purification Kit and migrated on agarose gel to ensure the presence of the specific DNA segment prior to sequencing. Purified LCR regions for HPV16 and HPV18 were then amplified using GeneAmp PCR System 9700. To ensure that the PCR was successful for each plaque, a negative control (H_2O) and a positive control (pHPV16, pHPV18) were used. Upon gel electrophoresis, a DNA ladder containing the specific migration distances for HPV16-LCR and HVP18-LCR was included to ensure that specific, strong bands of DNA were present for the respective samples. The samples were then sent to Genome Quebec for Sanger sequencing, along with primers for forward and reverse sequencing of each sample. The results were then verified using Chromas to compare the identified mutations in the forward and reverse sequences. The first ever identified sequence of each type's LCR is called the prototype. For HPV16 and HPV18, the prototypes are K02718.1and AY262282 respectively [120]. Our results were compared to the prototype sequences.

4.3 Variable Definitions and Measurements

Age: The exact age of each participant was calculated at the time of treatment, up to the ninth decimal. The variable was approximately normally distributed and was analysed as a continuous variable. We also explored the use of 40 years and 50 years of age as thresholds for dichotomising age since this was often done in the identified literature.

Parity: The questionnaire at Visit 2 included questions on the total number of pregnancies, followed by a breakdown of all of the different types of pregnancies

including full-term, pre-term, ectopic, spontaneous abortion, and planned abortion. Number of ectopic pregnancies, spontaneous abortions and planned abortions were very low in both cases and controls and could not be analysed as their own categories. It was of interest to exclusively look at births that passed through the cervix and caused a physiological change in the cervix when looking at the risk of recurrent cervical high-grade lesions as the outcome. In addition, hormonal changes occurring at 24 weeks of pregnancy could be a risk factor for treatment failure. The pregnancy data was therefore redefined as parity, which was a sum of the number of full-term and pre-term deliveries for each participant. The parity of this cohort was relatively low and non-normally distributed, so the variable was examined as three categories, comparing parity of 0, 1, and 2 or more.

Smoking: Eldridge et al. recently demonstrated that smoking may reduce one's immunity to infection with HPV16. In their study, being a current smoker was inversely proportional to the presence of antibodies against HPV16 [121]. It could be possible that the decreased immunity of heavier smokers also correlates to a higher incidence of treatment failure. The baseline questionnaire asked participants if they were never, ex-, or current smokers. Considering the relevant exposure window, the variable was converted to dichotomous, comparing non-smokers to current smokers at Visit 2.

Number of sexual partners in the last year: The questionnaire assessed the patient's number of sexual partners in the last year, including any current partner. Based on the distribution of the variable, with most of the study group having one current partner, the variable was dichotomised into 0 or 1 partner and 2 or more partners.

Condom use in the last year: Participants were asked how frequently they had used condoms for vaginal intercourse in the last year. The options were never (0%), rarely (1-25%), some of the time (26-75%), most of the time (75-99%) or always (100%). Most of the participants never used condoms, leaving very few participants in each of the categories from rarely through always. This was likely due to the fact that the group was

largely monogamous. The variable had to be dichotomised as a yes/no variable where yes=rarely + some of the time + most of the time + always and no=never.

Method of contraception: The method of contraception was analysed as dichotomous comparing hormonal methods to non-hormonal methods of contraception and their association with treatment failure. Hormonal types of contraception included oral contraceptives, hormonal IUD, injectable contraceptive, hormonal patch, and vaginal ring, or any non-hormonal method in combination with a hormonal method. Non-hormonal contraception included sterilization, the copper IUD, diaphragm, condoms, cervical cap and no contraception.

Margins: This described the presence of involved, or positive margins identified on the LEEP specimen. Positive margins signified that the lesion extended right to the edge of the excised tissue, meaning that the entire lesion may not have been successfully removed and that there could be remaining disease in the patient. The variable was analysed as dichotomous comparing negative margins to at least one positive margin.

Number of passes: Each surgeon decided the number of times the electrical loop was passed through the cervix to complete treatment. The variable was analysed as a dichotomous variable, comparing one pass (meaning the lesion and surrounding tissue were excised in just one block), to two or more passes.

Diagnosis on LEEP (conisation specimen): The diagnosis on conisation was defined as the severity of lesion identified in histopathology. The goal was to determine whether there was a difference in recurrence between those who were treated for CIN2 and those who were treated for the more advanced CIN3-CIS-AIS. Sometimes, the pathologist did not specify the exact type of high-grade lesion identified on the LEEP specimen. The diagnosis on LEEP was analysed as a categorical variable, comparing CIN2, CIN3-CIS-AIS and HSIL of unspecified severity.

Anti-HPV vaccination: Data on vaccination was collected via the questionnaire at Visit 2. Participants were asked whether or not they had previously received an anti-HPV vaccine, the name of the vaccine, the number of doses received, and time since the last dose. Based on the years of recruitment and the age of the participants, relatively few participants were vaccinated. Moreover, many were likely vaccinated after being infected with HPV and those who were vaccinated may have been recommended the vaccination due to higher risk behaviours, which would bias the association between anti-HPV vaccination and treatment failure. The association between prophylactic anti-HPV vaccination and treatment failure was therefore not analysed.

Genotype: Genotype was identified using Linear Array from Roche Diagnostics during a previous study of CoHIPP specimens by Nadège Andréa Zanré. Linear Array allowed for identification of 37 types of HPV DNA: 6, 11, 16, 18, 26, 31, 33, 34 (formerly 64 [122]), 35, 39, 40, 42, 44 (formerly 55 [122]), 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 71, 72, 73, 81, 82, 83, 84 and 89 [123]. Because of the clinical significance of HPV16 and HPV18, a binary variable was used to compare the association of treatment failure with the presence of HPV16 and/or HPV18 compared with all other types of HPV.

Number of types of HPV: Number of types was the sum of number of types detected per participant by Linear Array. Since infection with multiple types could be associated with treatment failure, number of types was analysed as dichotomous, comparing those with only one type to those with two or more types of HPV.

Viral load: Logarithmic transformation of the distribution of viral load created a variable with approximately normal distribution for HPV16. We analysed the log-transformed variable as continuous using Student's T-test in order to compare the mean HPV16 viral load between cases and controls. Logarithmic transformation did not normalise the distribution of HPV18 viral load and we therefore used Wilcoxon-Mann-Whitney U-test to compare cases and controls. We also explored dichotomising the variable, as most prior studies had. First, viral load was dichotomised into low and high loads according to the threshold that was determined to be most predictive of HSIL in HPV infected women

[124]. For both types, this was 0.11 viral copies per cell. Since this threshold may not have been predictive in an HSIL population, we also explored dichotomising viral load with thresholds of 0.22 copies/cell (doubling the threshold of 0.11 copies/cell) and the median of each type. Since only a subset of our study population had HPV16 or HPV18, and since pairs were not matched on genotype, we had to break the matching for our analysis of viral load. We performed unconditional logistic regression analysis on HPV16 viral load, but did not have a sufficient number of participants with HPV18 to do so.

Variant: The sequenced samples were classified as prototype if they had a 100% identity with the sequence of the prototype for their specific HPV type, as prototype-like if they had only one point mutation difference compared to the prototype and as non-prototype-like if they had more than one mutation compared to the prototype. We also estimated the association between specific variations of the HPV16 and HPV18 LCRs with HSIL treatment failure where frequencies within our population allowed. As previously explained for viral load, we had to break the matching for our analysis of HPV16 and HPV18 variants.

4.4 Statistical Analysis

All statistical analyses were conducted using the statistical analysis software SAS version 9.4 for Windows (SAS Institute, Cary Inc.).

4.4.1 Descriptive Statistics

Initially, descriptive statistics were estimated for all the risk factors of interest. This was done in order to observe the distribution of patient, behavioural, clinical and viral variables amongst both cases and controls. All variables were categorical, except for age which was continuous. The descriptive characteristics used for age were the median, the range, and the interquartile range in order to assure that there were no outliers who needed to be eliminated from the data set. For categorical variables, we included the crude number and proportion of cases or controls per category.

4.4.2 First objective

We used conditional logistic regression to estimate the association between potential predictors and HSIL treatment failure, since that is the statistical method of choice for nested case-control studies with matching [119]. ORs and 95% CI were estimated in separate models for each predictor.

We developed distinct models for each potential predictor. We first performed a separate univariable conditional logistic regression for each predictor. We then conducted a separate multivariable conditional logistic regression for each predictor. In order to identify appropriate confounders to include in each model, we used a correlation matrix between all predictor variables. Since all predictors could be associated with treatment failure, we then selected confounders for each model based on their correlation to the predictor of interest.

In order to proceed with an adjusted analysis of our predictors, we had to consider which confounders to include in the multivariable regression model for each potential predictor. Since each potential predictor had its own multivariable conditional logistic regression model, different confounders could be included for each variable of interest based on specific correlations. The confounders chosen to be included in each multivariable model had to be associated with the independent variable of interest and with HSIL treatment failure, and not be in the causal pathway. Due to the limited literature on predictors of treatment failure, there was an absence of known causal associations with HSIL treatment failure. Therefore, we could not use Directed Acyclic Graphs (DAG) to identify confounders for which to adjust. However, the essence of our study was that we suspected that any one of our variables of interest could be strongly associated with treatment failure. Any variables that were also associated to the predictor of interest in each model could therefore be considered a potential confounder. According to our analysis of the literature [34], we suspected that many of our predictors could be highly correlated. Neglecting to account for these correlations could have led to confounding bias.

We identified potential confounders between each predictor of interest and treatment failure using a correlation matrix of all the predictor variables (**Table 1**). We estimated Spearman correlations, as is appropriate for ordinal and dichotomised variables. For each predictor, we considered the correlation strong enough to be included in the model if the absolute value of the Spearman correlation coefficient was ≥0.2, as done previously [125]. If we considered that the correlated variables were in the causal pathway between the predictor of interest and HSIL treatment failure, we did not include them in the model. Age is an important predictor of treatment failure, as evidenced by previous studies and by our initial descriptive statistics, and it was therefore included as a confounder in all multivariable models, regardless of the correlation with the predictor of interest. An advantage of the method chosen to identify confounders is that it identified correlations specific to our study population, and without relying on previously identified causal associations.

We wanted to maintain the integrity of each variable's distribution as best as possible for the estimation of the Spearman correlations. We therefore wanted to include predictors in their "most continuous" form. Age was a continuous variable and was kept as such. Variables that could be considered as ordinal were included in the correlation analysis in that form, even if they had been dichotomised for the logistic regression analysis. For instance, the condom use variable, which was transformed to "ever vs. never" for its logistic regression models, was included in its original five-category ordinal form (never, rarely, sometimes, most of the time, always) for the Spearman correlations. Parity, number of sex partners, number of types and number of passes were included as discrete continuous variables. On the other hand, variables whose categories were not ordinal were included as dichotomous. The dichotomous variables in the correlation matrix included current smoking status, method of contraception, diagnosis on LEEP, genotype and margins. When included in the multivariable models as confounders, variables were included in their "most continuous" form.

Table 4.1. Spearman correlation coefficients for predictor variables

	Age	Parity	Current smoking status	Number of sex partners	Condom use	Method of contraception	Diagnosis on LEEP	Genotype	Number of types	Margins	Number of passes
Age	1.00	0.48	-0.01	-0.25	-0.22	-0.26	0.13	0.17	-0.30	0.06	0.01
Parity	0.48	1.00	0.09	-0.23	-0.28	-0.17	0.12	0.01	-0.19	0.10	0.16
Current smoking status	-0.01	0.09	1.00	0.07	0.07	-0.16	0.00	-0.10	0.03	0.00	0.22
Number of sex partners	-0.25	-0.23	0.07	1.00	0.34	0.09	-0.04	-0.04	0.19	-0.24	-0.12
Condom use	-0.22	-0.28	0.07	0.34	1.00	0.02	0.10	-0.11	0.06	-0.05	0.15
Method of contraception	-0.26	-0.17	-0.16	0.09	0.02	1.00	0.05	0.04	0.16	-0.10	0.04
Diagnosis on LEEP	0.13	0.12	0.00	-0.04	0.10	0.05	1.00	-0.06	0.01	0.14	0.12
Genotype	0.17	0.01	-0.10	-0.04	-0.11	0.04	-0.06	1.00	-0.23	-0.13	-0.07
Number of types	-0.30	-0.19	0.03	0.19	0.06	0.16	0.01	-0.23	1.00	-0.10	-0.05
Margins	0.06	0.10	0.00	-0.24	-0.05	-0.10	0.14	-0.13	-0.10	1.00	0.22
Number of passes	0.01	0.16	0.22	-0.12	0.15	0.04	0.12	-0.07	-0.05	0.22	1.00

Values in red are those with an absolute value ≥0.2, which were included as confounding variables in the predictor models for each individual variable.

Overall, there was little missing data for the variables of interest. All variables had less than 5% missing values except condom use, method of contraception and margins, which had 11.88% (14 cases; 10 controls), 11.39% (12 cases; 11 controls) and 20.79% (23 cases; 19 controls) missing values respectively. However, it was extremely important to avoid the loss of pairs due to missing values within confounding variables in order to maintain sample size in the multivariable analyses. Imputation of the most frequent value was used to replace missing values within variables included as confounders. To ensure that this did not overly bias the adjusted ORs in models, especially where condom use, method of contraception, or margins were included as confounders, ORs were generated with and without imputation for comparison, as a sensitivity analysis. Matched pairs with missing values were dropped from the analyses without imputation.

4.4.3 Second Objective

This second analysis included the results of our additional laboratory analyses of viral load and variant. Since infection with HPV16 or HPV18 was not part of the inclusion criteria for this study, not all of our study population was included in our analysis of HPV16 and HPV18 viral load and variants. Of the 101 cases and 101 matched controls, 134 (73 cases and 61 controls) had HPV16 and 11 (6 cases and 5 controls) had HPV18. We estimated descriptive statistics for these variables and performed unconditional logistic regression where numbers allowed. Since case-control pairs were not matched based on genotype, and frequently only one member of the pair had HPV16 and/or HPV18, the original matching had to be broken to allow for this analysis. Where multivariable analysis was possible, we adjusted for age.

4.4.4 Additional analyses

We hypothesised that smoking status could modify the oncogenic effect of HPV16 and/or HPV18, where the risk of treatment failure was further increased among current smokers. We also hypothesised that the association between HPV16 and/or HPV18 and treatment failure could be higher among those having positive margins. We created interaction terms Genotype x Smoking Status and Genotype x Margins. For both interaction variables, we performed a conditional logistic regression analysis to estimate the association with treatment failure (OR and 95% CI). We performed both univariable and multivariable analysis, adjusting simply for age

since age was forced into all predictor models. For Genotype x Smoking Status, being HPV16 and HPV18 negative and a non-smoker was used as the reference group. For Genotype x Margins, being HPV16 and HPV18 negative and having negative margins was the reference group. We compared the results with the OR for individual predictors (genotype, current smoking status and margins) in order to determine if there was any interaction. Although we knew that statistical power of these analyses would be limited, we decided to explore the possibility of effect modification since genotype seemed to be highly predictive of treatment failure. These additional analyses were also of interest since we were the first to conduct such an extensive study of the potential predictors of HSIL treatment failure. In addition, the analysis of potential effect modification of smoking and margins on genotype did not require any additional questionnaires to be administered, nor any additional laboratory tests to be performed.

Chapter 5 – Article

Predictors of High-grade Squamous Intraepithelial Lesion treatment failure

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Précis

Positive treatment margins and infection with HPV16/18 predict HSIL treatment failure.

Abstract

Objective To estimate the association between several risk factors and high-grade squamous intraepithelial lesions (HSIL) treatment failure in order to identify predictors.

Methods The study population included 1,548 Canadian women treated for HSIL who participated in a randomized control trial. HSIL treatment failure was the presence of histologically confirmed HSIL or worse during the two-year follow-up period. This nested-case control study included all 101 cases of treatment failure and controls that were matched 1:1 on treatment center and date of failure. Conditional logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) between each potential predictor and HSIL treatment failure. Independent variables that were examined included age, parity, smoking status, number of sexual partners, condom use, method of contraception, margins, number of passes, diagnosis on conisation, genotype and number of infecting types. Interactions between smoking and margins and genotype were evaluated.

Results Having positive vs. negative margins (adjusted OR=4.05, 95% CI 1.57-10.48) and being positive for *Human Papillomavirus* (HPV)16 and/or HPV18 vs. any other type (adjusted OR=2.69, 95% CI 1.32-5.49) were predictors of HSIL treatment failure in multivariable models. ORs suggested that older age, more severe lesions, and single-type infections may be at a higher risk of treatment failure but were not statistically significant. The ORs for smoking status, number of sexual partners, condom use, contraception, parity and number of passes were near the null value. We did not observe any evidence of interaction between smoking and genotype, nor between margins and genotype.

Conclusion Only positive margins and HPV16/18 positivity were predictors for being diagnosed with HSIL or worse within two years of treatment. However, we do not recommend automatic retreatment of those with positive margins because over 90% of those with positive margins did not fail treatment. The predictive value of HPV16 and HPV18 for HSIL treatment failure suggests that high coverage vaccination programs should contribute to a significant reduction in residual/recurrent disease.

Introduction

It is estimated that 1,350 Canadian women will be diagnosed with cervical cancer and 410 women will die of it in 2020 [12]. Globally, cervical cancer ranks fourth in terms of incidence and mortality amongst cancers affecting women [1]. Screening is a highly effective method of cervical cancer prevention [3, 4, 6, 9, 10]. Indeed, cervical cancer has a long pre-invasive phase that can be identified on histopathology, and the treatment of precancerous lesions, or High-grade squamous intraepithelial lesions (HSIL), reduces the incidence of cervical cancer [11]. Loop Electrosurgical Excision Procedure (LEEP) is the preferred treatment for cervical precancers or microinvasive cancers as it makes it possible to excise a limited and predetermined amount of cervical tissue and has the best success/side effect profile of conservative treatments [86].

Despite efforts to best treat women diagnosed with HSIL, 10-15% of those treated will have recurrent or residual disease, known as treatment failure [23]. Women who have been treated for HSIL have 4-5 times the risk of developing cervical cancer as women in the general population [63]. Those who experience treatment failure need to be retreated [24]. However, women who have undergone repeated excision of the cervix are at an increased risk of negative obstetric outcomes including second trimester pregnancy loss, preterm birth, and their offspring are at increased risk of low birth weight, complications of prematurity, and even neonatal death [24, 126, 127]. A large Danish cohort found that 33% of women with two conisations prior to pregnancy would experience preterm delivery [127]. In addition, preterm premature rupture of the membranes (pPROM) occurred in 92% of spontaneous preterm births in women with two prior conisations. Given the dire consequences, it is important to identify risk factors for treatment failure, as some may be modifiable and may help devise strategies to decrease treatment failure. Several patient, behavioural, clinical and viral risk factors for treatment failure have been studied [25-54]. Except for positive treatment margins that are strongly associated with treatment failure, evidence remains inconsistent for most risk factors. This is due to small sample sizes and a focus on a small number of potential risk factors simultaneously. Since exposures that are potentially associated with treatment failure are often highly correlated, results from past studies using univariate models may have potentially been biased, preventing the identification of independent predictors. Thus, the objective of our study was to explore a large

number of potential risk factors for HSIL treatment failure, in order to identify predictors of treatment failure.

Methods

Study population We used data collected in the Colposcopy vs. HPV testing to identify persistent precancers post treatment (CoHIPP) study, a randomized controlled trial (ClinicalTrials.gov Identifier: NCT01051895). CoHIPP methods have been described in detail previously [ref pending]. Briefly, women were recruited at the time of excisional treatment for HSIL (98% by LEEP); 1,548 had HSIL confirmed on the treatment specimen and were randomized to an HPV based follow-up strategy versus usual care. Participants in both groups were seen 6 months, 12 months and 24 months post treatment. All participants had exocervical and endocervical biopsies at 12 and 24 months.

For the present analysis, we performed a nested case-control study within the CoHIPP cohort. A total of 101 cases of treatment failure were identified within the study population. Cases included all participants who had histologically confirmed HSIL or worse at any point during the two-year follow-up. Controls were selected using incidence density sampling and matched 1:1 to cases by treatment center and by visit, which had to have occurred within 3 months of the case's identification. Controls were participants of the CoHIPP cohort who had not experienced treatment failure and who had not been censored at the time of matching. Of the 101 matched controls, one became a case at a later visit and nine were matched to two different cases.

CoHIPP received approval from the Research Ethics Committee of the Centre Hospitalier de l'Université de Montréal (CHUM). Participants provided free and informed consent to participate in CoHIPP and also consented to the use of their data and specimens for additional studies on HPV and cervical precancers.

Data collection Socio-demographic and behavioural data were collected using self-administered questionnaires at each visit. The randomisation visit questionnaire was used to measure baseline characteristics and exposures. Cervical specimens were also collected at each visit and banked for further biomolecular analysis. For this analysis, we used the randomization visit

questionnaire data and the specimens that were collected just prior to treatment for all biomolecular viral analysis.

Variables of interest Variables considered included age, parity, smoking status, number of sexual partners in the last year, condom use in the last year, method of contraception, positivity of margins, number of passes, diagnosis on LEEP, genotype and number of types. The age of each participant was calculated at the time of treatment and was analysed as a continuous variable. Parity for this study was low and was categorised as 0 (reference), 1, and 2 or more. We compared current smokers to current non-smokers (reference) at randomisation. Number of sexual partners in the last year included current partners and we compared having 2 or more partners to having 0 or 1 partner (reference). Condom use in the last year was analysed as a binary variable comparing ever vs. never (reference). The method of contraception was also binary, comparing hormonal contraception which included oral contraceptives, hormonal IUD, injectable contraceptive, hormonal patch, and vaginal ring, or any non-hormonal/hormonal combination, to non-hormonal or no contraception (reference), which included copper intrauterine device (IUD), diaphragm, condoms, cervical cap and no birth control. Margins on treatment specimen were analysed as a dichotomous variable comparing those with at least one positive margin to those with negative margins (reference). For number of loop passes done for treatment, we compared 2 or more passes to 1 pass (reference). Diagnosis on LEEP could have been the less severe Cervical Intraepithelial Neoplasia (CIN) 2, the more severe CIN3-Carcinoma in situ (CIS)-Adenocarcinoma in situ (AIS), or HSIL, not specified. Those with CIN2 were used as the reference group. HPV genotypes were detected on the cervical scrape collected immediately prior to treatment using Linear Array® assay from Roche Diagnostics, which identifies 37 types of HPV DNA [128]. Because of the clinical significance of both HPV16 and HPV18, a binary variable was created to compare those with HPV16 and/or HPV18 to infection with any other types (reference). Finally, we compared infection with 2 or more types to 1 type (reference).

Statistical analysis Statistical analyses were conducted using SAS version 9.4 for Windows (SAS Institute, Cary Inc.). Age was described by median, range and interquartile range. Other variables were described by the number and proportion of cases or controls in each category.

Odds ratios (OR) and 95%CI were estimated using conditional logistic regression models. Distinct univariable and multivariable models were developed for each potential predictor. The confounders included in each multivariable model had to be associated with both the independent variable of interest and the outcome variable. Due to the limited literature on predictors of HSIL treatment failure, there was an absence of known causal associations with our outcome. We therefore could not rely on the use of Directed Acyclic Graphs (DAG) to identify confounders that should be included in our multivariable models. In fact, we suspected that any one of our independent variables may be strongly associated with treatment failure. Any variable that was associated with the predictor of interest could then be considered as a potential confounder and included in the multivariable model. We suspected that many of our potential predictors were correlated [34] and neglecting to account for these correlations would have led to confounding bias. In order to determine the strength of these correlations, we estimated Spearman correlation coefficients between all of our independent variables. For each predictor, we included covariates for which the absolute value of the Spearman correlation coefficient was ≥0.2, as done previously [125]. Age was forced into all multivariable models, regardless of the strength of the correlation.

In order to avoid dropping of matched pairs from the analysis in cases where confounders contained missing data, we imputed the most frequent value among cohort members to replace missing data within the confounders of each model. However, in the main analyses of each variable of interest, the variable was left as-is in order to preserve the observed distribution. To ensure that this method did not overly bias the multivariable ORs, we conducted a sensitivity analysis comparing multivariable ORs and 95% CIs with and without the use of imputation in the confounder variables. Matched pairs with missing values were dropped from the analysis without imputation.

Results

Table 5.1 summarises the baseline characteristics of the study population. The median age was 35.28 for cases and 30.15 for controls. Over 50% of the population was nulliparous, specifically 48.51% of cases and 55.45% of controls. A slightly higher proportion of cases were current smokers than controls. The population was largely monogamous with over 70% of both cases

and controls reporting 0 or 1 sexual partner in the last year. Most participants never used condoms, likely because the group was largely monogamous. Distribution of condom use in the last year was almost the same for cases and controls, with 44.6% of cases and 47.5% of controls never using condoms. Fewer cases than controls used hormonal contraception. Far more cases had at least one positive margin (55.5%) compared to controls (33.7%). The distribution of number of passes was identical in cases and controls. More cases than controls had a more severe diagnosis of CIN3-CIS-AIS on LEEP, and HSIL type was not specified for 40.6% of cases and 34.7% of controls. Only 11.9% (24/202) of the study population had received an anti-HPV vaccine. Furthermore, given the age of the population and the recruitment period, most participants who were vaccinated received their first dose several years after initiation of sexual activity, when effectiveness is reduced [2, 129]. The variable was thus not analysed any further. As expected, more cases than controls had HPV16 and/or HPV18 at baseline. More controls than cases were infected with multiple types of HPV. All variables had less than 5% missing data except for condom use, method of contraception and margins, which had 11.9% (14 cases; 10 controls), 11.4% (12 cases; 11 controls) and 20.8% (23 cases; 19 controls) missing values respectively.

Table 5.2 shows the frequency of each HPV genotype in the LEEP specimens. High-risk types of HPV were detected more frequently than low-risk types among both cases and controls. HPV16 was the most frequent type in both cases and controls, followed by HPV31 and HPV52. The next most frequent types in cases were HPV53, HPV18 and HPV89, while in controls they were HPV33 and HPV62. 73 cases had HPV16 compared to 59 controls. There were only 11 participants infected with HPV18 (6 cases, 5 controls).

In our primary analysis (**Table 5.3**) we found that age, parity, being a smoker, number of sexual partners, use of condoms or hormonal contraception were not associated with the risk of treatment failure. As expected, having positive margins was a strong predictor of treatment failure (OR 4.05, 95% CI 1.57-10.48); however, the number of passes was not. Compared to a CIN2 diagnosis on LEEP, the ORs for treatment failure was 1.63 (95% CI 0.76-3.49) for CIN3-CIS-AIS and 2.12 (95% CI 0.81-5.55) for unspecified HSIL, but neither were statistically significant. Positivity for HPV16 or 18 vs. other HPV types at the time of LEEP was associated

with a higher risk of treatment failure. On the other hand, the OR for having infection with multiple HPV types vs. a single type was 0.43 with 95% CI 0.17-1.09. When analyses were restricted to pairs with complete data (i.e. covariate values not imputed), the results presented in Table 3 did not greatly change (**Supplementary table 5.1**). Imputation of the most frequent value decreased the variability of our data. Confidence intervals therefore tended to be slightly narrower in analysis with imputation compared to without.

Finally, we explored effect modification between key variables. Smoking may reduce one's immunity to infection with HPV16, decreasing the number of circulating antibodies [121]. We hypothesised that being a current smoker could modify the oncogenic effect of HPV16/HPV18 and increase the risk of HSIL treatment failure. However, no interaction was found between genotype and smoking status (**Supplementary table 5.2**). Similarly, we hypothesised that the odds of treatment failure amongst those with HPV16 and/or HPV18 could be increased by the presence of positive margins. Again, no interaction was identified.

Discussion

In our study of the potential risk factors for HSIL treatment failure, we found that positive margins and having HPV16 and/or HPV18 were significant predictors of HSIL treatment failure. The association between positive clinical margins and treatment failure was expected, and consistent with previous findings [26, 27, 30, 32, 38-40, 43, 46, 87, 96, 99, 105]. Overall, in CoHIPP, 10% of women with positive margins were diagnosed with HSIL within 2 years of treatment. Most likely these diagnoses represent persistent disease, as a positive margin indicates that some disease was left *in situ*. However, most women with positive margins will not have treatment failure. It is indeed possible for the disease to come to the edge of the cauterized region of the excised tissue, thus leading to a positive margin diagnosis, but for all the lesion to be removed. It is also possible that the immune reaction secondary to the treatment injury could lead to the clearance of the HPV infection and small residual disease.

For larger lesions, surgeons can opt to use a smaller loop and perform multiple passes in order to avoid an excessively deep excision that would result from using a larger loop [130, 131]. This type of procedure can make staging impossible if a small invasive cancer is found on multiple

pieces of the LEEP specimen, and thus would have a negative impact on the patient's treatment plan [106]. However, it is reassuring that treatment with multiple passes was not associated with an increased risk of treatment failure.

In addition, those with HPV16 and/or HPV18 identified on their LEEP specimen had a significantly higher odds of treatment failure than those with all other HPV types identified by Linear Array. Our findings add to evidence previously found by Wu et al. who showed that single-type infections with HPV16, 18, 33 and 45 were associated with an increased risk of biopsy proven residual/recurrent disease [51]. In contrast to our study, theirs was limited to patients with negative margins on treatment. Nonetheless, HPV genotype was not associated with the positivity of margins in our study population (Spearman correlation = -0.13).

In a previous study, infection with multiple HPV types vs. single type infection was significantly associated with a greater risk of recurrent/residual disease [51]. In contrast, our results suggested a protective effect of being infected with multiple types, though the OR was not statistically significant. We would have expected that adjusting for genotype would attenuate this effect, since infections with HPV16 and/or HPV18 were more likely to be single-type infections. However, the OR for infection with multiple types compared to one type was 0.43 with 95% CI 0.17-1.09, even after adjustment. It is possible that this result is unique to our study population. Overall, our results suggested that the type present was a more significant predictor of treatment failure than the number of types.

Advanced age, especially being 50 years of age and older, is generally considered a risk factor for treatment failure [34-37, 40, 42]. Our findings suggested that the odds of treatment failure may indeed increase with age. For example, in univariable analysis, participants 40 years of age and older had 2.62 (95% CI 1.31-5.25) times the odds of treatment failure compared to participants under the age of 30. However, the adjusted odds measured per five-year increase in age were not significant in our study population, possibly owing to a small number of women of older ages. We also explored using 40 and 50 years and older as thresholds but did not find any significant association with the risk of treatment failure (**Supplementary Table 5.3**).

Although parity and use of hormonal contraception have been associated with a higher risk of cervical cancer in HPV positive women [132] we, as others [37, 38, 42] have not found these characteristics to be associated with HSIL treatment failure. We should note that our study population was generally of low parity, with only 2% of women reporting more than 3 deliveries. As such, we could not investigate the potential impact of higher parity.

In our study, current smoking status was not associated with treatment failure. This finding is in agreement with a small study that found that being a smoker and number of cigarettes smoked were not significantly associated with relapse of CIN [53]. They found, however, that being a smoker in conjunction with HPV positivity after treatment increased the risk of relapse with a higher grade of disease (CIN3 and microinvasive cancer). It is important to note that HPV positivity post-treatment may act as a surrogate measurement for the outcome of interest, biasing their results. In addition, they did not adjust for confounders. In contrast, a more robust prospective study of 77 cases of treatment failure and 154 controls that investigated the association between smoking and treatment failure found that, not only does being a current smoker increase the odds of treatment failure (OR= 3.17, 95% CI 1.68-5.91), but there is an observable dose-response relationship [25]. They estimated that for every additional 10 cigarettes smoked per day (from 0-30 cigarettes), the odds of treatment failure increased by a factor of 2.58 (95% CI 1.70-3.91). Their estimates were adjusted for HPV infection post-treatment, but not for potential sociodemographic confounders. In our study, only 33% of participants were current smokers, a smaller proportion than the other study populations (52% and 54%). In addition, with the data at our disposal, we were not able to quantify smoking in terms of cigarettes or packs per day and therefore did not measure the effect of dose. On the other hand, we adjusted for behavioural confounders that were strongly correlated with smoking within our population (number of sex partners and method of contraception) but were not accounted for in the other studies that investigated smoking and treatment failure. Associations observed between smoking status and treatment failure in previous studies may simply have been the result of confounding bias.

Diagnosis on LEEP, or increased severity of the lesion did not show a positive association with treatment failure. However, the OR for those with CIN3-CIS/AIS does suggest the possibility of

an increased odds of treatment failure compared to those with CIN2. Judging by the even greater OR for the category HSIL not specified, we suppose that this group was primarily composed of participants with CIN3-CIS/AIS. Our analysis of this potential risk factor was limited by the proportion of subjects whose grade of lesion was not specified and this risk factor requires further investigation.

Despite being larger than most prior studies to have investigated predictors of treatment failure, this study was still limited by sample size, with only 101 cases of treatment failure identified in the CoHIPP cohort. This limits the statistical power of our analyses. The use of matched controls and conditional logistic regression should have produced estimates very similar to those that would have been obtained on the entire cohort. There was little missing data overall, however relative risks may have been biased for condom use, method of contraception and margins, which had 11.88%, 11.39% and 20.79% missing data respectively. In addition, our study was limited by the fact that the baseline questionnaire occurred 6 months after treatment. In fact, for condom use and number of sex partners, "in the last year" included 6 months prior to treatment and not a full year. This could have also affected measurement of current smoking status, since participants may have modified their behaviour since treatment. We also included a larger number of potential predictors than prior studies, which allowed us to control for potential confounding bias that had not been accounted for in other studies.

In conclusion, in this large cohort of unselected women who underwent treatment for HSIL, only having positive margins at treatment and being HPV16/18 positive were significantly associated with being diagnosed again with HSIL within two years. However, given that 90% of women with positive margins in CoHIPP did not experience treatment failure, and because of the risk to future pregnancies with repeated treatments [24, 126, 127] we do not recommend automatic retreatment of women with positive treatment margins. Rather, our results emphasise the importance of mechanisms to minimize losses to follow-up in this group. Finally, given the singular role of HPV16/18 in HSIL treatment failure, the implementation of high coverage HPV vaccine programs should lead to a significant decrease in re-treatments, and limit the associated adverse obstetric impacts.

Table 5.1. Baseline characteristics of study population

	Cases (N=101)	Controls (N=101)
Patient characteristics		
Age, median (range; IQR)	35.28 (21.66-62.10; 13.70)	30.15 (20.53-66.90; 9.27)
Parity, n (%)		
0	49 (48.5)	56 (55.5)
1	22 (21.8)	23 (22.8)
2 or more	30 (29.7)	20 (19.8)
Behavioural characteristics		
Current smoking status, n (%)		
Non-smoker	66 (65.4)	69 (68.3)
Smoker	35 (34.7)	31 (30.7)
Number of sexual partners*, n (%)		
0 or 1	75 (74.3)	71 (70.3)
2 or more	23 (22.8)	26 (25.7)
Condom use*, n (%)		
No	45 (44.6)	48 (47.5)
Yes	42 (41.6)	43 (42.6)
Method of contraception, n (%)		
Non-hormonal or none	52 (51.5)	36 (35.6)
Hormonal	37 (36.6)	54 (53.5)
Clinical characteristics		
Margins, n (%)		
Negative	22 (21.8)	48 (47.5)
At least one positive	56 (55.5)	34 (33.7)
Number of passes, n (%)		
1	61 (60.4)	61 (60.4)
2 or more	40 (39.6)	40 (39.6)
Diagnosis on LEEP, n (%)		
CIN2	18 (17.8)	31 (30.7)
CIN3-CIS-AIS	42 (41.6)	35 (34.7)
HSIL, not specified	41 (40.6)	35 (34.7)
Viral characteristics		
Anti-HPV vaccination, n (%)		
No	85 (84.2)	82 (81.2)
Yes	11 (10.9)	13 (12.9)
Genotype, n (%)		
No HPV16 or HPV18	22 (21.8)	37 (36.6)
HPV16 and/or HPV18	76 (75.2)	61 (60.4)
Number of types of HPV, n (%)		
1	52 (51.5)	40 (39.6)
2 or more	46 (45.5)	58 (57.4)

*In the last year

Missing: parity (2 controls), current smoking status (1 control), number of sexual partners (3 cases, 4 controls), condom use (14 cases, 10 controls), method of contraception (12 cases, 11 controls), margins (23 cases, 19 missing), anti-HPV vaccination (5 cases, 6 controls), genotype (3 cases, 3 controls), number of types (3 cases, 3 controls)

Abbreviations: IQR: Interquartile Range, LEEP: Loop Electrosurgical Excision Procedure, CIN: Cervical Intraepithelial Neoplasia, CIS: Carcinoma In Situ, AIS: Adenocarcinoma In Situ, HSIL: High-grade Squamous Intraepithelial Lesion, HPV: Human Papillomavirus

Table 5.2. Frequency of HPV genotypes in LEEP specimens

HPV Genotype	Cases (N=101)	Controls (N=101)
High-risk types	**137***	**144**
16	73	59
18	6	5
31	13	17
33	4	12
35	3	6
39	3	4
45	2	6
51	5	7
52	13	16
56	5	2
58	5	3
59	5	7
Low-risk types	**55**	**57**
6	3	1
34	0	1
40	1	1
42	2	4
44	1	3
53	7	3
54	6	6
61	4	1
62	6	12
66	2	1
67	2	4
68	1	0
69	0	1
73	1	3
81	2	2
82	3	3
83	3	2
84	5	4
89	6	5

*Total frequency of genotypes is greater than N due to the presence of co-infection, or infection with multiple types of HPV in one participant

Missing: 3 cases, 3 controls

Table 5.3. Univariable and multivariable analysis of potential predictors for HSIL treatment failure

Potential predictor	Crude OR (95% CI)	Adjusted OR (95% CI)	Covariates in adjusted model
Age, per 5-unit increase	1.28 (1.10-1.47)	1.28 (0.90-1.76)	Parity, number of sexual partners, condom use, method of contraception, number of types
Parity			
0	1	1	Age, number of sexual partners, condom use
1	1.07 (0.53-2.18)	0.49 (0.19-1.34)	
2 or more	1.63 (0.84-3.17)	0.87 (0.31-2.45)	
Current smoking status			
Non-smoker	1	1	Age, number of passes
Smoker	1.67 (0.62-2.19)	1.02 (0.51-2.05)	
Number of sexual partners*			
0 or 1	1	1	Age, parity, condom use, number of types, margins
2 or more	0.82 (0.40-1.67)	1.16 (0.41-3.29)	
Condom use*			
No	1	1	Age, parity, number of sexual partners
Yes	1.06 (0.55-2.01)	1.32 (0.56-3.09)	
Method of contraception			
Non-hormonal or none	1	1	Age
Hormonal	0.48 (0.24-0.96)	0.74 (0.34-1.61)	
Margins			
Negative	1	1	Age, number of sexual partners, number of passes
At least one positive	3.75 (1.72-8.18)	4.05 (1.57-10.48)	
Number of passes			
1	1	1	Age, current smoking status, margins
2 or more	1.00 (0.53-1.89)	0.94 (0.45-1.97)	
Diagnosis on LEEP			
CIN2	1	1	Age
CIN3-CIS-AIS	1.96 (0.94-4.07)	1.63 (0.76-3.49)	
HSIL, not specified	2.43 (0.95-6.23)	2.12 (0.81-5.55)	
Genotype			
No HPV16 or HPV18	1	1	Age, number of types
HPV16 and/or HPV18	2.21 (1.18-4.16)	3.03 (1.44-6.41)	
Number of types of HPV			
1	1	1	Age, number of sexual partners, genotype
2 or more	0.57 (0.29-1.12)	0.43 (0.17-1.09)	

*In the last year

OR: Odds Ratio, CI: Confidence Interval, LEEP: Loop Electrosurgical Excision Procedure, CIN: Cervical Intraepithelial Neoplasia, CIS: Carcinoma In Situ, AIS: Adenocarcinoma In Situ, HSIL: High-grade Squamous Intraepithelial Lesion, HPV: Human Papillomavirus

Supplementary Table 5.1. Multivariable analysis without imputation*

Potential predictor	Cases (N=101) n (%)	Controls (N=101) n (%)	Adjusted OR without imputation (95% CI)	Covariates in adjusted model
Age, per 5-unit increase	84 (83.2)	85 (84.2)	1.06 (0.97-1.15)	Parity, number of sexual partners, condom use, method of contraception, number of types
Parity				
0	44 (43.6)	51 (50.5)	1	Age, number of sexual partners, condom use
1	17 (16.8)	21 (20.8)	0.63 (0.19-2.04)	
2 or more	24 (23.8)	14 (13.9)	1.11 (0.30-4.17)	
Current smoking status				
Non-smoker	66 (65.3)	69 (68.3)	1	Age, number of passes
Smoker	35 (34.7)	31 (30.7)	1.02 (0.51-2.05)	
Number of sexual partners+				
0 or 1	63 (62.4)	62 (61.4)	1	Age, parity, condom use, number of types, margins
2 or more	22 (21.8)	24 (23.8)	0.85 (0.62-4.49)	
Condom use+				
No	44 (43.6)	44 (43.6)	1	Age, parity, number of sexual partners
Yes	41 (40.6)	42 (41.6)	1.20 (0.50-2.88)	
Method of contraception				
Non-hormonal or none	52 (51.5)	36 (35.6)	1	Age
Hormonal	37 (36.6)	54 (53.5)	0.74 (0.34-1.61)	
Margins				
Negative	22 (21.8)	42 (41.6)	1	Age, number of sexual partners, number of passes
At least one positive	48 (47.5)	28 (27.7)	3.71 (1.44-9.55)	
Number of passes				
1	19 (18.8)	16 (15.8)	1	Age, current smoking status, margins
2 or more	12 (11.9)	15 (14.9)	0.96 (0.47-2.03)	
Diagnosis on LEEP				
CIN2	18 (17.8)	31 (30.7)	1	Age
CIN3-CIS-AIS	42 (41.6)	35 (34.7)	1.63 (0.76-3.49)	
HSIL, not specified	41 (40.6)	35 (34.7)	2.12 (0.81-5.55)	
Genotype				
No HPV16 or HPV18	22 (21.8)	37 (36.6)	1	Age, number of types
HPV16 and/or HPV18	76 (75.2)	61 (60.4)	2.69 (1.31-5.49)	
Number of types of HPV				
1	51 (50.4)	40 (39.6)	1	Age, number of sexual partners, genotype
2 or more	44 (43.6)	56 (55.4)	0.53 (0.21-1.37)	

*Matched pairs with missing data were dropped from analysis

+In the last year

OR: Odds Ratio, CI: Confidence Interval, LEEP: Loop Electrosurgical Excision Procedure, CIN: Cervical Intraepithelial Neoplasia, CIS: Carcinoma In Situ, AIS: Adenocarcinoma In Situ, HSIL: High-grade Squamous Intraepithelial Lesion, HPV: Human Papillomavirus

Supplementary Table 5.2. Effect of the interactions between smoking and margins with genotype

Interaction	Cases n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted* OR (95% CI)
Genotype x Smoking				
No HPV16/18 x Non-smoker	16 (15.84)	28 (27.72)	1	1
HPV16/18 x Non-smoker	49 (48.51)	39 (38.61)	2.02 (0.97-4.22)	2.50 (1.11-5.63)
No HPV16/18 x Smoker	6 (5.94)	9 (8.91)	1.20 (0.33-4.36)	1.19 (0.31-4.60)
HPV16/18 x Smoker	27 (26.73)	19 (18.81)	2.06 (0.86-4.95)	2.05 (0.81-5.21)
Genotype x Margins				
No HPV16/18 x Negative	7 (6.93)	16 (15.84)	1	1
HPV16/18 x Negative	14 (13.86)	29 (28.71)	1.37 (0.44-4.28)	1.81 (0.53-6.23)
No HPV16/18 x Positive	10 (9.90)	9 (8.91)	3.43 (0.79-15.01)	4.32 (0.90-20.72)
HPV16/18 x Positive	44 (43.56)	23 (22.77)	6.15 (1.80-21.03)	9.18 (2.25-37.47)

*Adjusted for age
OR: Odds Ratio, CI: Confidence Interval, HPV: Human Papillomavirus
Missing: Genotype x smoking (3 cases, 4 controls), Genotype x margins (26 cases, 22 controls)

Supplementary Table 5.3. Age analyzed as a categorical variable

Potential predictor	Cases n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted OR* (95% CI)
Age (binary)				
<40	68 (67.33)	86 (85.15)	1	1
≥40	33 (32.67)	15 (14.85)	2.39 (1.25-4.56)	2.13 (0.72-6.30)
Age (decade)				
<30	32 (31.68)	48 (47.52)	1	1
30-<40	36 (35.64)	38 (37.62)	1.28 (0.67-2.46)	1.79 (0.59-5.46)
≥40	33 (32.67)	15 (14.85)	2.62 (1.31-5.25)	3.41 (0.81-14.29)
Age (binary)				
<50	96 (95.05)	98 (97.03)	1	1
≥50	5 (4.95)	3 (2.97)	1.67 (0.40-6.97)	0.68 (0.11-4.01)
Age (decade)				
<30	32 (31.68)	48 (47.52)	1	1
30-<40	36 (35.64)	38 (37.62)	1.28 (0.67-2.46)	1.68 (0.55-5.14)
40-<50	28 (27.72)	12 (11.88)	2.86 (1.32-6.18)	4.07 (0.91-18.28)
≥50	5 (4.95)	3 (2.97)	1.83 (0.43-7.86)	1.60 (0.20-13.11)

*Adjusted for parity, number of sex partners, condom use, method of contraception and number of types
OR: Odds Ratio, CI: Confidence Interval

Chapter 6 – Other Results

6.1 Viral load

We analysed viral load for cases and controls that had infections with HPV16 and/or HPV18 due to the clinical significance of these types. Based on genotyping results with Linear Array, HPV16 was detected in the specimens of 134 participants, 73 cases and 61 controls. Of those with HPV16, 5 specimens, 1 case and 4 controls, were not suitable for qPCR analysis, leaving us with a total of 129 specimens. The distribution of HPV16 viral load was unimodal, skewed to the right. For cases, the minimum viral load detected was 2.82×10^{-3} copies/cell and the maximum was 4928.82 copies/cell. The median was 3.20 copies/cell and the interquartile range was 19.11. For controls, the minimum viral load was 2.17×10^{-3} copies/cell and the maximum was 1877.21 copies/cell. The median was 3.54 copies/cell and the interquartile range was 29.51. While the maximum viral load was higher for cases than for controls, the median followed the opposite trend. We used a logarithmic transformation of HPV16 viral load in order to obtain an approximate normal distribution. This allowed us to estimate the difference in means between cases and controls using Student's T-test. For cases, the mean of the log-transformed viral load was 1.48copies/cell with a standard deviation (SD) of 2.62. For controls, the mean of the log-transformed variable was 1.56 copies/cell and SD=2.95. The results of the T-test did not contradict the null hypothesis of equal means (**Table 6.1)**.

HPV18 was detected in 11 participants, 6 cases and 5 controls, and all specimens were suitable for analysis via qPCR. Similar to HPV16, the distribution of HPV18 viral load was unimodal and skewed to the right. For cases, the minimum viral load was 3.78×10^{-3} copies/cell and the maximum was 117.40 copies/cell. The median was 0.70 copies/cell and the interquartile range was 38.46. For controls, the viral load ranged from a minimum of 5.17×10^{-3} copies/cell to a maximum of 7.07 copies/cell, with median equaling 0.32 copies/cell and interquartile range equaling 7.06. The maximum and mean were both higher for cases than for controls. Logarithmic transformation did not normalise HPV18 viral load, so we used Wilcoxon-Mann-Whitney non-parametric U-test on the untransformed variable. The null hypothesis was not rejected, and we did not observe a statistically significant difference between the mean scores **(Table 6.2).**

Table 6.1. Comparison of HPV16 viral load means after logarithmic transformation

Type	n	Mean (95% CI)	p-value*
HPV16			
Cases	72	1.48 (0.86-2.09)	0.87
Controls	57	1.56 (0.78-2.34)	

*Test: Student's T-test
CI: Confidence Interval

Table 6.2. Comparison of HPV18 viral load ranks

Type	n	Mean Score	p-value*
HPV18			
Cases	6	5.60	0.78
Controls	5	6.33	

*Test: Wilcoxon-Mann-Whitney U-Test, two-sided

We followed these tests with an unconditional logistic regression analysis to estimate the association between the log-transformed HPV16 viral load and HSIL treatment failure. The estimated association between log(HPV16 viral load) and treatment failure was precise and essentially null (**Table 6.3**).

Table 6.3. Logistic regression analysis of HPV16 viral load after logarithmic transformation

Type	Cases	Controls	Crude OR (95% CI)	Adjusted OR* (95% CI)
HPV16	72	57	0.99 (0.87-1.12)	0.97 (0.85-1.11)

*Adjusted for age
OR: Odds Ratio, CI: Confidence Interval

We next dichotomized the viral load variable into "low" and "high" categories and used a chi-squared test to compare the proportions between cases and controls, for both HPV16 and HPV18 (**Table 6.4**). Using 0.11 copies/cell as the threshold to differentiate high from low viral load, the vast majority of those with HPV16 had a high viral load: 93.06% of cases and 89.47% of controls. Most of those with HPV18 also had a high viral load: 66.67% of cases and 60.00% of controls. For both genotypes, the proportion of high viral load was not significantly different

between cases of treatment failure and controls. Small numbers limited the power of the chi-squared test, especially for HPV18, but it is evident from **Table 6.4** that the proportion of high viral load was similar between cases and controls. We also explored doubling this threshold and using the median of each type as the threshold (**Annexes 1-4**). However, we were still unable to discern any significant association between viral load and treatment failure.

Table 6.4. Viral load dichotomized for HPV16 and HPV18

Type	Viral load (copies/cell)	Cases n (%)	Controls n (%)	p-value
HPV16	≤0.11	5 (6.94)	6 (10.53)	0.47
	>0.11	67 (93.06)	51 (89.47)	
	Total	72	57	
HPV18	≤0.11	2 (33.33)	2 (40.00)	0.82
	>0.11	4 (66.67)	3 (60.00)	
	Total	6	5	

6.2 Variants

6.2.1 HPV16 Variants

Of the 134 specimens that were positive for HPV16, we were unable to purify an adequate amount of DNA for 1 case and 7 controls, leaving 126 to be sequenced. In our study, we identified 19 different variants of HPV16, including the prototype sequence (**Table 6.5**). The most common variant overall was the prototype, the reference sequence of the HPV16 LCR, making up 40.48% of HPV16+ specimens. The prototype was also the most frequent sequence in the controls (46.30%). On the other hand, the most frequent variant in the cases was MTL-16-LCR-75 (48.61%), which has a point mutation at position 7521 of an adenosine instead of a guanine. The nucleotide positions of variations in the HPV16 variants identified are described in **Table 6.6**. Compared to the prototype, all variations that we identified were single nucleotide polymorphisms (SNP). Non-prototype variants had between 1 and 10 SNPs, with most having just 1 (54.17% of cases and 27.78% of controls) (**Table 6.7**).

Table 6.5. Frequency of HPV16 Variants

HPV16 Variant	Cases (N=72) n (%)	Controls (N=54) n (%)
Prototype	26 (36.11)	25 (46.30)
MTL-16-LCR-03	1 (1.39)	3 (5.56)
MTL-16-LCR-34	1 (1.39)	
MTL-16-LCR-38	1 (1.39)	
MTL-16-LCR-39	1 (1.39)	
MTL-16-LCR-47	2 (2.78)	1 (1.85)
MTL-16-LCR-61		2 (3.70)
MTL-16-LCR-62	1 (1.39)	
MTL-16-LCR-75	35 (48.61)	13 (24.07)
MTL-16-LCR-77	1 (1.39)	
MTL-16-LCR-78	1 (1.39)	1 (1.85)
MTL-16-LCR-79		2 (3.70)
MTL-16-LCR-87	1 (1.39)	1 (1.85)
MTL-16-LCR-111		1 (1.85)
MTL-16-LCR-112		2 (3.70)
MTL-16-LCR-113		1 (1.85)
MTL-16-LCR-114		1 (1.85)
MTL-16-LCR-115		1 (1.85)
U34099	1 (1.39)	

*LCR: Long Control Region

Table 6.6. HPV16 LCR Polymorphism

HPV16 Variant	Phylogenetic branch	Lineage	Position in Nucleotide Sequence																				Number of mutations
			7485	7489	7496	7507	7521	7526	7549	7568	7669	7689	7729	7743	7764	7784	7786	7792	7799	7826	7834	7837	7839	
Prototype*	Eur	A	A	G	T	A	G	A	C	T	C	C	A	T	C	C	C	C	G	G	G	A	A	
Mutations			C	A	C	C	A	G	T	G	T	A	C	G	T	T	T	G	A	A	T	G/C	G	
MTL-16-LCR-03	AA1	D3	C	A			A				T	A	C	G	T		T							9
MTL-16-LCR-34	Eur	A								G														1
MTL-16-LCR-38	Eur	A							T															1
MTL-16-LCR-39	Eur	A			C		A																	2
MTL-16-LCR-47	Eur	A																G						1
MTL-16-LCR-61	NA	D1	C	A			A				T	A	C		T		T							8
MTL-16-LCR-62	NA	D1	C	A			A				T	A	C		T	T	T							9
MTL-16-LCR-75	Eur	A					A																	1
MTL-16-LCR-77	Eur	A					A											T						2
MTL-16-LCR-78	Eur	A				C	A																	2
MTL-16-LCR-79	NA	D1	C	A			A				T				T		T							6
MTL-16-LCR-87	AF1	B1		A			A					A			T		T				T			6
MTL-16-LCR-111	Eur	A	C	A			A				T	A	C		T		T				T			9
MTL-16-LCR-112	Eur	A								G												G		2
MTL-16-LCR-113	Eur	A													T									1
MTL-16-LCR-114	AF1	B1		A			A	G				A			T		T				T			7
MTL-16-LCR-115	Eur	A					A												A					2
U34099	AF2	C	C	A			A				T				T		T			A	T	C	G	10

*Prototype GenBank Identifier K02718.1
The mutation in red was identified in our population and had not been previously identified in the literature.

Table 6.7. Number of mutations identified in HPV16 variants

Number of Mutations	Cases (N=72) n (%)	Controls (N=54) n (%)
0	26 (36.11)	25 (46.30)
1	39 (54.17)	15 (27.78)
2	4 (4.17)	4 (7.41)
6	1 (1.39)	3 (5.56)
7		1 (1.85)
8		2 (3.70)
9	2 (2.78)	4 (7.41)
10	1 (1.39)	

The identified variants were classified into 5 phylogenetic branches including European (Eur), African 1 (Af1), African 2 (Af2), Asian-American (AA) and North American (NA). European variants were, by far, the most common in both cases and controls (**Table 6.8**).

Table 6.8. Phylogenetic classification of HPV16 variants

Phylogeny	Cases (N=72) n (%)	Controls (N=54) n (%)
Eur	68 (94.44)	45 (83.33)
AA1	1 (1.39)	3 (5.56)
Af1	1 (1.39)	2 (3.70)
Af2	1 (1.39)	
NA	1 (1.39)	4 (7.41)

Eur: European, AA1: Asian-American 1, Af1: African 1, Af2: African 2, NA: North American

We also classified the variants on the basis of their similarity to the prototype. Prototype-like variants had only one point variation from the prototype, and non-prototype-like variants had more than one. The prototype comprised 36.11% of cases and 46.30% of controls, while prototype-like variants comprised 54.17% of cases and 27.78% of controls. In univariate logistic regression, those with prototype-like variants had 2.5 times the odds of treatment failure compared to those with the prototype (**Table 6.9**). When we adjusted for age, the odds ratio was essentially unchanged. The estimated increase was

statistically significant in both univariable and multivariable analysis. We decided to further explore this by looking at the effect of specific variations. Of all the variations in our study population, the substitution at the 7521 position was the only one that occurred with enough frequency to estimate associated risk using logistic regression. Those with the sequence variation at position 7521 had 1.65 (95% CI 0.78-3.47) times the risk of treatment failure of those without this variation in multivariable analysis (**Table 6.10**).

Table 6.9. Association between infection with prototype-like and non-prototype-like variants and treatment failure

HPV16 Variant	Cases (N=72) n (%)	Controls (N=54) n (%)	Crude OR (95% CI)	Adjusted OR* (95% CI)
Prototype	26 (36.11)	25 (46.30)	1.00	1.00
Prototype-like	39 (54.17)	15 (27.78)	2.50 (1.11-5.62)	2.49 (1.07-5.83)
Non-prototype-like	7 (9.72)	14 (25.93)	0.48 (0.17-1.39)	0.51 (0.17-1.152)

*Adjusted for age

Table 6.10. Association between the substitution at position 7521 and treatment failure

Variation	Cases (N=72) n (%)	Controls (N=54) n (%)	Crude OR (95% CI)	Adjusted OR* (95% CI)
No variation at 7521	29 (40.28)	29 (53.70)	1.00	1.00
Variation at 7521	43 (59.72)	25 (46.30)	1.72 (0.84-3.50)	1.65 (0.78-3.47)

*Adjusted for age

6.2.2 HPV18 Variants

Our study included 11 participants who were positive for HPV18, including 6 cases and 5 controls. Of those, we were unable to purify the DNA for 4 controls, leaving only 1 control to sequence. We identified 5 distinct variants of the LCR of HPV18, including the prototype (**Table 6.11**). The exact polymorphisms are presented in **Table 6.12**. All polymorphisms that we observed were SNPs. The most frequent positions for these point mutations were 7529 (cytosine for adenosine), 7567 (adenosine for cytosine) and 7670 (adenosine for tyrosine), each of which occurred in 3 different variants. The number of mutations in comparison to the prototype ranges from 1 to 5 (**Table 6.13**).

Table 6.11. Frequency of HPV18 Variants

HPV18 Variant	Cases (N=6) n (%)	Controls (N=1) n (%)
Prototype	2 (33.33)	
MTL-18-LCR-01		1 (100.00)
MTL-18-LCR-28	2 (33.33)	
MTL-18-LCR-29	1 (16.67)	
MTL-18-LCR-37	1 (16.67)	

Table 6.12. HPV18 LCR Polymorphisms

HPV18 Variant	Phylogenetic branch	Position in Nucleotide Sequence							Number of mutations
		7486	7529	7563	7564	7567	7670	7717	
Prototype*	Eur	C	C	G	A	A	A	A	
Mutation		T	A	A	C	C	T	C	
MTL-18-LCR-1	Eur		A		C	C	T		4
MTL-18-LCR-28	Eur	T	A			C	T		4
MTL-18-LCR-29	Eur	T	A	A		C	T		5
MTL-18-LCR-37	AsAl							C	1

*Prototype GenBank Identifier AY262282.1

Table 6.13. Number of mutations identified in HPV18 Variants

Number of Mutations	Cases (N=6) n (%)	Controls (N=1) n (%)
0	2 (33.33)	
1	1 (16.67)	
4	2 (33.33)	1 (100.00)
5	1 (16.67)	

We classified the variants by phylogeny. All identified sequences were of European (Eur) origin except for one case that was of Asian-Amerindian (AsAi) origin (**Table 6.14**). Just as we did for HPV16, we also categorized the HPV18 variants based on their similarity to the prototype. Out of all 6 sequenced LCRs, 4 were non-prototype-like (**Table 6.15**).

Table 6.14. Phylogenetic Classification of HPV18 Variants

Phylogeny	Cases (N=6) n (%)	Controls (N=1) n (%)
Eur	5 (83.33)	1 (100.00)
AsAi	1 (16.67)	

Eur: European, AsAi: Asian-Amerindian

Table 6.15. Similarity to prototype of HPV18 Variants

HPV18 Variant	Cases (N=6) n (%)	Controls (N=1) n (%)
Prototype	2 (33.33)	
Prototype-like	1 (16.67)	
Non-prototype-like	3 (50.00)	1 (100.00)

Chapter 7 – Discussion

7.1 Summary of results

The objectives of our study were to identify predictors of HSIL treatment failure and to estimate the association between HPV16 and HPV18 viral load and variants and treatment failure. In our study population, cases and controls presented similar baseline distributions for several of the investigated risk factors. However, cases were older than controls at baseline. While the majority of the study population was nulliparous, we observed more cases than controls who were multiparous. Our study population also had more cases than controls who were smokers at baseline, who did not use hormonal contraception, who had at least one positive margin, who had CIN3-CIS-AIS (Cervical Intraepithelial Neoplasia 3-Carcinoma in situ-Adenocarcinoma in situ) or HSIL not specified (compared to CIN2), who had HPV16 and/or HPV18, and who had only one type of HPV.

In our investigation of the potential risk factors for HSIL treatment failure, we confirmed that positive margins and having HPV16 and/or HPV18 were predictors of HSIL treatment failure. Though the identified predictors are not modifiable, they do have clinical and public health implications. Specifically, women treated for HSIL who have positive margins or whose infections include HPV16 and/or HPV18 should be monitored more closely during follow-up since they are at a higher risk of treatment failure. In addition, since a large proportion of HSIL treatment failure could be attributed to infection with HPV16 and/or HPV18, we can expect that anti-HPV vaccination programs will significantly decrease the incidence of treatment failure in vaccinated populations.

Our results suggested a positive association between age and the odds of treatment failure, though without statistical significance. They also suggest that being infected by a single type of HPV may increase the risk of treatment failure as opposed to being infected with multiple types. We did not observe a statistically significant OR for the association between diagnosis on LEEP and HSIL treatment failure. However, the OR for those with CIN3-CIS-AIS compared to CIN2 did suggest a possible positive association

with treatment failure and the OR for HSIL not specified was even greater. We suppose that this group of unspecified severity was primarily composed of participants with CIN3-CIS-AIS. Finally, we did not observe any significant or strong association between parity, current smoking status, number of sexual partners, condom use, method of contraception or number of passes and HSIL treatment failure.

7.2 Comparison with the literature and discussion of results

The literature on risk factors for HSIL treatment failure is largely inconsistent. Many different definitions of treatment failure have been used to define persistent, recurrent or residual disease after treatment, including post-treatment abnormal cytology, persistent infection with HPV, persistent type-specific infection with HPV, detection of >1RLU using HC2, identification of any grade of lesion on histopathology, or identification of HSIL or worse on histopathology. Our study defined HSIL treatment failure as the identification of HSIL or worse on histopathology. Not only does this definition represent the most reproducible one, it is also the result of most clinical significance, as it would lead to a second treatment.

In the literature review presented in Chapter 2, having positive margins was the most consistently identified risk factors for residual/recurrent disease after treatment for cervical precancers. The association that we identified between positive clinical margins and HSIL treatment failure was expected and was consistent with previous findings [26, 27, 30, 32, 38-40, 43, 46, 87, 96, 99, 105]. In CoHIPP, only 10% of participants with positive margins were diagnosed with treatment failure. The cases of HSIL treatment failure identified amongst women with positive margins likely represented cases of persistent disease, rather than recurrent disease, because positive margins indicate that there is remaining lesion *in situ.* However, it is also possible for the disease to come all the way to the edge of the cauterized region even if all of the lesion is successfully removed, producing a positive margin with no residual disease. It is also possible that the treatment itself, which causes injury to the cervix, produces a secondary innate immune response that is capable of clearing the HPV infection and small residual disease [133].

When treating larger lesions, surgeons can opt to use a smaller loop and execute multiple passes in order to avoid excessively deep excisions that result from the use of a larger loop, although some experts have recommended using a single pass whenever possible.[130, 131]. Multiple passes can make staging the excised disease impossible if a small invasive cancer is found on multiple pieces of the LEEP specimen, and, in turn, can have a negative impact on the patient's overall treatment plan[106]. However, it is reassuring that we did not find any association between number of passes and HSIL treatment failure. As we mentioned in Chapter 4, LEEP with multiple passes can also lead to the identification of positive margins, even if the entire lesion was successfully excised in the end. In our study population, margins and number of passes were indeed correlated. We therefore adjusted for margins in the multivariable analysis of number of passes, and vice versa.

In addition, those with HPV16 and/or HPV18 identified on their LEEP specimen had significantly higher odds of treatment failure than those with all other HPV types identified by Linear Array, in both univariable and multivariable analysis. When we controlled for age and for number of types, the magnitude of the OR increased. The five most common genotypes in our population were HPV16 (65.34%), HPV31 (14.85%), HPV52 (14.36%), HPV62 (8.91%) and HPV33 (7.92%). These genotypes rank within the most common types found in CIN2 and CIN3 according to a Canadian study by Coutlée et al., except for HPV62 [134]. HPV18 was more frequent in their study than in ours. Wu et al. had previously shown that single-type infections with HPV16, 18, 33 and 45 were associated with an increased risk of biopsy proven residual/recurrent disease[51]. They controlled for the impact of margin status by restricting their study population to patients with negative margins on treatment. Nonetheless, HPV genotype was not associated with margins in our population (Spearman correlation = -0.13). The association we identified between genotype and HSIL treatment failure applied to those with both negative and positive margins. Wu et al. only provided p-values, with no estimate of relative risk. We estimated multivariable ORs, but not for individual genotypes. In other studies, infection with multiple types had been significantly associated with an even greater risk of recurrent/residual disease than single-type infections[51]. Our multivariable model for

genotype controlled for potential confounding by number of types. When we considered number of types as a potential predictor for treatment failure, we did not find that being infected with multiple types of HPV increased the odds of treatment failure. Rather, our results suggested that a single-type infection was more likely to lead to treatment failure. The crude OR for multiple types compared to one type was 0.57 (95% CI 0.29-1.12) and the adjusted OR was 0.43 with 95% CI 0.17-1.09. Thus, the suggestive decreased risk with multiple vs. single HPV type was stronger after adjusting for genotype, along with age and number of sexual partners. Since genotype, specifically HPV16/18, and age are associated with an increased risk of treatment failure in our study population, this direction of confounding is expected. According to a study published in Nature (Oncogene), most high-grade cervical lesions are monoclonal, resulting from the integration of a singular type of HPV[135]. In addition, cells expressing mRNA transcripts from integrated viral genome copies seem to have a selective growth advantage over cells with only episomal-derived transcripts. Integration of the viral genome is likely an important factor in the progression of HPV infection to neoplasia and invasion. Studies have found that multiple integration sites in a clonal population seem to be very rare[135, 136]. This supports our result that single-type infections are associated with a higher risk of treatment failure than multiple-type infections since they are more likely to integrate into the cell's genome. Overall, the evidence from our study more strongly supported the influence of HPV type vs. number of types as a predictor of treatment failure. In addition, our findings compliment studies that found that persistent infection with HPV16 [45, 57] or HPV18 [57] after treatment was significantly associated with residual/recurrent disease compared to other types. We believe that genotype at treatment, rather than persistence of specific genotypes after treatment, is a more clinically useful risk factor in the prediction of treatment failure since it allows for risk stratification before the start of follow-up. In addition, persistence of certain HPV types is, of course, associated with treatment failure, since all cases of treatment failure will necessarily involve HPV infection. Considering the significance of margins and completeness of excision, it makes sense that a large portion of cases of treatment failure results from the persistence of pre-treatment infection or lesion. Persistence of specific

genotypes acts more like a measure of outcome than a measure of exposure and should be treated as such.

Advanced age is generally considered to be a risk factor for HSIL treatment failure. Three studies had identified being 50 years of age or older as a significant risk factor for treatment failure[32, 39, 51]. Two studies that analyzed age as a continuous variable found that as age increased, the risk of residual, recurrent or invasive disease after treatment increased [46, 50]. Our results do indeed suggest that the odds of treatment failure increase with age, though the crude and adjusted ORs per 5-year increase in age were not statistically significant. We further explored age, using 40 years and older and 50 years and older as thresholds. In both cases, dichotomizing the variable did not result in a significant association between age and HSIL treatment failure. We believe that our sample size, and the limited number of older women in our study population (4% 50 years or older), prevented us from identifying a statistically significant association between age and treatment failure, but that a positive association is indeed present. Others also found that age was not significantly associated with HSIL treatment failure, even if a positive relationship was observed [26, 28, 31, 34, 36, 37, 41, 43, 101]. Ghaem-Maghami et al. suggested that the significance of older age in other studies was due to confounding caused by the location of the upper limit of the lesion, which has often receded into the endocervix in older women[34]. They explained that a sufficiently deep excision in older women would successfully remove the entire lesion and produce a low risk of treatment failure. We did not have data on the depth of the excision in our study and therefore could not explore this theory. The covariates we included in the adjusted model were indeed correlated with age (Chapter 4, Spearman correlation matrix). However, they did not act as true confounders since the estimated crude OR and adjusted OR were identical.

Although parity and use of hormonal contraception have been associated with a higher risk of cervical cancer in HPV positive women [132], we did not find any association between these risk factors and HSIL treatment failure. Other studies had also found no association between parity and hormonal contraception and treatment failure [37, 38, 42,

45, 46, 53]. For parity, the shape and direction of the association changed when we controlled for age, number of sex partners and condom use. In univariate analysis, the estimated risk increased as parity increased. However, in multivariable analysis, we observed a U-shaped relationship between parity and treatment failure. In order to better understand this change, we estimated ORs including only one of the covariates at a time (**Annex 5**). We found that age was responsible for the change in direction and shape of the relationship. With increased age, women are more likely to be multiparous. Our results for increasing parity were rather in the protective direction. This contradicts our hypothesis that hormonal and physiological changes to the cervix that occur later in pregnancy and during birth could increase the risk of treatment failure. While the distribution of parity within our population was not surprising considering that Canadian women overall have a low fertility rate (1.54 live births per woman in 2016 [137]), we must note that parity was low in our study population. Only 2% of women reported more than 3 deliveries, therefore preventing us from investigating the effect of higher parity.

We had suspected that being a current smoker might influence the risk of HSIL treatment failure. However, our results showed no association. Our findings are consistent with a few small studies that found that being a smoker and number of cigarettes smoked were not significantly associated with residual/recurrent disease [46, 50, 53]. Zivadinovic et al. found, however, that being a smoker in conjunction with HPV positivity within one year of treatment increased the risk of relapse with CIN3 and microinvasive cancer ($p<0.01$). We believe that it is important to note that HPV positivity post-treatment may act as a surrogate measurement for recurrence, biasing their interpretation. In addition, they did not adjust for confounders. A larger (77 cases, 154 controls), more robust study that investigated smoking and treatment failure found that being a current smoker was significantly associated with treatment failure (OR= 3.17, 95% CI 1.68-5.91) and observed a dose-response relationship [25]. In their study, an increase of 10 cigarettes smoked per day (from 0 to 30 cigarettes) was associated with an increased odds of treatment failure (OR=2.58, 95% CI 1.70-3.91). They controlled for HPV infection post-treatment, but not for sociodemographic or behavioral confounders. We did not have data that would have permitted us to investigate the dose-response effect that was observed in

this prior study. On the other hand, we adjusted for behavioral confounders that were strongly correlated to smoking in our population (number of sex partners and method of contraception). The results of our adjusted analysis (OR=1.02, 95% CI=0.51-2.05) tended towards the null value compared to our unadjusted analysis (OR=1.67, 95% CI=0.62-2.19), which supports the presence of confounding. Behavioral confounders were not accounted for in any prior study of smoking and HSIL treatment failure. It is therefore possible that the positive association observed by other investigators was the result of confounding bias.

We did not find a significant association between diagnosis on LEEP and HSIL treatment failure. However, the OR for CIN3-CIS-AIS compared to CIN2 suggested that the more severe disease might be associated with an increased risk of treatment failure. Our results were limited by the proportion of participants for whom the severity of HSIL was not specified (41% of cases and 35% of controls). We suspect that the majority of HSIL that were not specified represented more severe disease since the OR for this group compared to CIN2 was even greater than what was observed for CIN3-CIS-AIS. Often, CIN2 are actually over-diagnosed CIN1 and not true precancers. Whereas the newer HSIL classification requires pathologists to further examine CIN2 for the presence of the p16 biomarker and to downgrade CIN2 p16- to LSIL and classify CIN2 p16+ as true HSIL[55]. Our data indirectly supports the use of the HSIL terminology for the staging of disease. Estimates for both of our comparison groups were dampened when we adjusted for age, which was expected since, based on the natural history of the disease, older women are more likely to have more severe or invasive lesions and are more likely to fail treatment.

Our results do not support the use of HPV16 or HPV18 viral load as a predictor for HSIL treatment failure. We explored several ways of analyzing the variable (continuous, dichotomized with a threshold of 0.11copies per cell, 0.22 copies per cell, and each type's median number of copies per cell). We noticed that, while the initial threshold of 0.11 copies per cell may have been predictive of HSIL in another study [124], it may not have been optimal in a population with confirmed HSIL. We explored the effects of doubling

the threshold to 0.22 copies per cell, using the median of each type as the threshold (HPV16: 4.46 copies/cell, HPV18: 0.32 copies/cell), and analyzing the variable as continuous after a logarithmic transformation. In all cases, no association was found. There were 10 previous studies estimating the association between viral load and treatment failure. All prior studies used semi-quantitative HC2 in order to measure viral load in Relative Light Units (RLU). Our study was the first to use quantitative qPCR which is more sensitive and more specific than HC2 [138]. The latter produces less precise typing results and is less sensitive, failing to detect low concentrations of HPV [138]. In addition, qPCR takes into account the number of cells in the tested specimen and provides a number of copies per cell and is type-specific, whereas HC2 does not account for the number of cells present and detects the presence of multiple hr-HPV types at once. This means that HC2 results may be skewed when multiple high-risk types are present. Despite the different measures used, our results are consistent with four previous studies that did not observe an association between viral load and treatment failure[28, 43, 99, 101]. Six other studies found that there was a positive association between high pre-treatment viral load or high pre- and post-treatment viral load and treatment failure [26, 35, 37, 47, 114, 115]. The identified studies used a wide range of different threshold to distinguish those with high from low viral load. The chosen threshold did not seem to influence the likelihood of obtaining significant results. High HPV viral load is associated with acute infections and can trigger the host's immune response [139]. Latent infection and chronic infection with HPV have been characterized but are not yet fully understood. It is possible that that some HPV infections maintain a low copy number in order to establish themselves as a persistent latent infection and avoid the host's immune response. In addition, the integration that favors abnormal cell proliferation and oncogenesis is usually monoclonal[135, 136]. Technically, a single integrated copy of the virus could be associated with oncogenesis and treatment failure.

We did not find any prior investigations of HPV variants and treatment failure. Our results suggested that prototype-like variants of HPV16 were associated with an increased odds of treatment failure. Prototype-like variants were also the most common in our population with HPV16, compared to the prototype and non-prototype-like

variants. We suppose these types may have acquired the ability to evade the body's immune response more effectively. The substitution of adenosine for guanine at position 7521 was the most frequent variation from the reference sequence (43 cases and 25 controls). An adjusted OR of 1.65 (95% CI 0.78-3.47) suggested that this particular variation in the LCR may increase the risk for treatment failure compared to the reference sequence. This association has never been reported and should be investigated in other populations. We did not find evidence to support any association between variants of HPV18 and treatment failure. It is of note, however, that our analysis was limited to only 6 cases and 1 control and is not at all conclusive on the matter.

7.3 Strengths

A total of 30 studies that investigated HSIL treatment failure were identified, most having very small sample sizes and analyzing a limited number of risk factors. Of those studies, only three had a larger sample size than our study. One of those was a large Swedish database cohort (N=132 493) that only estimated the association between age and long-term survival after treatment and controlled only for the time period of the diagnosis and time since diagnosis [48]. They concluded that the long-term risk of dying from cervical or vaginal cancer after treatment for CIN3 is strongly increased in women older than 60 years of age and that treatment at a later age enhances this increased risk. While we did not have such long-term data and did not have such a wide range of ages within our study population, we did find that risk of treatment failure seemed to increase with age. Another study had N=3385 and had 417 cases but used only univariate analysis to estimate the association of a few risk factors with treatment failure and only provided p-values [32]. They concluded that women 50 years of age and older ($p<0.01$), along with those with positive margins ($p<0.01$) were at a higher risk for recurrent CIN. The third had just under 2500 participants and 189 cases but only focused on clinical risk factors of treatment failure and used a combination of histological and cytological diagnoses to define treatment failure[34]. Their main conclusions were that a higher grade of CIN on treatment and involvement of the endocervical canal were the factors most significantly associated with high-grade disease after treatment. Amongst those whose lesion extended into the endocervical canal, insufficiently deep biopsy and incomplete excision/positive

margins were the most important predictors of the same outcome. Of these clinical risk factors, we only studied margins. Our results also pointed to the significance of margins, though regardless of the involvement of the canal. In comparison to the literature, our study looked at a very wide range of risk factors, controlling for all correlations between those risk factors, and had a larger sample size than most studies.

Our source population, the CoHIPP cohort, was recruited in 13 treatment centers across Canada and the participation rate was high. A strength of our study is therefore that it should be representative of women treated for HSIL in Canada. In addition, our investigation used precise laboratory tests and protocols. The use of Linear Array to genotype and qPCR to quantify viral load provided much more precise data than HC2 used in prior studies. We also had access to a highly experienced lab that specialized in HPV, which limits issues of contamination or other technical problems [140].

7.4 Limitations and bias

7.4.1 Precision

Despite being larger than most prior studies to have investigated predictors of treatment failure, this study was still limited by sample size, with only 101 cases of treatment failure identified in the CoHIPP cohort. This limits the statistical power of our findings. The nested case-control design should have produced estimates very similar to those that would have been obtained on the entire cohort. There was little missing data overall, however results may have been biased for variables with higher proportions of missing data such as condom use (11.88%) method of contraception (11.39%) and margins (20.79%). In the case of some potential risk factors, condom use for instance, variables could not be used in their original questionnaire format because of very low frequencies of certain categories and had to be recategorized in order to perform the logistic regression. Loss of information is inevitable when forced to combine categories. In addition, our study was limited by the fact that the baseline questionnaire occurred 6 months after treatment. In fact, for condom use and number of sex partners, “in the last year” included 6 months prior to treatment and not a full year. This could have also affected measurement of current smoking status, since participants may have modified

their behavior since treatment. In addition, our measurement of smoking status is very crude and may have been more informative if we had dose-related measures such as pack-years. Finally, our analysis of the diagnosis on LEEP was limited by the proportion of subjects for whom the exact grade of lesion was not specified.

7.4.2 Selection bias

Firstly, the CoHIPP cohort had an extremely low rate of loss to follow-up (2%), limiting the selection bias in our source population. Selection bias was also taken into account during the planning stage of this study. The study design, a nested case-control, calls for sampling from within a very well-defined cohort. Cases and controls both came from the same source population and should not have been different on the basis of exposure. This source population was recruited in treatment centers across Canada and participants were matched on treatment center and on time. This should have limited biases that could be caused by different protocols and follow-up between provinces and over time.

7.4.3 Information bias

Since our questionnaire data came from a prospective cohort, and baseline questionnaires were answered before the onset of treatment failure, we did not have to contend with the possibility of recall bias. However, the questionnaire used to measure baseline exposures was administered six months after the treatment visit, at the first follow-up visit. This may have influenced the exposure levels reported by study participants who may have modified their behaviors since their treatment. In the case of condom use and number of sexual partners, patients were asked to report their behavior in the last year. Because of the timing of the questionnaire, this did not include the full year prior to treatment, but 6 months pre- and 6 months post-treatment. The potential bias due to the timing of the questionnaire was likely non-differential since participants did not know if they had treatment failure at the time. Certain factors related to sexual history, such as number of sexual partners in the last year, are associated with social stigma, which may lead to underreporting. However, since questionnaires were administered before the onset of recurrent disease, cases should have reported any erroneous information at approximately the same rate as controls. In addition, questionnaires were administered in the presence of

a nurse at the time of a medical visit, when patients may be more likely to be honest about sensitive topics as it pertains to their health. Finally, laboratory technicians did not know the status of specimens being analyzed. All specimens were analyzed in a certified lab, following the same protocols.

7.4.4 Confounding and Interactions

Causal associations with treatment failure have not yet been identified, which made it difficult to choose appropriate confounders for our models. In our study, we supposed that any one of our independent variables could be associated with treatment failure. True confounders must be associated with both the predictor of interest and HSIL treatment failure. We included confounders in each multivariable model that were associated with the predictor of interest. In this way, we evaluated only one axis of confounding and assumed that the identified confounders were also associated with the outcome. This allowed us to control for confounding that was specific to our study population. However, we may have over-adjusted in certain cases where an overly large number of confounders were included in some models compared to the sample size. This resulted in a loss of precision with no decrease in net bias, such as in the adjusted model for age. In the case of age, adjusting for correlated variables did not change the OR and confounding was therefore not truly present, but the 95% CI became slightly wider indicating a loss of precision compared to the crude estimate.

In addition, we suspected that smoking status or that margin involvement may modify the effect of genotype on HSIL treatment failure. We hypothesized that being a current smoker may increase the effect of being HPV16+ and/or HPV18+. We also hypothesized that having positive margins may increase the effect of being HPV16+ and/or HPV18+. However, we did not observe any effect modification for either of these variables.

7.5 Conclusions

In our nested case-control study we estimated the association between various risk factors and HSIL treatment failure. We identified positive margins and genotypes HPV16 and/or HPV18 as significant predictors of HSIL treatment failure. In addition, it is possible that prototype-like variants of HPV16, especially those with a substitution at position 7521, are associated with a higher risk of treatment failure than the prototype and non-prototype like variants. Further studies on variants are required in order to confirm or refute this possibility. We did not have the sample size to produce estimates for HPV18 variants, despite the type's clinical significance.

The predictors identified in this study were not modifiable, but instead have clinical and public health implications. The identification of predictors of HSIL may allow for risk stratification of patients after treatment. This could allow for closer surveillance of those at high risk, while reducing visits and interventions for those at a very low risk of treatment failure. In turn, this could also allow for better allocation of the health system's resources, both financial and personnel. This study emphasizes the importance of closer follow-up for those with positive treatment margins. However, considering the high proportion of patients with positive margins who do not experience treatment failure and the serious future obstetrical risks from repeat treatment, we do not recommend automatic repeat treatment for those with positive margins. Considering the important role that HPV16 and HPV18 play in HSIL treatment failure, high coverage vaccination programs should play an important part in reducing the incidence of treatment failure.

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Annexes

Annex 1. Association between dichotomized viral load and treatment failure by logistic regression, doubling the threshold

Type	Viral load (copies/cell)	Cases n (%)	Controls n (%)	p-value
HPV16	≤0.22	10 (13.89)	7 (12.28)	0.79
	>0.22	62 (86.11)	50 (87.72)	
	Total	72	57	
HPV18	≤0.22	2 (33.33)	2 (40.00)	0.82
	>0.22	4 (66.67)	3 (60.00)	
	Total	6	5	

Annex 2. Logistic regression analysis of HPV16 viral load, doubling the threshold

Type	Viral load (copies/cell)	Crude OR (95% CI)	Adjusted OR* (95% CI)
HPV16	≤0.22	1	1
	>0.22	0.87 (0.31-2.44)	0.81 (0.28-2.36)

*Adjusted for age

Annex 3. Association between dichotomized viral load and treatment failure by logistic regression, median as threshold

Type	Viral load (copies/cell)	Cases (N=72) n (%)	Controls (N=57) n (%)	p-value
HPV16	≤4.46	35 (48.61)	29 (50.88)	0.087
	>4.46	37 (51.39)	28 (49.12)	
	Total	72	57	
HPV18	≤0.32	3 (50.00)	2 (40.00)	0.90
	>0.32	3 (50.00)	3 (60.00)	
	Total	6	5	

Annex 4. Logistic regression analysis of HPV16 viral load, median as threshold

Type	Viral load (copies/cell)	Crude OR (95% CI)	Adjusted OR* (95% CI)
HPV16	≤4.46	1	1
	>4.46	1.10 (0.55-2.19)	1.04 (0.50-2.16)

*Adjusted for age

Annex 5. Sensitivity analysis of parity and its covariates

Predictor	Adjusted OR (95% CI)	Covariates in model
Parity		Age, number of sexual partners, condom use
0	1	
1	0.49 (0.19-1.34)	
2 or more	0.87 (0.31-2.45)	
Parity		Age and number of sex partners
0	1	
1	0.59 (0.22-1.43)	
2 or more	0.81 (0.32-2.10)	
Parity		Age and condom use
0	1	
1	0.64 (0.28-1.46)	
2 or more	0.85 (0.35-2.03)	
Parity		Number of sex partners and condom use
0	1	
1	1.01 (0.45-2.27)	
2 or more	1.79 (0.76-4.20)	
Parity		Age
0	1	
1	0.66 (0.29-1.47)	
2 or more	0.85 (0.38-1.91)	
Parity		Number of sex partners
0	1	
1	1.17 (0.55-2.51)	
2 or more	1.79 (0.83-3.82)	
Parity		Condom use
0	1	
1	0.98 (0.47-2.04)	
2 or more	1.48 (0.70-3.13)	