HPV GENOTYPE DISTRIBUTION AND ONCOGENE EXPRESSION IN HIV-POSITIVE ADULTS AND THE UNDERLYING RISK FACTORS FOR ANAL, ORAL AND GENITAL MALIGNANCY:

AN ATLANTIC CANADA PROSPECTIVE COHORT STUDY.

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ABSTRACT

BACKGROUND:

Human Papillomavirus (HPV) is the most common sexually-transmitted agent. Infections with oncogenic HPV types 16 & 18 are causally linked to the development of cervical cancer, as well as a proportion of anal, oropharyngeal, vulvar, vaginal and penile cancers and their associated precancerous lesions. Immune suppression increases the likelihood of HPV-related diseases, and people with human immunodeficiency viral (HIV) infection or with HIV-positive partners are at a higher risk of precancerous lesions and cancers, as well as genital warts.

OBJECTIVES:

Determine the prevalence and distribution of high risk (HR) oncogenic HPV genotypes in HIV-positive adults in Atlantic Canada. Examine association between HR HPV genotypes and premalignant lesions and incident malignancy; Examine association between premalignant lesions and malignancy and patients' demographics and underlying risk factors.

METHODS:

This prospective cohort study was designed for four years in Atlantic Canada HIV care clinics. Total 300 were enrolled in the study and of them, 263 were included in the final analysis. Participants were required to complete a confidential questionnaire to obtain demographic and risk factor data. Annual collection of oropharyngeal and anal swab specimens from all participants and an additional cervical specimen from females were obtained. All specimens were tested for cytologic abnormalities, HPV DNA and HPV genotyping. The ASIR of the incident cancers was calculated using the Canadian general population as reference.

RESULTS:

Of 263 patients 93.2% were males. The mean (SD) age of the study population at the enrollment time was 46.9 (9.4) years and 51.3 (9.1) years at the study's end. A total of 227 (86.3%) participants were positive for HPV infection. Of these, 88.1% had HPV infection at one body site and 11.9% had HPV genotypes detected at two body sites simultaneously. Up to 50 HPV genotypes were detected, of which 32 (63%) were HR oncogenic types. Eight (16%) HPV types were significantly associated with the confirmed 31 (11.8%) cases of precancerous lesions and 8 (3.3%) incident cases of malignancy. The precancerous lesions

significantly associated with patients CD4 cell count < 200 cells/mL (p=0.034), smoking (p=0.007), history of anogenital warts (p=0.002) and genital herpes (p=0.007).

CONCLUSIONS:

The overall incidence of cancer was 3.3%, all of them diagnosed in males. The ASIR (95%CI) of anal cancer is 535/100,000 (30-970) and ASIR (95%CI) of oral cancer is 533/100,000 (30-970).

KEY WORDS:

Human papillomavirus infection, anal cancer, cervical cancer, head and neck cancer, squamous cell carcinoma, HPV genotyping, HPV prevalence and incidence, HPV and malignancy, HPV risk factors, HIV-HPV co-infection, prevalence of cancer in MSM.

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List of Abbreviations

ACHIVE Atlantic Canada HIV/AIDS Education

AGC Atypical Glandular Cells

AIDS Acquired Immune Deficiency Syndrome

AIN Anal Intraepithelial Neoplasia

AIRN Atlantic Interdisciplinary Research Network

ASC Atypical Squamous Cells

ASCCP American Society for Colposcopy and Cervical Pathology

ASC-H Atypical Squamous Cells, cannot exclude HSIL

ASC-US Atypical Squamous cells of Undetermined Significance

ASIL Anal Squamous Intraepithelial Lesion

CC Cervical Cancer

CDC Centres for Disease Control

CD4 A major classification of T lymphocytes, referring to those that carry the CD4 antigen, also called CD4 T lymphocytes

CI Confidence Interval

CIN Cervical Intraepithelial Neoplasia

CIS Carcinoma *in situ*

CMAJ Canadian Medical Association Journal

DNA Deoxyribonucleic Acid (HPV DNA)

EUROGIN European Research Organization on Genital Infection and Neoplasia

E6/E7 Oncogene lineages of Human Papillomavirus type 16 E6, E7 in pre-invasive and invasive cervical squamous cell carcinoma

E6-AP E6-Associated Protein

GI Gastro-Intestinal

HAART Highly Active Antiretroviral Therapy

HGAIN High-Grade Anal Intraepithelial Neoplasia

HIV Human Immunodeficiency Virus

HIV RNA Plasma viral load with HIV Ribonucleic Acid

HNSCC Head & Neck Squamous Cell Carcinoma

HPV	Human Papillomavirus
HR	High Risk
HRA	High Resolution Anoscopy
HSIL	High-Grade Squamous Intraepithelial Lesion
IARC	International Agency for Research on Cancer
ICH	International Committee on Harmonization
ID	Infectious Disease
IR	Incidence Rate (Unadjusted)
ISH	In situ Hybridization
IU	International Unit
JAMA	Journal of the American Medical Association
LA	Linear Array Assay
LabNet	Laboratory Network
LBP	Liquid-Based Preparation
LSIL	Low-Grade Squamous Intraepithelial Lesion
MB	Manitoba
MLR	Multivariate Logistic Regression
MSM	Men who have sex with men
NBM	New Brunswick, Moncton
NBSJ	New Brunswick, Saint John
NCI	National Cancer Institute
NLSJ	Newfoundland and Labrador, St. John's
NML	National Microbiology Laboratory, Winnipeg, MB
NSH	Nova Scotia, Halifax
NYS DOH	New York State Department of Health
OR	Odds Ratio
OSCC	Oral Squamous Cell Carcinoma
P	P-value
Pap	Papanicolaou test or smear
PCR	Polymerase Chain Reaction

PEI	Prince Edward Island
PHAs	People living with HIV/AIDS
PHAC	Public Health Agency of Canada
PHL	Public Health Laboratory in St. John's, NL
PGMY/GP	Primers that were used in Luminex-Based Assay
PR	Prevalence Rate
RN	Ribonucleic Acid, HIV viral genome
RR	Relative Risk
SIR	Standardized Incidence Ratio
STI	Sexually Transmitted Infection
TBS	The Bethesda System
TZ	Transformation or Transitional Zone
ULR	Univariate Logistic Regression
VL	Plasma Viral Load

CHAPTER 1

INTRODUCTION

1.1 Introduction to the Thesis

Human Papillomavirus (HPV) infection is believed to be one of the most common sexually transmitted infections (STI) in Canada and around the world.

The Centre for Disease Control (CDC) estimates that at least half of all sexually active individuals will acquire HPV at some point in their lives (CDC Fact Sheet, 2011); however this viral infection usually clears by itself and causes no signs or symptoms.

The greatest risk factors for infection with HPV in the general population are young age and sexual activity. Besides these factors, other risk factors for HPV infection and clinical sequelae of infection include high number of sexual partners, smoking, and co-infection with *Chlamydia trachomatis* and/or *Herpes Simplex Virus* (HSV2) which is also called *Human Herpesvirus 2* (HHV-2) (Canadian Cancer Society (CCS), Risk Factors for Cervical Cancer, 2015). HPV transmission occurs directly through genital-to-genital, orogenital, and anogenital contact, and infrequently through hand-to-genital contact (Ryan DP et al, 2000). HPV can also be contracted through vertical (from mother to child) and fomite (via inanimate objects such as sex toys) transmission (Mayeaux EJ Jr & Spigener SD, 1997). Condoms do not prevent transmission since HPV may infect the base of the penis or the upper thigh areas not covered by condoms (Goldstone S, 1999). The incubation period of HPV is usually 6 weeks to 8 months, but can be as long as several years. Most HPV infections are subclinical; hence asymptomatic men may act as an HPV reservoir because the virus can live in latent form in the urethra or prostate gland (Frega A et al, 2013).

While HPV infection is mostly transient in the majority of individuals and does not lead to disease, immune suppression increases the risk of HPV (CCS, 2015). Those with human immunodeficiency viral (HIV) infection or with HIV-positive partners are at a higher risk of precancerous lesions and cancers as well as genital warts (Kreuter A. and Wieland U., 2009). Furthermore, for those who have HIV infection, there is a heightened risk of rarer and/or more aggressive forms of cancer which tend to be more advanced, occurring in younger age with poorer prognosis. They are also more likely to spread to unusual sites (Jensen et al., 2007; Lillo & Uberti-Foppa, 2006; Nicol et al., 2005; Palefsky et al., 1999; Schlecht et al., 2005).

1.2 Epidemiology of HIV-HPV Co-Infection

Transmission of HPV infection occurs primarily by skin-to-skin sexual contact and HPV is prevalent in all sexually active populations. Epidemiologic studies indicated that the risk of contracting HPV infection in the general population is influenced by: sexual activity itself; sexual activity at an early age; multiple sexual partners at any time of life, or being the partner of someone who had multiple sexual partners; and personal history of other sexually transmitted diseases. The primary immune response to HPV infection is cell-mediated; therefore, conditions that impair cell-mediated immunity such as human immunodeficiency viral disease (HIV) should increase the risk of acquisition and progression of HPV infection. Compared to HIV-seronegative men, infection with HIV is an additional risk factor for developing anal cancer, with relative risks (RR) for men seropositive for HIV of about 60 for *in situ* anal cancers, and about 40 for invasive anal cancers (Frisch et al., 2000).

It also was reported that compared to men who have sex with men (MSM) seronegative for HIV, those who are seropositive have a 2-fold higher risk of anal cancer (Goedert et al., 1998). Several studies examined the changes in the incidence of anal cancer and the introduction of highly-active antiretroviral therapies (HAART) (Bower et al., 2006; Hessol et al., 2007; D'Souza et al., 2008; and Piketty et al., 2008). The trends reported in the studies were consistent in that HAART therapy did not appear to have reduced the occurrence of anal cancer, as it did for other AIDS-related malignancies such as Kaposi's sarcoma and non-Hodgkin lymphoma (Bower M et al, 2004; Diamond C et al, 2005). In the largest study (Chiao EY et al, 2005) involving general population-based cancer registries, anal cancer incidence increased from 0.6 to 0.8 per 100,000 between 1973 and 2001. In the HAART era, there was a significant increase in incidence rates in both men and women, although more so in men. In 2006, Lampinen and colleagues reported that the increased risk of anal cancer among HIV-positive MSM can be as high as 140-fold when compared to HIV-positive men who are not practicing sex with men. The risk of HPV-associated anal cancer is 163-fold greater in young men with HIV than in young HIV-negative men (Breese P. et al, 1994). The progression of atypical squamous intraepithelial lesion (ASIL) to invasive anal cancer is influenced by a number of factors including: HIV seropositivity, infection with multiple HPV serotypes, and a high level of DNA of high-risk HPV genotypes (Uronis & Bendell, 2007). Cervical cancer is by far the most frequently recognized HPV-associated cancer, with an association with HPV 16 and HPV 18 (Clifford et al., 2003; Pretet et al., 2008). Many studies have shown that HIV-positivity is associated with an increased prevalence of cervical HPV infection and cervical intraepithelial neoplasia (CIN) (Palefsky J, 2006).

Women with HIV or in a relationship with HIV-positive individuals are at increased risk for anal and cervical cancer as well as genital warts. In women who are HIV-HPV co-infected, lesions tend to be high-grade with a shortened interval between infection and invasive cancer (Apgar BS and Brotzman G, 1999). HIV-HPV co-infection is thought to increase the risk of anal carcinoma by 30 times (Sobhani et al, 2004), and these women have a 6.8-fold greater risk of invasive anal cancer when compared with HIV-negative women (The National Cancer Institute Women's Health Report, 2007).

HPV 16 is found in an even higher proportion of anal cancers than cervical cancers (Fox P, 2006). Likewise, anal cancer may be preceded by a series of precancerous lesions, known as anal intraepithelial neoplasia (AIN). The incidence of anal cancer is elevated in HIV-negative MSM and is even higher among HIV-positive MSM (Chin-Hong PV & Palefsky JM, 2005). A recent review conducted by the International Agency for Research on Cancer (IARC) concluded that while cervical cancer is virtually entirely related to HPV, other sexually-transmitted rare cancers are also associated with HPV to a varying extent: penile cancer at 40%, anal at 90%-95%, vulvar/vaginal at 40% and oropharyngeal at 12% (Munoz N. et al, 2006). The current estimated worldwide burden of cancer cases caused by HPV, and by HPV 16 and 18, is 5.2% and 3.7%, respectively (Parkin DM, 2006).

As a result of this evidence, a number of studies have recommended that all HIV-infected individuals should be screened for HPV-related disease for early detection and treatment given the heightened risk of persistent HPV infection, malignant transformation, widespread disease and frequent recurrences (Palefsky JM, 2005).

1.3 Laboratory Definition of HPV infection

Human Papillomavirus (HPV) is a virus that can lead to abnormal tissue growth (warts) and result in changes to the affected cells. Persistent infection with certain types of HPV can lead to cervical cancer, as well as anal, vaginal, vulvar, penile and oropharyngeal cancers (National Cancer Institute (NCI) Dictionary, http://nci.nih.gov/dictionary/).

The HPV family is ubiquitous in the human population and more than 140 virus types have been identified (de Villiers et al., 1997). The viruses are small double-stranded DNA viruses with a genome of approximately 8kb that specifically target the basal cells of the epithelial mucosa (zur Hansen & de Villiers, 1994) and the metaplastic cells at the squamocolumnar junctions of the cervix (transitional zone - TZ) and anus (above dentate line). Additionally, HPV may infect the glandular epithelium of the endocervix, resulting in neoplasms, such as adenocarcinoma *in situ* or invasive adenocarcinoma. Visually-detectable HPV infections may manifest as warts. A history of anal warts (Condyloma Acuminata) increases the risk of anal squamous cell carcinoma 10-fold (Bonnez W & Reichman RC), and approximately 50% of patients with anal squamous cell carcinoma also have a history of anal warts (Ryan DP et al, 2000).

Low-risk HPV types **(6, 11, 34, 40, 42, 44, 53, 54, 55, 57, 61, 70, 71, 72, 81, 84**) are associated with benign lesions such as warts (*Condyloma Acuminata*), while infections with probable high-risk **(26, 53, 66, 68, 73, 82)** and high-risk HPV types (**16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59**) have the potential to progress into malignant lesions.

There is also a large group of Unknown Effect HPV genotypes (**30, 74, 85, 86, 87, 89, 90, CP6108 and IS39).**

1.4 Natural History and Pathogenesis of HPV infection

The overwhelming majority of patients with ano-genital cancer show serological, histopathological or molecular evidence of prior infection with HPV, and viral DNA sequences can be detected in their tumor tissue. Clinical pathological studies also provide strong causal evidence for HR HPV DNA in high-grade anal dysplasia and cancer (Abbasakoor F & Boulos PB, 2005; Gervaz P et al, 2006). In case-control studies using polymerase chain reaction (PCR), a highly sensitive assay for HPV DNA, between 80% and 100% of anal biopsy specimens contained HR HPV DNA, primarily HPV 16 (OHTA Series, 2007).

There is also molecular evidence that high-risk HPV viruses integrate into anal cells (Zbar AP et al, 2002; Martin F & Bower M, 2001). High-risk viruses encode for at least three oncoproteins with growth-stimulating and transforming properties: E5, E6, and E7. The "E" designation indicates that these primary oncogenes are expressed early in the HPV life cycle. Integration of the HPV DNA results in a break in the E1 and E2 regions of the viral genome, leading to a loss of the E2 protein function and subsequent increased gene expression of E6 and E7, whose cooperation is needed to maintain the malignant cell expression *in vitro*. The products of these two genes alter the host cell metabolism to favor neoplastic development. Werness et al (1990) showed that the E6 proteins from HPV 16 and HPV 18 are capable of binding to p53 protein of the host cells. This binding promotes the degradation of p53 via the ubiquitin pathway (Scheffner et al., 1990 & 1993). Subsequent work has shown that the E6-mediated degradation of p53 is dependent upon a cellular protein, called E6-associated protein or E6-AP (Huibregtse et al., 1991 & 1993). An effect of this targeted degradation is to prevent apoptosis of the infected host epithelial cells. The host cells telomerase is also activated, further augmenting oncogenic changes.

A natural consequence of the E6-induced degradation of p53 is the inhibition of both p53 growth and apoptotic functions of the normal cell cycle. Once squamous and squamocolumnar tumor cells metastasize, mutations within p53 become more frequent (Crook & Vousden, 1992). This seems to indicate that the presence of mutant p53 gives cells a competitive advantage over cells in which p53 activity is not abrogated by E6 proteins. This supports the idea that mutants of p53 can have a dominant-negative phenotype. In order to have a productive infection, HPV types must infect keratinocytes in the basal layers of the epidermis. HPV undergoes vegetative replication only in differentiating epithelium, and the virus requires cellular DNA replication proteins in order to replicate its own DNA. Both of these proteins (E6 & E7) are expressed in anal neoplasia (Da Costa MM et al, 2002). The premalignant changes seen in cervical high-grade dysplasia (HSIL) and the greater degree of angiogenesis and apoptosis (natural cell death) than there is in the normal tissue are also seen in high-grade anal dysplasia (Little VR et al, 2000; Mullerat J et al, 2003).

The major steps in the carcinogenesis pathway have been summarized by Moscicki AB and colleagues in 2006. Initially it requires an infection with one or more HR HPVs, and then viral persistence occurs rather than clearance, it follows by clonal progression of persistently infected epithelium to precancers, and finally, leads to invasion into the underlying tissue. The low- and high-risk HPVs differ in their sites of DNA replication within the differentiating epithelium (Doorbar et al., 1997). The low-risk HPV types generally replicate only in the lower levels of the stratified epithelium where the keratinocytes are still undergoing normal cell division. In contrast, the high-risk HPVs replicate their genomes in the higher levels of the epithelium where the keratinocytes would have normally entered the process of terminal differentiation and switched off DNA replication. Thus, the high-risk HPV types stimulate cells to replicate DNA in a more unnatural environment than the low-risk HPVs.

1.5 Tumorigenic Potential of HPV

Human Papillomavirus (HPV) infection is a significant source of morbidity and mortality worldwide. Management entails removal of discrete lesions and monitoring for recurrences. Prophylactic vaccines have become available and hold promise to significantly reduce the burden (morbidity and mortality) associated with HPV infections. A bivalent vaccine (Cervarix) has been formulated to protect against the two most common high-risk HPV types for cervical cancer, HPV 16 and 18. The second, a quadrivalent vaccine (Gardasil) targeting HPV 16 and 18 and the two most common low-risk types, HPV 6 and 11, is widely available in Canada. The vaccines contain papillomavirus-like particles (empty shells of viral structural proteins) that produce a neutralizing antibody response, which is believed to prevent papillomavirus from infecting host cells. The Society of Obstetricians and Gynecologists of Canada estimates that 10% to 30% of the Canadian female adult population is infected with HPV. This is in line with research from the US and Europe which has shown that 10% to 40% of sexually active women are infected by HPV at any one time (http://www.hpvinfo.ca/health-care-professionals/what-is-hpv/incidence-and-prevalence-of-hpv-in-canada/).

Approximately 6.2 million new HPV infections occur in the United States every year. In 2004 alone, approximately more than 20 million individuals were infected (Centers for Disease Control and Prevention, Genital HPV Infection: CDC Fact Sheet, CDCP 2004).

Almost all cases of invasive cancers of the cervix, most other ano-genital tract cancers, and approximately 20%-25% of head and neck cancers contain oncogenic HPV viruses (predominantly types 16, 18, 31, and 45 for cervical and other ano-genital tract cancers, and type 16 for oropharyngeal cancers) (Zur Hausen, 1996; Munos et al., 2003).

In the "Study to Understand the Natural History of HIV/AIDS (SUN) in HIV-positive adults" by Vellozzi C and others (2009), the prevalence of HPV in the cervix and anus was 86% and 93% respectively, and for high-risk HPV types the prevalence rates were 68% and 85%, respectively. A history of anal sex was not predictive of an abnormal anal cytology. These results, although not completely independent of a history of anal intercourse, are explained by the anatomical proximity of the anus and the cervix. Squamous tumors of the anogenital region have similar histological, epidemiological, and pathogenetic properties (Melbye M & Sprogel P, 1991; Dujovny N et al, 2004). The cervix, like the anus, has a transitional or transformational zone with an increased risk of dysplasia. In this area of transition, there is active changeover of columnar epithelium to squamous epithelium through the process of squamous metaplasia. Potentially-precancerous precursors of the epithelium, or dysplasia; are referred to as anal intraepithelial neoplasia or AIN when developing in the anus, and cervical intraepithelial neoplasia or CIN when developing in the cervix. Dysplastic cells have abnormal changes, however they do not show evidence of invasion into surrounding tissue. The most severe form is called carcinoma *in situ*, where the cells appear like cancer cells, but have not invaded beyond the basement membrane (membrane separating epithelium from tissue below).

Intraepithelial neoplasia has been characterized into various grades, low (LSIL) and high (HSIL) based on their potential to progress toward invasive cancer. This process can be accelerated by trauma, healing and repair, such as might be expected to occur in receptive anal intercourse.

Although several malignant forms can occur, squamous occurs most commonly (Rousseau Jr DL et al, 2005).

1.5.1 HPV-related HNSCC (Head & Neck Squamous Cell Carcinoma)

The involvement of HPV in oral and oropharyngeal carcinogenesis was first proposed by Syrianen and colleagues in 1983. Several studies have reported HPV DNA in normal, pre- and malignant oral mucosa, although many of them were small hospital-based cross-sectional studies (Hodge et al., 1985; Hoshikawa et al., 1990; Blot et al., 1994). More recently, larger studies of HPV DNA prevalence in the head and neck mucosa have shown that HPV may be an additional independent risk factor for a subset of HNSCC (Schwartz et al., 1998; Smith et al., 1998, 2004; Herrero et al., 2003; Hansson et al., 2005). Other studies suggested that despite the majority of cases of oral and oropharyngeal cancer being attributed to tobacco and alcohol usage, there may be differences between the tumors that develop in smokers/drinkers and those that develop in non-smokers/non-drinkers (Koch et al., 1999; Wiseman et al., 2003).

A meta-analysis of cases from 1982 to 1997, examining the risk of HPV detection in normal, pre- and cancerous oral tissue, showed that the probability of HPV being detected in mucosa increased along with the degree of dysplasia (Miller and Johnstone, 2001). In a total of 4680 samples from 94 studies, these investigators reported that the pooled probability of detecting HPV in normal oral mucosa was 10% (95%CI 6.1-14.6), in benign leukoplakia was 22% (95%CI=15.7-29.9), in oral intraepithelial neoplasia 26.2% (95%CI 19.6-33.6%), in verrucous carcinoma 29.5% (95%CI 23.0-36.8), and in oral squamous cell carcinoma 46.5% (95%CI 37.6-55.5). HPVs 16 and 18 were detected in 30% of oral squamous cell carcinomas (OSCC), while other high-risk HPV genotypes were detected in less than 1% of head and neck tumors.

There was substantial heterogeneity in detection rates between studies which may be attributed to several factors, including: variations in prevalence between geographic locations of the

studies, between head and neck anatomical sites (Kreimer et al., 2005), and multiple HPV detection methods (polymerase chain-reaction [PCR], *in situ* hybridization [ISH], and others).

Two large studies strengthened the correlation between HPV-associated ano-genital cancers and HNSCC. The study by Frisch and Biggar in 1999 was designed to determine whether there was a risk of tonsillar or other HNSCCs among patients with HPV-associated ano-genital cancers. The risk of tonsillar cancer (RR=4.3, 95%CI 2.7-6.7) or other HNSCCs (RR=2.3, 95%CI 1.7-3.0) was significantly increased in these patients. Patients with cancers unrelated to HPV had a relative risk (RR) close to 1. The study by Hemminki et al. in 2000 investigated the occurrence of second primary cancers in the upper aero-digestive tract among 135,386 women (Sweden Family Cancer Database) who were initially diagnosed with cervical cancer *in situ or* cervical carcinoma. The occurrence of first primary cancers among their husbands was also assessed. The overall standard incidence ratios (SIR) for females with carcinoma *in situ* was 1.86 with the highest SIR attributed to the larynx; and for females with invasive cervical cancer, the overall SIR was 2.45 with the highest SIR attributed to the hypopharynx. Husbands of women with carcinoma *in situ* and with invasive cervical cancers had an overall SIRs of 1.43 and 1.37 respectively, with the highest SIR attributed to the tonsils. Men are slightly more prone to HPV-related oral tumors than women. A research published in the *Journal of Clinical Oncology* in February 2008 reported that the incidence of HPV-related oral squamous cell carcinomas in the United States increased significantly from 1973 to 2004, particularly among white men at younger ages. The increases might have been the result of changing in these young men's sexual behavior (Harrington K, 2015).

The literature associating oral SCC and HIV infection is limited to a few case series, which show a younger age group (median age 40–45 years), more advanced local disease, and a higher tumor stage compared with non-HIV-positive oral SCC patients (Singh B et al, 1996; Roland JT Jr et al, 1993). An epidemiological study conducted by UK researchers in Kenya (Butt FMA et al, 2012) identified 200 HIV-positive patients with an orofacial malignancy, of whom16 (8.0%) had oral SCC. The female-to-male ratio was approximately 1:1, and the age range was 17 to 43 years (mean age 31.7 years). The majority (68.8%) of their patients denied using tobacco or alcohol, while the remainder (31.2%) used one or both. The oral cavity sites affected were the tongue or the floor of the mouth (62.5%), the buccal mucosa (12.5%), the lower lip (12.5%) and the maxillary or mandibular alveolus (12.5%). The majority (62.5%) of HIV-positive patients had TNM stage III or IV disease (Tumor, Node, Metastasis system for cancer staging created by the American Joint Committee on Cancer [AJCC]), while the rest (37.5%) had stage I or II disease. Evidence supports the idea that HNSCC is a multifactorial disease with at least two pathways, one driven by smoking and alcohol consumption, with another driven by HPV. Although these pathways are possibly distinct, HPV infection and smoking are not mutually exclusive (Braakhuis et al., 2004; Ragin et al., 2004; Ferris et al., 2005).

1.5.2 Anal Cancer in HIV-positive Adults

Anal cancer is similar to cervical cancer biologically, including a causative association with human papillomavirus (Hoots, Palefsky et al., 2009). Within the anal canal the squamocolumnar junction (TZ) is anatomically very similar to the cervical squamocolumnar junction (TZ) on the cervix; these junctions are typically areas of squamous metaplasia, which are also present on the cervix.

These areas are especially susceptible to the oncogenic effects of HPV. It is in these areas that the basal cells are often closest to the surface which can facilitate infection by HPV.

The equivalent of high-grade CIN, high-grade anal intraepithelial neoplasia (HGAIN), is known to progress to anal cancer (Watson et al., 2006). Several recent population-based studies showed that anal cancer rates have been increasing, and that the trend has been particularly dominant in urban populations, particularly those centres with high concentrations of homosexual males or men who have sex with men (MSM). Increasing rates have been reported in Copenhagen (Frisch M, et al 2003), London (Newsom-Davis T & Bower M, 2010) and San Francisco (Cress RD & Holly EA, 2003; Palefsky JM et al, 2005). The highest increase in anal cancer was reported in San Francisco, with rates in men aged 40 to 64 years increasing from 3.7 to 20.6 per 100,000 from 1996 to 1999 (Chris RD & Holly EA, 2003). Human papillomavirus (HPV), a common sexually-transmitted disease that is almost universal in men infected with human immunodeficiency virus (HIV), is associated with the development of anal squamous cell carcinoma (Frisch M et al, 1993; Bower M et al, 2004; Cress RD & Holly EA, 2003 and Palefsky JM et al, 2005). The incidence of anal squamous cell carcinoma in HIV-positive men who have sex with men doubles that of HIV-negative MSM (Diamond C et al, 2005; Patel HS et al, 2007). HIV-positive MSM who practice receptive anal intercourse are twice as likely to develop anal squamous cell carcinoma as HIV-negative MSM. The incidence of HPV-associated anal cancer is high among HIV-positive MSM, and possibly in HIV-positive women (Kreuter A. et al., 2008, Shack L. et al, 2014). The risk of anal cancer compared with the general population, is elevated 24-fold in HIV-infected women, 32-fold in HIV-infected men, and 52-fold in HIV-infected MSM (Chaturvedi AK et al, 2009 & Shiels MS et al, 2009).

For women, the occurrence of anal cancer is linked to their risk for other cancers in the anogenital region. Cancer occurring anywhere in the anogenital region puts women at increasing risk for other primary or secondary cancers in the region, a phenomenon referred to as a *field cancerization* (Slaughter DP et al, 1953). Two larger cancer registry-based studies, one in the UK (Evans HS et al, 2003) involving 145,621 person-years of follow-up from 1960 to 1999, and one in Sweden (Hemminki K et al, 2000) that followed 135,386 women from 1958 to 1996, found significantly increased risks for other genital cancers after an initial diagnosis of cervical cancer. In the UK study, rates for secondary primary cancers after an initial diagnosis of cervical cancer were increased for the vagina (SIR=8.0, 95%CI 4.4-13.5), anus (SIR=6.3, 95%CI 3.7-10.0), and vulva (SIR=1.9, 95%CI 1.0-3.3). In the Swedish study, increased rates for second cancers in the anogenital region after a primary cervical cancer were also reported, with the highest being for anal cancer (SIR=4.8, 95%CI 3.7-6.0).

A broad-based cancer and acquired immune deficiency syndrome (AIDS) registry linkage study examining the relationship of all HPV-related cancers in patients with AIDS reported significantly increased risks of HPV-related cancers in men and women (Frisch M et al, 2000). The RR for anal cancer was significantly higher for men than for women for invasive lesions (RR=37.9, 95%CI 33.0-43.4 vs. RR=6.8, 95%CI 2.7-14.0), *in situ* precursor lesions (RR=60.1, 95%CI 49.2-72.7 vs. RR=7.8, 95%CI 0.2-43.6) and anal cancers. Although homosexuals with HIV exposure had the highest RR for anal cancer (RR=59.5, 95%CI 51.5-68.4), both male (RR=5.9, 95%CI 2.7-11.2) and female (RR=7.3, 95%CI 1.5-21.4) intravenous drug users also had an increased RR for anal cancer.

Highly active antiretroviral therapy (HAART) was introduced for widespread use in 1996, and since then the incidence of anal cancer has dramatically increased in the HIV-positive population, now exceeding the highest incidence of cervical cancer among women reported anywhere in the world (Palefsky JM. et al, 2005).

Three previously conducted studies reported a high incidence of anal cancer among HIV-positive MSM since 1996. One study was conducted by Piketty et al. and showed an incidence of anal cancer from a cancer registry in France of 75/100,000 person-years among HIV-positive MSM since 1999 (French Hospital Database). Patel and colleagues showed an incidence of 78/100,000 person-years among HIV-positive MSM from a "Surveillance, Epidemiology, and End Results Program" - HIV registry match in the United States since 2000. D'Souza and colleagues showed an incidence of 137/100,000 person-years among HIV-positive MSM since 1996 among men participating in the Multicenter AIDS Cohort Study. The trends reported in the studies were consistent in that HAART therapy did not appear to have reduced the occurrence of anal cancer, as it did for other AIDS-related malignancies such as Kaposi's sarcoma and non-Hodgkin lymphoma. In the largest study (Chiao EY et al, 2005) involving general population-based cancer registries, anal cancer incidence increased from 0.6 to 0.8 per 100,000 between 1973 and 2001. Two studies, one in the United Kingdom (Bower M et al, 2004) and one in the United States (Diamond C et al, 2005) reported dramatically increased anal cancer rates in HIV populations after the introduction of HAART. In the UK study, the incidence increased from 35 to 92 per 100,000 people with HIV. The overall incidence in the HIV cohort compared to the general population was 60/100,000 vs. 0.52/100,000. In the US study, rates in the general population among men aged 25 to 64 years increased from 0 to 224/100,000 from 1991 to 2000. The rate of anal cancer in the HIV cohort of men compared to men without HIV increased from 98 to 352 per 100,000.

The prevalence of anal HPV infection in HIV-negative MSM of all age groups is high (19.8%), and does not decrease with age in HIV-negative men who remain sexually active with multiple sexual partners (Chin-Hong et al., 2004 The "EXPLORE" study).

The data from a cohort of 1,409 HIV-negative MSM, aged 18 to 89 years and recruited from four US cities suggested that individuals might be susceptible to reinfection at least transiently, following re-exposure. This suggests that immunity to specific HPV types does not persist. It is considered that these men are repeatedly clearing and then becoming reinfected with HPV, giving rise to low- and high-grade anal intraepithelial neoplasia (AIN) which in many cases is transient. This fact would explain the absence of an age effect on the prevalence of AIN. The overall prevalence of HPV infection was 57% and was similar across all age groups. The "EXPLORE" group also reported factors significantly associated with risk of HGAIN (high grade anal intraepithelial lesions): increasing number of male sexual partners ($p=0.047$), and anal infection with increasing HPV types ($p<0.001$).

The absence of high-risk HPV at a single time point and from a single body site cannot guarantee that the virus is not present at another site or that the individual might not become infected at a later date (Fox et al., 2005). One problem here is the method of identifying the HPV. There is a probability that it may not always be picking up the infection; that perhaps individuals with HIV have a lower clinical threshold for infection than the general population. If this is true, the method of HPV detection becomes critical as the current assays include clinical thresholds which may not be fully applicable to the HIV-positive population. HIV-positive patients other than MSM can have AIN even where there has been no history of receptive anal intercourse; however the risk for these other groups appears to be much lower.

The study by Wilkin and colleagues in 2004 showed that 18% of men with no history of receptive anal intercourse had AIN based on cytology, compared with 65% for those who practiced receptive anal intercourse. The difference still remains significant based on histological findings, at 23% compared to 52%.

There is also an interpretive factor (or bias) that needs to be accounted for in both the histological and cytological interpretations. From 1980 to 2005, of the 20 533 estimated anal cancer cases, 1665 (8.1%) were HIV-infected. From 2001 to 2005, the proportion of anal cancer cases with HIV infection was the highest - 1.2% (95%CI 0.93%-1.4%) among females and 28.4% (95%CI 26.6%-29.4%) among males (USA, Shiels MS et al., 2012). The increasing anal cancer incidence rates in the US were strongly influenced by the HIV epidemic in males but were independent of HIV infection in females.

It is also well established that the risk of both prevalent and incident high-grade AIN increases as CD4 cell T count falls below 200 cells/mL (Kiviat et al., 1993; Palefsky et al., 1998). Evidence suggests that unlike most other malignancies occurring in the HIV-positive population, anal cancer is potentially preventable, using methods similar to those used to prevent cervical cancer in women (Palefsky J.M., 2009).

1.5.3 Cervical Cancer in HIV-positive Women

In 1993, the definition of AIDS (acquired immunodeficiency syndrome) was revised to include women with invasive cervical cancer (ICC) (Maiman M et al, 1993 & Maiman M et al, 1997). This decision was somewhat controversial as the incidence of cervical cancer had not yet increased among HIV-infected women. Nonetheless, the high prevalence of HPV coinfection and the increasing incidence of CIN lesions in HIV-infected women were of concern, strongly

suggesting that the risk of cervical cancer would rise over time. In fact, as shown in subsequent epidemiologic studies, a statistically-increased risk of ICC has been demonstrated among HIV-infected women (Dorrucci M et al, 2003; Maiman M et al, 1994). Worldwide cancer of the cervix (CC) is the second most common cancer among women with an estimated 529,409 new cases and 274,883 deaths in 2008. About 86% of the cases, representing 13% of female cancers, occur in developing countries.

Worldwide, mortality rates of CC are substantially lower than incidence with a ratio of mortality/incidence of 52% (IARC, *GLOBOCAN* 2008). In the last few decades, the incidence of cervical cancer has significantly declined with the introduction of cervical cancer screening to identify and treat women with cervical cancer precursor lesions (high-grade CIN or CIN II-III, and particularly CIN III). The treatment of high-grade CIN through a variety of modalities has also substantially reduced the incidence of cervical cancer. HPV is one of the most common infections of the female genital tract, and it is also one of the most costly. HPV-associated health care costs include routine Pap tests, treatment of genital warts, follow-up of cytological abnormalities, and management of cervical malignancies. High-risk oncogenic HPV types 16 and 18 are associated with 99.7% of all cervical cancers, as well as cytological abnormalities which carry significant health care costs and psychosocial morbidity. There is now considerable evidence that HPVs that are primarily transmitted through sexual contact are found in over 99% of the cases of invasive cervical cancer. Canadian researchers found that there was a long latency period between primary infection and cancer; the authors suggest that additional risk factors are involved in the process of tumor development (Mougin C and colleagues, 2001). These risk factors may include younger age, lower education, nutritional status, multiple sexual partners, younger age at both first sexual experience and first pregnancy, and multiple pregnancies ($p<0.003$) (Bell MC. et al in 2011).

Also associated were recreational drug use, current smoking and history of sexually transmitted diseases. Although 10% to 40% of women in the general population can be infected by HPV during their sexual life, only a small minority of them is at risk for developing cancer.

The first population-based study to investigate the prevalence of HPV types in all grades of cervical neoplasia, as observed in a large sample of high-risk population, was conducted by Herrero R et al in 1999. As observed for HSILs, HPV 16 was the most common type (11.8%) followed by HPV 52 (5.6%) and HPV 51 (5.4% of positive subjects). Each tested precancerous lesion had at least one high-risk HPV type; however most were associated with multiple HPV types. In Canada, women account for 17.3% (11,191 cases) of the 67,442 positive HIV test results reported since November 1st, 1985, and represent a growing proportion of new HIV diagnoses (26.2% in 2008 compared with 11.7% before 1999) (www.phac-aspc.gc.ca/aidssida/publication/Survreport/2008/dec/index-eng.php).

Women who are HIV-positive are at an increased risk for human papillomavirus infection, precancerous and cancerous lesions, as compared to HIV-negative women (Saslow D et al. 2002; Chin KM et al. 1998; Massad LS et al. 1999; Maiman M et al. 1998; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans Human Papillomaviruses, 2007). Canadian researcher Pamela Leece in her 2010 retrospective cohort study "Cervical cancer screening among HIV-positive women" wrote: "33% (42 of 126) of the HIV-positive women who underwent cervical screening had at least 1 abnormal test." Abnormal results were not significantly related to viral load; however, there was a significant relationship between lower recent CD4 T cell count (<200 cells/μL vs. ≥200 cells/μL) and having one or more abnormal Pap test results (OR=6.64, p=0.04). Rates of cervical screening in HIV-positive women in Ontario are estimated to be 68.6% during a 3-year period, indicating that HIV-positive women might

receive less screening than the general population (OCSP, Cancer Care Ontario, www.cancercare.on.ca/documents/OntarioBethesda2001.pdf - 2006). HIV treatment guidelines recommend annual Papanicolaou (Pap) test for HIV-infected women. The US study conducted by Oster AM and colleagues in 2009 assessed screening prevalence and associated factors among HIV-infected women. Of 2417 women, 556 (23.0%) did not report receiving a Pap test during the previous year. Not having a Pap test was associated with increasing age (adjusted odds ratio (AOR) = 1.3 per 10 years, 95%CI 1.1 to 1.4), and most recent CD4 count of less than 200 cells/mL (AOR = 1.6, 95%CI 1.1 to 2.1) or unknown (AOR = 1.4, 95%CI 1.1 to 1.7; both vs. CD4 count of > or =200 cells/mL). Odds of a missed Pap test increased for women whose most recent pelvic exam was not performed at their usual source of HIV care (AOR = 2.6, 95%CI 2.1 to 3.2). Nearly 1 in 4 women did not receive an annual Pap test. The researchers concluded that HIV care providers should ensure that HIV-infected women receive annual Pap tests, recognizing that missed Pap tests are more likely among older women and women with low CD4 cell counts. Although there is a trend towards the association between older age and decreased likelihood to adhere to Pap smear screening in previous studies among HIV-positive women, in the current study, younger women were more likely to demonstrate non-adherence to cervical cancer screening (Oster AM et al, 2009; Baranoski AS et al, 2011; Keiser O et al, 2006). Detecting cervical cancer in its earlier stages is life-saving. For instance, cervical cancer diagnosed at an early stage has a 92% 5-year survival rate (Saslow D et al, 2011). Given the increased cervical cancer risk among HIV-positive female smokers in particular, health care providers should give emphasis to the continuity of gynecologic care across women's life cycles. Likewise, Oster and colleagues noted that attention should be given to ensure that women of all ages equally recognize the importance and benefits of Pap smear screening.

Researchers from the UK (Kuhn L et al, 2010) conducted a randomized clinical trial of two screen-and-treat strategies among 6555 women in Cape Town, South Africa, among whom 956 were HIV-positive. Women were randomized to screen-and-treat utilizing either HPV DNA testing, a visual inspection (colposcopy) with acetic acid as the screening method, or placed in a control group. They were then followed for 36 months after randomization with colposcopy and biopsy to determine the study endpoint of CIN II or higher. In the control group, HIV-positive women had higher rates of CIN II or higher, detected by 36 months (14.9%), than HIV-negative women (4.6%, p = 0.0006). Screen-and-treat utilizing HPV DNA testing significantly reduced cervical intraepithelial neoplasia grade II or higher through 36 months in both HIV-positive (RR=0.20, 95%CI 0.06-0.69) and HIV-negative women (RR = 0.31, 95%CI 0.20-0.50). Reductions in the visual inspection with acetic acid-and-treat group were less marked.

The clinical presentation of cervical cancer in HIV-positive women tends to be more aggressive than in the general population, with many patients presenting with advanced-stage disease (Klevens RM et al, 1996). Diagnosis is often delayed due to misinterpretation, as many of the systemic signs of cancer, such as unexplained weight loss, low-grade temperatures and/or lymphadenopathy, may initially be attributed to the underlying HIV or another infection. In a study of 16 HIV-seropositive women with ICC, comparisons were made with 68 HIV seronegative women. The HIV-infected women were more likely to have high-grade tumors, lymph-node involvement and squamous cell pathology. While the stage of cervical cancer did not correlate with CD4 T levels, the CD4 status did influence treatment outcome. Patients with CD4 counts greater or equal to 500cells/mL demonstrated a more favorable response to treatment. Nonetheless, the median survival for the HIV-infected women was only 9 months and ultimately, more women died from cervical cancer than from AIDS (Schiffman M. et al, 2007).

During the pre-study and study periods, more than thousand of relevant articles have been reviewed and systematically updated in Introduction Chapter. More than 200 of them are listed in the Reference section of the thesis. Table 1 shows findings from some Canadian and international studies that investigated risk and incidence of HPV-associated precancerous lesions and cancers.

Table 1: Findings from Canadian and International Studies on Risk and Incidence of HPV-associated Precancerous Lesions and Malignancy

Author, Country, Journal & Year of publication	Title of the Study	Key Findings
Shack L., Lau H.Y., Huang L. et al. Alberta, Canada *CMAJ*, 2014	**Trends in the incidence of human papillomavirus-related noncervical and cervical cancers in Alberta, Canada: a population-based study**	**The annual percentage of the SIR increased for each 5-year interval of the study period:** **For oropharyngeal cancers (men – 3.4, p<0.001; women – 1.5, p=0.009)** **For anal cancers (men – 1.8, p=0.008; women – 2.2, p<0.001)** **For cervical cancer (among women 75-84 years – 3.5, p=0.04)**
Shiels M.S., Pfeiffer R.M., Chaturvedi A.K, et al. USA *J Natl Cancer Inst*, 2012	**Impact of the HIV Epidemic on the Incidence Rates of Anal Cancer in the United States**	**During 1980–2005, of the 20 533 estimated anal cancer cases, 1665 (8.1%) were HIV-infected. During 2001–2005, the proportion of anal cancer cases with HIV infection was the highest—1.2% (95% CI = 0.93 to 1.4%) among females & 28.4% (95% CI = 26.6 to 29.4%) among males.**
Gaisa M., Sigel K., Hand J. et al. London, England *AIDS*, 2014	**High rates of anal dysplasia in HIV-infected men who have sex with men, women and heterosexual men**	**Among 728 HIV+ people:** **Anal SCC (OR (95%CI)=2.2 (1.3-3.7)** **Anal HSIL in 32% of MSM, 26% of women, 23% of heterosexual men**
Berry J.M., Jay N., Cranston R.D. et al. USA *Intern. Journal of Cancer (IJC)*, 2014	**Progression of anal high-grade squamous epithelial lesions to invasive anal cancer among HIV-infected men who have sex with men**	**During 1997-2011, 138 HIV-infected MSM were diagnosed with anal & perianal SCC.** **Anal cancer incidence is 80 times higher in HIV+ MSM than men in the general population. In 2012 this incidence was 131/100,000 in North America**
Moscicki A.B., Palefsky J.M. USA. *Journal of Low Genital Tract Disease*, 2011	**Human Papillomavirus in Men: An Update**	**90% of anal cancer due to HPV 16 & 18** **70% of cervical cancer due to HPV 16 & 18**
Chaturvedi A.K., Madeleine M.M. et al. USA *J Natl Cancer (JNCI)*, 2009	**Risk of Human Papillomavirus-Associated Cancers among Persons with AIDS**	**From 1996 to 2004:** **SIR (95% CI) of all HPV-associated cancer *in situ*=8.9 (8.0-9.9)** **SIR of anal cancer=34.6 (30.8-38.8)** **SIR of oropharyngeal cancers=1.6 (1.2-2.1)**
Silverberg M.J., Lau B., Justice A.C. et al. USA *Clin Infect Dis*, 2012	**Risk of Anal Cancer in HIV-Infected and HIV-Uninfected Individuals in North America**	**SIR (95%CI) of anal cancer=80.3 (42.7-151.1) for HIV+ MSM and** **SIR (95%CI) of anal cancer=26.7 (11.5-61.7) for HIV+ men compared with HIV-uninfected men**

1.6 Problem Statement and Hypothesis

The risk of HPV associated malignancies is genotype-dependent. However, from the standpoint of HPV epidemiology, there is a lack of information on HPV genotype distribution and epidemiology of HPV-associated anal, oropharyngeal and cervical cancer among those living with HIV/AIDS (PHAs) in Atlantic Canada. This prospective cohort study, involving PHAs treated at the Infectious Diseases clinics in Atlantic Provinces aimed to reduce this information gap. While HPV prevalence is likely to be high in the target population, testing for the high risk (HR) HPV genotypes and associated cytological abnormalities should identify those at increased risk of malignancy. Moreover, determining the HPV genotype will be beneficial in assessing the relative risk and detecting the malignancy earlier, which will also be quite useful as a part of ongoing HIV disease management.

The main research hypothesis in this study is that the incidence of HPV-associated anal, oropharyngeal and cervical cancers is higher among HIV-infected adults living in Atlantic region of Canada than their incidence in the Canadian general population. The aims of this study were (1) to reduce the information gap on HPV genotype distribution and epidemiology of HPV-associated cancer among those living with HIV/AIDS in Atlantic Canada (2) to examine and quantify the relationship between precancerous lesions and cancers (anal, oropharyngeal and cervical) and HR HPV genotypes (3) to examine the relationship between precancerous lesions and cancers and predictors of HPV-related diseases. The following components were measured (i) self-reported history of unprotected sex (ii) self-reported history of sexually-transmitted infections (iii) self-reported number of sexual partners during the previous year (iv) self-reported history of smoking (v) annual prevalence of HPV genotypes over a four-year period (vi) annual levels of CD4 T cell count and plasma HIV RNA viral load over a four-year period.

1.7 Research Questions, Objectives and Purpose of the Study

The author of this thesis studied exposure to HPV infection of HIV-infected individuals living in Atlantic Canada from 2009 to 2015. The study intended to answer the following questions: (1) What was the prevalence of HR oncogenic HPV genotypes in HIV-positive adult population in Atlantic Canada at baseline and three years of follow-up? (2) Were the HR oncogenic HPV genotypes associated with underlying anal, oropharyngeal and cervical premalignant lesions and malignancy? (3) Were the detected premalignant lesions and malignancy associated with patients' demographics and behaviors as well as with patients' health status?

The objectives of the study were:

To determine the prevalence of HR HPV genotypes in HIV-positive adults at baseline and throughout the years of observation

To investigate association between these HPV genotypes and diagnosed precancerous lesions and cancers

To investigate association between precancerous lesions and cancers and their potential predictors such as HIV markers (CD4 T cell count and HIV RNA plasma load), smoking, history of STIs, number of sexual partners, number of HPV types in a specimen, and history of unprotected sex.

Atlantic Canada is currently poised to effectively establish an HIV-HPV surveillance network. This capacity comes from the two Atlantic Canada initiatives: the Atlantic Interdisciplinary Research Network (AIRN) formed in 2005, and the Atlantic Canada HIV Education Network (ACHIVE) established in 2002. From the standpoint of the strength of the existing Atlantic Canada networks, it is a sound prospect to establish an "Atlantic Canada HIV-HPV Surveillance Network" which could provide valuable information and serve as a model

to the rest of Canada. The study data and results might also be quite useful and included as a part of ongoing HIV patient care and management. They can potentially initiate some changes in the primary care policies with follow-up recommendations for the annual anal screening of all HIV-infected adults, regardless of age and gender. The screening procedure would advisedly include both a visual inspection of the perianal region, and a digital rectal/anal examination (DRE/DAE) with anal specimen collection for the cytology evaluation and HPV genotyping. Furthermore, determining HPV genotype prevalence is beneficial in assessing the risk of acquired malignancy and will provide useful information in the era of genotype-specific HPV vaccination. Finally, findings from this study contribute to the national data on genotype distribution and add to the existing body of knowledge on HIV-HPV co-infection.

1.8 Thesis Outline

Chapter 1 presents an introduction to the research, a rationale for the study, the research hypothesis and the specific research questions. Chapter 2 provides information about the study design, settings and population, and describes all techniques, tools, guidelines and classifications that were used for the research purpose. Chapter 3 presents the results from the analyses. Chapter 4 provides a discussion of the study key findings, policy implications, the study limitations and potential areas for future research. Chapter 5 provides the conclusions that were drawn from the study findings.

1.9 Statement of My Role in the Project

I hold M.D. from the Azerbaijan State Medical University and worked as a primary healthcare physician for more than 15 years. My participation in this study started in November 2008, the time when applications for ethical approval were being drafted and submitted to HREAs at all research sites. For almost two years, from January 2009 to December 2010, I was working with a multidisciplinary team as a part-time Research Assistant II (CIHR-PHAC fellowship supporting my Master's program). In January 2011, I was appointed as a Research Coordinator of this project and carried out the study logistics until its end in April 2015. I recruited and sought consent from participants in the ID clinic at the NLSJ site, coordinated the communication among the sites, initiated research-related discussions both on-line and at the annual ACHIVE gatherings, provided literature review and critical appraisal of evidence, assembled and shipped out the study supplies, collected data from the sites and laboratories, sent the cytology results to the site investigators, followed-up the patients' referrals to specialists, created and maintained datasets, analyzed and interpreted data, prepared and presented posters and oral abstracts at national and international conferences, and with my supervisor, eventually published an abstract with the preliminary findings in *Annals of Epidemiology* (2013). I also prepared and submitted annual reports to PHAC, as well as renewals and amendments to HREA. Currently, I am finalizing my PhD thesis and drafting an article with the final findings.

CHAPTER 2

METHODOLOGY

2.1 Study Design

This prospective cohort study was carried out from June 2009 to April 2015.

2.2 Study Settings

The study centers in Atlantic Canada were located in St. John's, NL, Saint John, NB, Moncton, NB and Halifax, NS. Patients from Prince Edward Island (PEI) were mainly seen in Halifax. A study poster with the PI's contact info was distributed at the local HIV clinics (Appendix A). Enrolment began in June 2009 and was ended in September 2012.

All HIV-positive patients seen through the participating Infectious Disease (ID) clinics were approached by the clinic physicians or nurses to request participation in the study. They explained the study to the potential participants and obtained written consent. Consent was obtained using ethics board-approved consent forms with the clear understanding that the patients' unique identification numbers (IDs) will be retained in order to conduct future patient follow-ups (Appendix B). Consent was also obtained to annually access the patient's medical record information, such as current values (at the time of annual observation) of HIV RNA viral load, CD4 T cells count, treatment status, and history of sexually transmitted infections. This was done in order to correlate these factors with disease outcomes. All consenting participants were enrolled during a three-year period and were followed up, per usual care, for up to three years. During initial interviews, participants were administered a 26-item self-reported deidentified confidential Patient Questionnaire to obtain demographic and risk factor data (Appendix C).

The questionnaire was completed by the patient in a private room at the research site, and was then given to a research nurse in a sealed envelope. The sealed envelope was mailed to the PHL in St. John's, NL along with the patient's paperwork and specimens (Appendix H).

At the time of enrolment, the clinic physician/nurse completed the 12-item Clinic Baseline Questionnaire with the current tests results related to the HIV markers and patient health status (Appendix D). The 9-item Annual Clinic Follow-up Questionnaire was completed for all study participants by their treating physician/nurse with the latest data for the year of observation during the three years of follow-up visits (Appendix E). They also completed the patient's enrolment card in order to register the dates of the specimen collection at baseline and during the follow-ups (Appendix F).

2.3 Study Population

Inclusion Criteria:

All HIV-positive adults who attended ID clinics in the Atlantic Canada provinces from June 2009 to September 2012.

Exclusion Criteria:

HIV-positive people ≤ 18 years of age

HIV-positive adults who are involved within other ongoing research projects

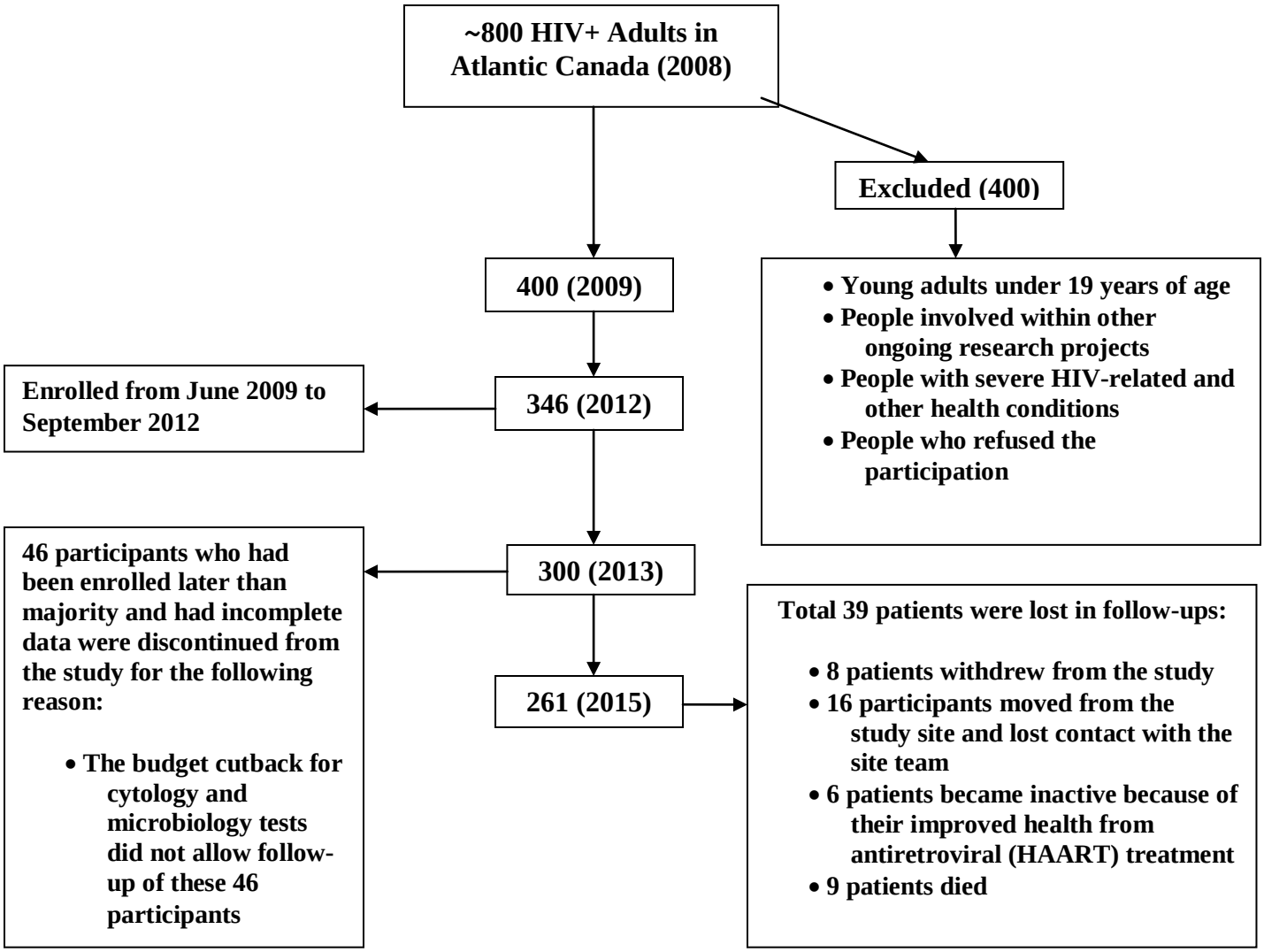
HIV-positive adults with severe HIV-related and other health conditions

Sample Size Considerations and Calculation: All HIV-positive adults treated through the Infectious Diseases clinics participating in the research were approached by the clinic physicians or nurses to request their participation in the study. In 2008, Atlantic Canada had approximately 800 routinely followed HIV-positive adults. Of them, 346 were enrolled in the study and of those, 263 were included in the final analysis. The flow chart below displays the changes in the sample size throughout the years of observation.

Biostatistician was consulted and the recommendation was to retrospectively calculate the sample size in order to have enough power to detect clinically important differences between groups. The sample size (N) calculation was carried out by the SAS 9.4 Proc Power procedure. The incidence information, which was required in the calculation, was from the 2015 CDC "Fact Sheets" (http://www.cdc.gov/.../hpvcancer/). According to the CDC, in women 30 years of age and older the 10-year cumulative incidence of cervical cancer caused by the combination of HPVs 16 & 18 was 39% as compared to the 1.7% cumulative incidence of cervical cancer not caused by the HPVs 16 & 18.

We observed our participants for 4 years which is almost half of the study time in the CDC report. Therefore, we assumed that our 4-year incidence of cancer caused by both HPV 16 and HPV 18 would be 20% as compared to 1% of the 4-year incidence of cancer being negative for HPVs 16 & 18. The calculated risk difference (RD) was (20% - 1%) = 19%. Based on our data, we observed the proportion of HPVs 16 & 18 being simultaneously positive in 44 (17%) patients. We needed to recruit 102 participants (N=102) in order to have Power=80%, β=0.20 and α=0.05. In our study, 263 participants were included in the final multivariate regression analysis.

CHART 1: The Flow Chart of the Study Population throughout the Years of Observation



2.4 Study Milestones and Timeframe

September 2008 – May 2009
Acquiring the ethics clearance at all study sites

June 2009
Enrollment was started at all study sites with targeted 400 participants; the considered study due date was December 31, 2013

September 2012, ACHIVE in Halifax, NS
Recruitment was finished with total 346 participants

January-March 2013
Negotiation with the Cytology & Microbiology laboratories; the expected study due date was extended to December 31, 2014

September 2013, ACHIVE in Moncton, NB
Number of the participants was reduced from 346 to 300 with the consequently reduced number of the specimens that still needed to be tested

September 2014, ACHIVE in Saint John, NB
The study due date was extended to April 30, 2015

April 30, 2015
The study was officially closed and the specimens' collection was finalized

May-September 2015
Collection of the remaining materials from all of the project's centers and both laboratories, data cleaning, statistical analyses, interpretation of the results

September 2015, ACHIVE in Terra Nova, NL
Presentation of the final report with the study findings to all study co-investigators

October 2015 – December 2015
Dissemination of the study results, drafting of the articles for the publication

2.5 Specimen Collection and Centralization of the Data

The labels for each study site, the consent forms, enrolment cards and questionnaires were designed and printed prior to specimen collection. The study supplies were purchased and accumulated in the PHL storage space. Each province had its particular label color: New

Brunswick-red labels, Newfoundland-blue and Nova Scotia-green labels. Each label included the site name, patient's personal identification number (PIN), and the type of specimen collected (A, O, C). For example, NLSJ 001 and checked A & O squares on the label indicated that this patient was a male (only anal and oropharyngeal specimens have to be obtained), his PIN in the research was 001 and he was from Newfoundland, St. John's. In May 2009, the personal research kits were assembled and shipped out to each study center. The same procedure was repeated yearly during the three follow-up years.

The personal kit for the screening year included (i) labeled paper-work (consent form, 26-item Patient's Questionnaire, 12-item Clinic Baseline Questionnaire, and laboratory requisition form with the enrollment card) (ii) three Pap vials with a liquid media for the obtained specimens (iii) packs with Dacron and sterile cotton swabs (for anal and oropharyngeal specimens, respectively), including blue cervical brush for female participants (iv) paper bag and plastic biohazard bags for each collected specimen that was shipped back to the PHL in St. John's, NL.

The personal kit for the follow-up years included (i) 9-item Clinic Follow-up Questionnaire (ii) laboratory requisition (iii) two or three Pap vials (depending on the participant's gender) (iv) packages with Dacron and cotton swabs, and/or cervical brush (v) paper and plastic biohazard bags. The shipment services were provided by FedEx Canada. In order to standardize collection among the study sites, the detailed guidelines for specimen collection (NYS DOH Guidelines recommendations on anal pap smears -Appendix G) were sent to the research sites at the beginning of the study. Trained personnel collected an oropharyngeal and anal swab specimens from all consenting males and females. Females were asked to provide an additional cervical specimen. This study used SurePath™ Liquid-Based Pap test (BD Diagnostics) supplies for specimen collection: SurePath™ vials with 10 ml of ethanol-based media, and blue cervical

brushes with the detachable end (http://www.bd.com/tripath/physicians/). The cervical transitional zone (TZ) is the site of origin of most cervical neoplastic lesions, and as in sampling for cervical cytology, was targeted in the study for exfoliated cells collection. Anal cytology samples were collected by rotating a water-moistened Dacron swab in the anal canal without direct visualization (blind or non-guided method) above the squamocolumnar transitional or dental zone (TZ), which is approximately 2 cm above the anal verge (NYS DOH AIDS Institute's HIV quality-related, http://hivguidelines.org/Content.aspx). The oropharyngeal specimen was collected from the back side of the patient's throat using a sterile cotton swab.

The end sites of the collection devices were individually placed in a SurePath collection Pap vial. The sample-handling for all three specimens was similar. The resulting solution was stored at room temperature in the PHL and later used for the preparation of thin-layer slides for cytologic analysis. All specimens and completed paperwork from the study centers were sent to the Public Health Laboratory (PHL) in St. John's, NL. The specimens were shipped under conditions that protected sample integrity (WHO Guidance on regulations for the transport of infectious substances) (http://www.who.int.csr/resources/biosafety/WHO HSE EPR 2008 10/html). In the PHL, the vial's content was divided in two parts and sent to different laboratories: one-third to the Eastern Health Regional Cytopathology Laboratory in St John's, and two-thirds to the National Microbiology Laboratory (NML) in Winnipeg, MB for HPV DNA detection and HPV genotyping.

The cytology reports were forwarded to the study physicians through the lead principal investigator and the study coordinator who analyzed and prepared milestone reports to the Public Health Agency of Canada (PHAC) in Ottawa. The detailed study Flow Chart is shown in Appendix J.

2.6 Brief Description of the Existing Tests to Detect HPV Infection - WHO HPV Laboratory Network (LabNet) Data (July 2010).

There are two tests available to detect the presence of HPV viral DNA in a cell: the Hybrid Capture II test and the DNA PCR test (Chin-Hong PV & Palefsky JM, 2002). The Hybrid Capture II test is a more general test that can detect the presence or absence of the high-risk forms of the virus, but cannot specify their subtypes. Its advantages are that it is quick and less expensive compared to PCR tests. The PCR test can detect the type of HPV present, yet its sensitivity varies by the type of PCR system used. It is also generally more expensive and requires the presence of a greater viral load (A Global Review, 2008; BCCA Vancouver Centre; Canadas MP et al, 2004).

WHO HPV Laboratory Network (WHO HPV LabNet) developed the manual on existing tests to detect HPV infection based on knowledge and experience gained through its International collaborative studies over the past several years. HPV cannot be cultured by conventional methods and is a cell-associated virus; therefore, HPV infection is monitored indirectly by detection of HPV DNA in a cellular sample obtained from a particular anatomic site. Lysis with or without extraction is required to release the viral DNA from the sample. Cellular DNA is also released at the same time and can serve as a control for the sample adequacy. Human papillomavirus (HPV) can be found in human epithelia in two forms, either individually or in a combination with episomal or extrachromosomal HPV particles. It can also be integrated into the human genome (Cooper K & Herrington CS et al, 1991). It was shown in previous studies that HPV DNA is present in three morphologically distinct forms in the nuclei of cervical precancerous and cancerous lesions by non-isotopic *in situ* hybridization (NISH) (Cooper K, et al, 1991). These forms were referred to as NISH signals types 1, 2, & 3, where a type 1 signal is diffused and present throughout the nucleus and represents episomal HPV virus.

A type 2 signal is punctuated and represents integrated HPV virus, and a type 3 signal is a combination of both forms. Therefore, a pattern regarding the physical state of the HPV DNA in cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC) is that of episomal HPV virus predominating in the early stages of CIN and SCC, with integrated virus being detected more frequently in HPV-related high grade CIN and SCC. Furthermore, the latter may or may not contain episomal forms as well (Lehn H et al, 1988; DiLuca D et al, 1989). It should be kept in mind that detection of HPV DNA usually indicates current infection, but surface contamination cannot be excluded. Similarly, failure to detect HPV DNA does not exclude HPV infection as low-level infections or sampling errors, and infections at other anatomic sites need to be excluded.

HPV infection is not treated, so current uses of HPV testing in screening and clinical diagnosis are directed towards detection of HPV-associated precancers that are treated, rather than to diagnose infection *per se*. HPV cannot be easily propagated by standard *in vitro* culture systems, and in malignant tissue there are little or no infectious HPV particles. For these reasons, methods are based on the detection of HPV nucleic acids, in most assay formats, HPV DNA. Molecular methods for HPV detection can be grouped into two main categories (1) those that rely on signal amplification to detect the targets (2) those that rely on target amplification itself.
The WHO HPV LabNet has performed a series of proficiency testing studies since 2007. In total, 81 datasets with HPV typing data were returned to World Health Organization for evaluation. These different assays are detailed in Table 2.
In our study, we used the Roche Linear Array which was the most widely-used assay with results reported by 15 laboratories. The WHO HPV LabNet has agreed that a laboratory that performs HPV DNA detection and typing be considered proficient if it is able to detect 50 International

Units (IU)/5µL of HPV16 and HPV18 DNA and 500 genome equivalents (GE)/5µL of other HPV types. In addition, it should not give more than one false-positive result (FP) in the panel. It was recommended that genotyping assays should detect, at a minimum, the fourteen most common high-risk (HR) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 & 68) and the two low-risk (LR) HPV types targeted by a current quadrivalent HPV vaccine Gardasil - HPVs 6 and 11 (Meijer CJ. et al, 2009).

In view of the variety of HPV DNA detection and genotyping assays being used by laboratories worldwide, it is necessary to validate the assays both qualitatively and quantitatively in order to determine their following properties: (1) Sensitivity/Limit of Detection (2) Specificity (3) Accuracy (4) Reproducibility (5) Robustness (6) Linearity (7) Analytic Range based on ICH policies and procedures

(International Committee on Harmonization, ICH Validation of analytical procedures: text and methodology, http://www.emea.europa.eu/pdfs/human/ich/038195en.pdf).

Table 2: The WHO HPV LabNet Dataset

HPV Assay Type	Number of Datasets	HPV Region Targeted (Primers)
All Assays	**81**	**L1/E1/E6/E7**
1. *Linear Array (Roche)**	**15**	**L1 (PGMY)**
2. PGMY – RBH	**7**	**L1 (PGMY)**
3. In-house Type-Specific PCR	**7**	**L1/E6/E7**
4. In-house 16/18 Specific PCR	**6**	**E6/E7**
5. InnoLiPA (Innogenetics)	**6**	**L1 (SPF10)**
6. CLART (Genomica)	**6**	**L1 (PGMY)**
7. DNA Chip (Biocore)	**4**	**L1**
8. In-house Lineblot	**4**	**L1 (GP)**
9. In-house PCR Luminex	**4**	**L1 (GP or modified GP)**
10. In-house PCR Luminex	**4**	**E6/E7**
11. In-house Microarray	**3**	**L1/E7**
12. PCR – RFLP	**3**	**L1**
13. Microarray (Genetel)	**2**	**L1**
14. DEIA LiPA Assays	**2**	**L1 (SPF10)**
15. In-house PCR E/A	**2**	**L1**
16. Microarray (Papillocheck)	**1**	**E1**
17. Type-specific PCR (GenoID)	**1**	**L1**
18. *In-house PCR Luminex**	**1**	**L1 (PGMY – GP)**
19. PCR Luminex (Multimetrix)	**1**	**L1 (GP)**
20. PCR E/A (GenoID)	**1**	**L1**
21. In-house PCR Sequencing	**1**	**L1 (PGMY – GP)**

*: **HPV assays in *italics* (#1 & #18) were used in the study**

2.7 HPV DNA and Genotyping Tests used in this Study

The National Microbiology Laboratory in Winnipeg, MB conducted HPV DNA and Genotyping analysis for our study. Two HPV assay types were used (#1 and #18 in Table 1) for these purposes: The Linear Array Genotyping Test (#1) and The Luminex®-Based Genotyping Assay (#18 – In-house PCR Luminex L1 (PGMY-GP)). The laboratory supplies were provided by the Roche Molecular Diagnostics, which operates in the U.S. as the legal entity Roche Molecular Systems, Inc. (Roche Molecular Diagnostics Global website: http://molecular.roche.com/assays). Roche research assays and PCR technology have been widely used in landmark epidemiology studies around the world to characterize the incidence and distribution of HPV genotypes and for classification of the HPV types, as they relate to cervical cancer.

The Linear Array HPV Genotyping Test (LA) is a qualitative *in vitro* test for the detection of human papillomavirus in clinical specimens. The test utilizes amplification of target DNA by the polymerase chain reaction (PCR) and nucleic acid hybridization, and detects 37 HPV DNA types in cervical cells collected in PreservCyt solution (PreservCyt is a registered trademark of Cytyc Corporation, owned by Hologic). The Linear Array HPV Genotyping Test is registered for use in the European Union for detection of 37 high- and low-risk human papillomavirus genotypes, including those considered a significant risk factor for high grade squamous intraepithelial lesions' (HGSIL or HSIL) progression to cervical cancer (numbers typed in bold). HPV genotypes include: 6, 11, **16, 18**, 26, **31, 33, 35, 39**, 40, 42, **45, 51, 52**, 53, 54, 55, **56, 58, 59**, 61, 62, 64, **66,** 67, **68,** 69, 70, 71, 72, **73** (MM9), 81, **82** (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108.

In summary, the Linear Array assay has superior ability to detect HPV DNA with low and high β-globin references lines; is capable of detecting HPV genotypes in a multiple infection, which can occur in up to 35% of patient samples (Van Hamout D et al, 2009); has superior ability to detect HPV DNA and individual types that may be attributed to the use (Coutlee F et al, 2006) of (i) Standardized, quality-controlled reagents (ii) Primer concentrations that minimize competition due to coamplification.

The Luminex®-Based Genotyping Assay was developed by a team of specialists from NML; PHAC, Winnipeg, MB; Cadham Provincial Laboratory; Manitoba Health and Healthy Living, Winnipeg, MB; PHL, St. John's, NL; and Department of Medical Microbiology, University of Manitoba, Winnipeg, MB. This assay can simultaneously identify 45 mucosal HPV genotypes and was evaluated with the Roche Linear Array (LA) test. The study conducted prior to this project amplified single-stranded HPV DNA carrying a biotin tag that was generated using primers PGMY (Gravitt PE et al, 1998) and GP5+/GP6+ (Husman AM et al, 1995) in a nested PCR reaction. They used a set of 45 Luminex microspheres coupled with 45 unique HPV probes for detection and typing. A total of 149 cervical specimens collected in PreservCyt were utilized in the study. The Luminex method identified 45 vs. 37 mucosal HPV types either with or without cross hybridization, as compared to the Linear Array. It showed a higher sensitivity than LA test, 85 vs. 73 positive samples, and 171 vs. 164 total HPV types detected, with 47 multiple infections detected with both methods. On the other hand, the LA test showed slightly better sensitivity for detection of multiple infections with 3 or more types. Discordant samples included 12 Luminex positive/LA negative results and 36 multiple infections in which the list of types was partially different between the two methods.

Four of these samples contained types not detected by the LA probes. No sample was completely discordant for HPV typing. The overall distribution of HPV types was similar between the two methods, with the exception of HPV 52, which was less frequently detected by the Luminex method compared to LA (8 vs. 18, respectively).

In conclusion, the tested Luminex assay, when compared to the Linear Array (LA) test, offers more flexibility, lower cost and less hands-on time (Goleski VA et al, 2008). In our study, the NML administration kindly offered us the use of both PCR assays *gratis* (Linear Array and Luminex) during the screening year and only Luminex assay during the follow-up observations.

2.8 Cytopathology and Histopathology Tests

Screening for cervical or anal intraepithelial lesions involves the two-stage procedure: the first is Pap test for abnormal cytological findings, followed by a referral for an anoscopic examination (similar to a cervical colposcopic examination), and biopsy if necessary.

In our study, The 2001 Bethesda System (TBS 2001) terminology was used to report the anal, oropharyngeal and cervical specimens' cytology test results. Forty-four international organizations with interest in cervical cytopathology cosponsored the Bethesda System 2001 Workshop along with the National Cancer Institute (NCI) in April 2001. The goal of the Bethesda System has always been to promote effective communication of relevant cytology findings between the laboratory and clinician to provide optimal patient care. The Bethesda System was developed primarily for cervical cytology specimens, and both the terminology and morphologic criteria reflect these. However, specimens from other body sites such as the throat, vulva, vagina, and anal/rectal samples may be reported using similar terminology (Solomon D & Nayar R, 2001).

The Bethesda System's Second Edition provides a clearer indication of adequacy; specimens are now designated as "Satisfactory" or "Unsatisfactory" for evaluation: (1) For "Satisfactory" specimens, information on transformation zone (TZ) sampling and other adequacy qualifiers are included (2) For "Unsatisfactory" specimens, information on whether or not the laboratory has processed/evaluated the slide are included (whether the specimen was rejected or processed and examined, but deemed unsatisfactory for evaluation because of obscuring blood, etc.)

An adequate liquid-based preparation (LBP) should have an estimated minimum of at least 5000 well-visualized/well-preserved squamous cells. For interpretation of adequacy for anal specimens, at least 8 nucleated squamous cells had to be visualized at high magnification (40X) to be considered adequate (Scholefield JH, et al, 1998; http://iris.ucl.ac.uk/research). This was derived using the ThinPrep criteria of 4 cells per HPF, and doubling it to accommodate the diameter of the sample being smaller for SurePath. If the specimen shows a cytologic abnormality, it is not necessary to report a specimen as unsatisfactory as the abnormality is reported independent of the cellularity when an abnormality is found. Studies of anal cytology have not found the presence of metaplastic or glandular mucosa necessary to reflect sampling of the transformation zone in contrast to cervical cytology where there needs to be at least two groups of 5 metaplastic or glandular cells.

The 2001 Bethesda System maintains equivocal category atypical squamous cells (ASC) and simplifies its qualifiers to realistically reflect the inability of pathologists to accurately and reproducibly interpret these specimens (the reproducibility of ASC as an interpretation is around 40%). All interpretations of ASC should be qualified as "Of Undetermined Significance" (ASC-US) or "Cannot Exclude HSIL" (ASC-H). ASC-US is expected to comprise more than 90% of ASC interpretations in most laboratories. ASC-H is a designation reserved for the

minority of ASC cases (expected to represent less than 10%) in which the cytological changes are suggestive of HSIL and require clinical investigation such as high resolution anoscopy (Solomon D et al, 2001).

Squamous intraepithelial lesion (SIL) encompasses the spectrum of noninvasive cervical squamous epithelial abnormalities associated with HPV. In TBS, this spectrum is divided into low-grade (LGSIL or LSIL) and high-grade (HGSIL or HSIL) categories. Low-grade lesions encompass the cellular changes variously termed "HPV cytopathic effect" (koilocytosis) and mild dysplasia or cervical intraepithelial neoplasia 1 (CIN I). High-grade lesions encompass moderate dysplasia, severe dysplasia, and carcinoma *in situ* or CIN II and CIN III.

Conceptually, HPV-associated abnormalities can be divided into transient infections that generally regress over the course of 1 to 2 years (mean is 18 months) and HPV persistence that is associated with an increased risk of developing a cancer precursor or invasive cancer (Bosch FX et al, 2002).

These abnormality categories, along with the recommended management are demonstrated in Table 3. This Table was revised in January 2007 with support from the Nova Scotia Gynecological Cancer Screening Program, and Ontario's Laboratory Proficiency Testing Program Guidelines. Consultation was held with leading pathologists and physicians in the Province of Newfoundland and Labrador, and was endorsed by the NL Medical Association (Cervical Screening Initiatives Program 2007, Clinical Management Guidelines). These guidelines were used for follow-up and management of all (anal, oropharyngeal and cervical) detected cytologic abnormalities. As it was clearly demonstrated in the Table 3, epithelial cell abnormalities require further histopathology investigation such as colposcopy with biopsy for cervical histopathology, high resolution anoscopy (HRA) with biopsy for anal histopathology,

and the biopsy of oral lesions. The correspondence of the findings from cytopathology laboratory with those from histopathology laboratory is necessary for the confirmation of the type of lesion or stage of malignancy to develop the strategy of their further treatment. The study main outcomes were precancerous lesions and cancers. Their management and necessity of further investigation (biopsy) was determined by specialist based on a severity of lesion.

Table 3: Clinical Management Guidelines (The 2001 Bethesda System)

RESULT	RECOMMENDED MANAGEMENT
Specimen Adequacy Statement	
Satisfactory	**Routine Screening** at **annual** intervals (unless the specimen adequacy statement is accompanied by a qualifier and subsequent recommendation).
Unsatisfactory	Repeat smear **after 12 weeks.**
Negative	
NIL Negative for Intraepithelial Lesion	Routine screening* If specific pathogen is present, treat as clinically appropriate. *In the presence of a gross abnormality with a negative Pap test, patient should be referred for Colposcopy
Epithelial Cell Abnormalities	
ASC-US Atypical Squamous Cells of Undetermined Significance	Women < 30 years of age: A repeat Pap test in six months is recommended; If abnormal, refer for Colposcopy. If negative, repeat in six months. After two negative Pap tests, return to routine screening Women > 30 years of age: **HPV Positive* Colposcopy and Biopsy** Women > 30 years of age: **HPV Negative* Routine annual screening** *HPV Testing will be done through the laboratory automatically for ASCUS results in women over 30 years. A combined report will be issued.
ASC-H Atypical Squamous Cells cannot exclude HSIL	**Colposcopy and Biopsy.**
LSIL Low Grade Squamous Intraepithelial Lesion	**Colposcopy and Biopsy.**
HSIL High Grade Squamous Intraepithelial Lesion	**Colposcopy and Biopsy.**
AGC Atypical Glandular Cells	**AEC** – Atypical Endocervical Cells – Colposcopy and Endocervical Curretage (ECC) For women over 35, endometrial sampling is also recommended. **AEMC** – Atypical Endometrial Cells – Colposcopy and Endometrial Sampling (EM) **NOS** – Not Otherwise Specified – Colposcopy, ECC and EM Sampling **FN** – Favor Neoplastic – Colposcopy, ECC and EM Sampling.
AIS Adenocarcinoma In Situ Squamous Cell Carcinoma Adenocarcinoma	**Colposcopy, Biopsy and endocervical curretage** as recommended. **Colposcopy and Biopsy.** **Colposcopy and Biopsy.**
Other	
Endometrial Cells in a woman over 40 (or a younger woman with unexplained vaginal bleeding)	These findings should be interpreted in light of the clinical scenario. Clinical correlation is advised. **Endometrial** biopsy is recommended if **post-menopausal** or patient has abnormal pre-menopausal bleeding.

In our study we used The Bethesda 2001 System and CIN Classifications to coordinate cytological and histological findings (Table 4).

Table 4: Correspondence of Cytological and Histological Findings

(TBS 2001 & CIN Classifications were used in this study)

Cytological classification (used for screening)		Histological classification (used for diagnosis)	
Pap	Bethesda system	CIN	WHO descriptive classifications
Class I	Normal	Normal	Normal
Class II	ASC-US ASC-H	Atypia	Atypia
Class II	LSIL	CIN 1 including flat condyloma	Koilocytosis
Class III	HSIL	CIN 2	Moderate dysplasia
Class III	HSIL	CIN 3	Severe dysplasia
Class IV	HSIL	CIN 3	Carcinoma in situ
Class V	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma

Table 5 below provides description of the management procedures for the study participants who were diagnosed with the cell abnormalities in their specimens. If the Pap test abnormalities were persistent in the follow-ups, these patients would have had annual anoscopy or colposcopy with biopsy.

Table 5: Management Procedures of the HIV-infected Study Participants with Pap test Abnormalities

Type of Specimen	Site-specific Collection of Pap smear	Management of Pap test abnormalities
Anal	**Non-visualized specimen collection from anal canal using Dacron swabs**	• **ASC-US & HPV+** **Referral to GI surgeon for HRA & possible biopsy of lesion** • **ASC-H, LSIL & HSIL** **High Resolution Anoscopy (HRA) with biopsy, follow-up (treatment or repeat anal Pap smear in 3 months)** • **SCC *in situ*, Invasive cancer Treatment**
Oropharyngeal	**Specimen collection from back site of the throat using sterile cotton swab**	• **ASC-US & HPV+** **Referral to ENT specialist for oral examination & possible biopsy of lesion** • **ASC-H, LSIL & HSIL** **Biopsy of lesion, follow-up (treatment or repeat oral Pap smear in 3 months)** • **SCC in *situ*, Invasive cancer Treatment**
Cervical	**Cervical swab using cervical blue brush**	• **ASC-US & HPV+** **Referral to Gynecologist for colposcopy & possible biopsy of lesion** • **ASC-H, LSIL, HSIL** **Colposcopy with biopsy, follow-up (treatment or repeat cervical/vaginal Pap smear in 3 months)** • **SCC *in situ*, Invasive cancer Treatment**

Anal cancers were defined using International Classification of Diseases for Oncology, 3rd edition (ICD-O-3), topography codes C210 (anus, not otherwise specified) and C211 (anal canal).

Anal cancers were classified by histology as squamous cell carcinomas (ICD-O-3 codes 8050–8089), adenocarcinomas (ICD-O-3 codes 8140–8309), carcinomas not otherwise specified (ICD-O-3 code 8010), and other histological subtypes.

2.9 Statistical Analyses

2.9.1 Data collection

Data was collected from the following documents: (1) The self-reported 26-item Patient's Questionnaire (PQ) (Appendix C) administered to all participants at enrollment (2) The 12-item Clinic Baseline Questionnaire (Appendix D) which was completed by the study co-investigators (physicians and/or nurses) at the enrollment (3) The 9-item Clinic Follow-up Questionnaires (CQ) from the three consecutive years of follow-up (4) The annually conducted laboratory tests' results (Cytology, HPV DNA & Genotyping) (5) The annual measurements of the HIV markers (CD4 T cell count and HIV RNA load) (6) Histopathology reports

SAS version 9.4 was used for the statistical analyses. All tests were two-sided with the significance at $\alpha < 5\%$.

2.9.2 Dependent Variables

In this longitudinal project, the **exposure** of the HIV-positive population to HPV infection was investigated. The dependent variables (Ys) in this study were incident HPV-related cancers (anal, oropharyngeal and cervical) and prevalent precancerous lesions. Those dependent variables were the study **primary outcomes**.

2.9.3 Independent Variables

The following independent variables (Xs) were extracted from the patient and clinical questionnaires and laboratory tests results used in this project:

Patient's self-reported age, sex, education level, number of sexual partners, history of unprotected sex, history of sexually transmitted infections, smoking status; and annual measurements of CD4T cell counts and plasma HIV RNA viral load. They also included the detected HPV genotypes. Variables described as **Predictors** of the persistent HPV infection and its progression to neoplasia in the model included smoking status, HIV laboratory markers, and patient's sexual behavior. Variables described as **Cofactors** in the model included age, sex, number of sexual partners, history of STIs and others.

2.9.4 Descriptive Statistics

Descriptive statistics were conducted to describe the characteristics of the sample studied stratified by age, sex, and the study sites. As there were four study sites (St. John's, Halifax, Moncton and Saint John), the difference between groups was examined by using Chi-Square test for categorical variables and t-test for continuous variables to determine the level of significance. A preliminary analysis of patients' age through Histogram 1 illustrated data that was normally distributed. Therefore, Mean (SD) and Frequency (Proportions) were provided for continuous and categorical variables respectively. The outliers (data points that are greater or less than 3 standard deviations from the mean) can influence the average. The median as descriptive measure of CD4 T cell counts and HIV RNA load levels is more accurate than the mean to describe the central tendency of the data and to compare the variability of the data.

Incidence Rate (Unadjusted) based on person-time (person years [PYs])) and Age Standardized Incidence Ratio (ASIR) using the Canadian general male population as a reference group were calculated. Their tabulated calculations with formulas are presented on pages

2.9.5 Logistic Regression Models

Logistic Regression (Univariate and Multivariate) was used to determine whether the patient's self-reported variables were predictors of the HPV-associated cancers and precancers.

The dependent variables were dichotomous or having only two outcomes (presence of lesion/cancer 1= yes, 0= no). The advantage of using logistic regression is that the estimates of the coefficients in the equations (βn) can be interpreted easily as they are presented as odds ratios (ORs). Logistic regression is part of generalized linear models or GLM and allows one to predict a discrete outcome from a set of independent variables that may be continuous, discrete or dichotomous. In this study, the regression models used the Maximum Likelihood Estimation method (MLE), as the distribution of the response variables was binomial (Munroe B., 2003).

Steps in the building a Main Effects Model was built using the purposeful selection of variables. **Univariate Logistic Regression** ($Y = \beta 0 + \beta 1X1$) was carried out for each of the independent variables. All independent variables which were significant at 0.20 ($p \leq 0.020$) and non-significant but clinically important independent variables were included in the multivariate logistic regression model. **Multivariate Logistic Regression** model ($Y = \beta 0 + \beta 1X1 + \ldots + \beta nXn + \varepsilon$) allows us to estimate the association between given independent variable and the outcome holding all other variables constant (i.e., when the remaining independent variables are held at the same value or are fixed). MLR model included all the significant ($p < 0.20$) and clinically important variables. Then, variables which were not significant at 0.05 were removed from the model. We run the Likelihood Ratio test to examine the significance of the variables that were removed in the above step. Then we assessed the confounding effects by dropping one variable at a time to estimate the changes in Beta (β) coefficients (≥ 15%). Finally, we checked linear assumptions of continuous variables. If they did not meet the linearity assumptions, they were replaced with categorical variables. **Steps in building the Interaction Effects**: Interaction terms which should be clinically plausible were identified. A multivariate model included main effects and one interaction term on at a time; non-significant terms were removed from the model. Our Final Model includes main effects and all significant interaction terms.

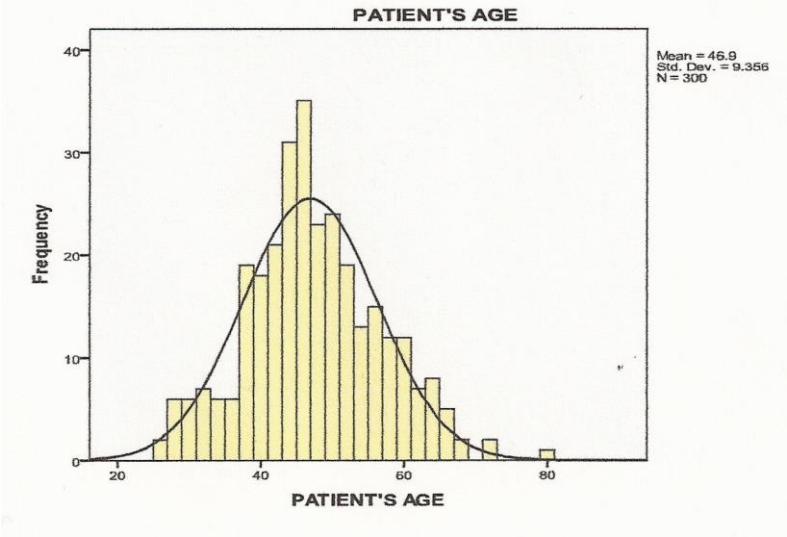
CHAPTER 3

STUDY RESULTS

3.1 Descriptive Statistics

The population mean age (SD) was 46.9 (9.3) years. The distribution of the study population by age is shown in Histogram 1. Of 300 patients at the baseline, 91.7% were males. At the end of the study, of 263 participants 93.2% were males.

Histogram 1
Distribution of the Study Population by Age (in years)



The population distribution by age categories and gender among the provinces is shown in Table 6. The differences among the provinces were compared using Chi-Square test (Fisher's Exact p value when the expected count of cell was less than 5):

Table 6: Distribution of the Study Population by the Site, Age Categories and Gender at the Baseline (N=300)

Variable	NLSJ N (%) =44 (14.7%)	NBM N (%) =90 (30.0%)	NSH N (%) =150 (50.0%)	NBSJ N (%) =16 (5.3%)
Age Categories N (%)*				
25 – 39	**8 (18.2%)**	**21 (23.3%)**	**25 (16.7%)**	**7 (43.8%)**
40 – 59	**33 (75.0%)**	**63 (70.0%)**	**105 (70.0%)**	**9 (56.3%)**
60 & >	**3 (6.8%)**	**6 (6.7%)**	**20 (13.3%)**	**0 (0.0%)**
Gender N (%) **				
Male	**34 (77.3%)**	**85 (94.4%)**	**142 (94.7%)**	**14 (87.5%)**
Female	**10 (22.7%)**	**5 (5.6%)**	**8 (5.3%)**	**2 (12.5%)**

***: P=0.0814 from Chi Square test (not significant)**
****: P= 0.0018 from Chi Square test (significant difference)**

Our findings from Table 6 showed that the distribution of the study population by age categories did not differ significantly across the study sites. The distribution by gender showed significant difference among the study sites with the highest female proportion at the NLSJ site (22.7%).

A total of 39 (13%) participants were lost during the follow-up years with 87% of attrition rate: (1) Eight (20.5%) patients withdrew from the study (2) Sixteen (41%) participants moved from the study site and lost contact with the site team (3) Six (15.4%) patients became "inactive" because of their improved health from HAART (Highly Active Anti-Retroviral Therapy). The research visits were adjusted to the patients' regular clinical schedule; therefore, these six patients did not have their follow-up visits for research purpose (4) Nine or 23.1% patients died. The change in the total number of participants by the study sites during the follow-up observations is shown in Table 7:

Table 7: Comparison of the Loss by the Study Sites throughout Follow-up Years

Study Site	Baseline N	1st year F-up N	2nd year F-up N	3rd year F-up N	Total Loss N (%)
NLSJ	44	43	42	42	2 4.5% at NLSJ 5.1% within total loss
NBM	90	88	86	83	7 7.8% at NBM 17.9% within total loss
NSH	150	138	127	121	29* 19.3% at NSH 74.4% within total loss
NBSJ	16	16	16	15	1 6.3% at NBSJ 2.6% within total loss
Total	300	285	271	261	39 100% within total loss 13.0% within cohort

***: Chi Square test, p value<0.05**

Our findings showed a significant difference in the loss to follow-up rates among the four study sites. The highest rate was observed at the NSH site (19.3%) and the lowest was observed at the NLSJ (4.5%). This might be explained by the facts that Halifax was the largest site and that this site experienced relocation of the research staff during the study years. Also, HIV-infected participants from Prince Edward Island were mainly treated in the Halifax ID clinics and had a tendency to move from one clinic to another. From 16 participants who moved from the study site and lost contact with the site team, twelve (75%) came from the NSH site.

Participants lost to follow-up and participants retained in the cohort were compared in Table 8. Baseline characteristics were stratified by these two groups.

Table 8: Comparison of Baseline Characteristics between Participants Lost to Follow-up and Participants Retained in the Cohort

Characteristic	Lost to Follow-up (39) N (%)	Remained in the Cohort (261), N (%)	P value*
Age, mean (SD)	**45.7 (10.6)**	**47.2 (9.1)**	**0.374**
Gender (males)	**32 (82.1%)**	**243 (93.1%)**	**0.029**
Smoking (yes)	**19 (48.7%)**	**111 (42.5%)**	**0.492**
Male Partners			
0	**15 (38.5%)**	**103 (39.5%)**	
1 or 2	**14 (35.9%)**	**106 (40.6%)**	**0.479**
≥3	**10 (25.6%)**	**52 (19.9%)**	
Unprotected Oral Sex (yes)	**17 (43.6%)**	**128 (49.0%)**	**0.607**
Unprotected Anal Sex (yes)	**8 (20.5%)**	**47 (18.0%)**	**0.663**
Ever on anti-HIV therapy	**31 (79.5%)**	**240 (92.0%)**	**0.036**
Currently on ARV therapy	**30 (76.9%)**	**230 (88.1%)**	**0.074**
CD4 cells count (<200 cells/mL)	**9 (23.1%)**	**75 (28.7%)**	**0.701**
History of Anogenital Warts (yes)	**13 (33.3%)**	**95 (36.4%)**	**0.615**

***: t test for continuous variable (age) and chi square test for all categorical variables**

There was a significant difference between two groups by their gender distribution with the higher proportion of males among the retained participants (p=0.029). The proportion of patients who have been on antiretroviral therapy prior to the study was higher among the retained participants as well (p=0.036).

The causes of nine deaths were analysed, leading to two patients who reached the study endpoint (cancer) but died during the follow-up period being included into the final analysis. This brought the total to **263** participants. The two cases that were included despite the patients' deaths were:

Male patient who died of Tongue Cancer in 2010 with HPV 16 in his oropharyngeal specimen, and combination of HPVs 16 & 52 in his anal specimen, and male patient who died of Kidney Failure in 2011 but was previously diagnosed with Anal Invasive Squamous Cell Carcinoma with the combination of HPVs 11, 16, 39, 52 & 74 in his anal specimen.

3.1.1. Descriptive Statistics for 300 Participants at the Baseline

Data from the 26-item Patient's Questionnaire was analyzed:

Of 300 patients at the baseline, 271 (90.3%) had reportedly been on anti-HIV treatment sometimes previous to the study, and 260 (86.7%) were on antiretroviral therapy at the enrolment time. Of 263 patients at the end of the study, 232 (88.2%) have been receiving a combined antiretroviral therapy (ARVT). The majority (221(73.7%)) of participants did not have an AIDS-defining illness previous to the study; 292 (97.3%) of them did not have any AIDS-defining events at the enrolment time, and almost all of them (261(99.2%)) were AIDS-free at the end of the follow-ups.

The majority of the study participants (245 (81.7%)) had anal sex with condoms. The number of patients who had oral sex with condoms (or did not practice oral sex), and who did not use condoms during oral sex was almost equal (146 (48.7%) & 154 (51.3%), p=0.437).

All 25 female participants had been screened for cervical cancer prior to the study within different time intervals from their last Pap test. Of those 25 females, 7 (28%) were in the "less than 6 months ago"; 4 (16%) were in "6 months to less than 12 months" and 6 (24%) females in "from 1 year to less than 3 years ago" categories. Four (16%) females had had it "from 3 years to less than 5 years"; one (4%) woman was last screened "5 and more years ago;" and three (12%) of them did not recall the date of the last Pap test.

The most important demographics and other patients' characteristics are presented in Tables 9 and 10 below:

Table 9: Patients Demographics and Other Important Characteristics at Baseline (N=300)

Variable	N (%)
HAART (yes)	**271 (90.3%)**
AIDS-defining Illness (yes)	**79 (26.3%)**
HPV vaccination (yes)	**0 (0.0%)**
Undergraduate and Graduate Education (yes)	**147 (49.0%)**
Smokers (yes)	**132 (44.0%)**
History of Anal Pap Test (yes)	**33 (11.0%)**
Number of Male Sexual Partners	**IQR=1 male partner/year**
History of Unprotected Anal Sex (yes)	**55 (18.3%)**
History of Unprotected Oral Sex (yes)	**154 (51.3%)**
History of STIs (yes)	**193 (64.3%)**
History of Hepatitis C (yes)	**31 (10.3%)**
History of Anogenital Warts (yes)	**106 (35.3%)**
History of Genital Herpes (HHV-2) (yes)	**46 (15.3%)**

Table 10: Patients' Baseline Demographics and Behavior Characteristics by Gender, and the most frequently observed HPV genotypes stratified by Body Site and Gender (N=300)

Characteristic	Males (N=275)	Females (N=25)	P value*
Age, mean (SD)	**46.7 (9.1)**	**41.6 (7.3)**	**0.006**
Unprotected Vaginal Sex (N/%)	**8 (2.9%)**	**8 (32.0%)**	**<0.0001**
Unprotected Oral Sex (N/%)	**139 (50.5%)**	**6 (24.0%)**	**0.012**
Unprotected Anal Sex (N/%)	**53 (19.3%)**	**2 (8.0%)**	**0.277**
Male Partners			
0	**111 (40.4%)**	**7 (28.0%)**	**0.031**
1 or 2	**104 (37.8%)**	**16 (64.0%)**	
≥3	**60 (21.8%)**	**2 (8.0%)**	
Anal HPVs			
16	**91 (33.1%)**	**2 (8.0%)**	**0.01**
18	**41 (14.9%)**	**1 (4.0%)**	**0.13**
45	**48 (17.5%)**	**2 (8.0%)**	**0.22**
52	**49 (17.8%)**	**2 (8.0%)**	**0.21**
Oral HPVs			
16	**5 (1.8%)**	**0 (0.0%)**	**N/A**
35	**2 (0.7%)**		
45	**2 (0.7%)**		
72	**3 (1.1%)**		
Cervical HPVs			
16	**N/A**	**4 (16.0%)**	**N/A**
18		**1 (4.0%)**	

***: t test for continuous variable (age) and chi square test for all categorical variables**

The findings from Table 10 showed that at the enrolment time the cohort males were significantly older than females (p=0.006). Higher proportion of females reportedly practised unprotected vaginal sex (p<0.0001); while a higher proportion of males reported history of unprotected oral sex (p=0.012). The proportion of women who had 1 or 2 male sexual partners per year was higher than the proportion of men who reported the same number of male sexual partners per year (64.0% vs. 37.8%). The proportion of men with anal HPV 16 infection was significantly higher than proportion of women infected by the same HPV genotype and at the same body site (p=0.01).

3.1.2. Descriptive Statistics for the Study Sites

The tables below from 11 to 14 compare our findings among the study sites. This was a collaborative multicenter cohort study and the study co-investigators wanted to know the site-related statistics as well. The comparison between the study sites was carried out by Chi Square test.

Table 11: Patients' Demographics by the Study Sites at the Baseline (N=300)

PARAMETER	NLSJ (St. John's)	NBM (Moncton)	NSH (Halifax)	NBSJ (Saint John)	P value*
Patients, N (%)	**44 (14.7%)**	**90 (30.0%)**	**150 (50.0%)**	**16 (5.3%)**	
Age, mean (SD) (min, max)	**45.2 (7.4) 28-62**	**45.9 (9.7) 26-80**	**48.7 (9.4) 27-72**	**41.8 (9.0) 26-57**	**0.0044**
Country of origin, N (%) (Canada)	**43 (97.7%)**	**87 (96.7%)**	**134 (91.3%)**	**16 (100%)**	**0.2291**
Race, N (%) (White Caucasian)	**43 (97.7%)**	**88 (97.8%)**	**142 (89.3%)**	**15 (93.8%)**	**0.5402**
Education, N (%)					
•None/Elementary	**1 (2.3%)**	**4 (4.4%)**	**7 (4.7%)**	**0 (0.0%)**	**0.1034**
•High School/Diploma	**21 (47.7%)**	**25 (27.8%)**	**35 (23.0%)**	**5 (33.3%)**	
•College/University/+	**22 (50.0%)**	**61 (67.8%)**	**108 (72.3%)**	**11 (66.7%)**	
Children (None), N (%)	**32 (72.7%)**	**83 (92.2%)**	**139 (92.7%)**	**13 (81.3%)**	**0.0026**

***: t test for continuous variable (age) and chi square test for all categorical variables**

The findings from Table 11 showed that the average age was different across the study sites (p=0.0044) with the lowest mean age of 41.8 years at the NBSJ site, and the highest mean age of 48.7 years in NSH. We also found a significant difference in the proportion of the HIV-positive participants with children across the study sites (p=0.0026) with the highest in St. John's, NL (27.3%) and the lowest in Halifax, NS (7.3%).

Table 12: Distribution of the Risk Factors for HPV associated Malignancy by Provinces (N=300)

RISK FACTOR	NLSJ N=44 (34 males)	NBM N=90 (85 males)	NSH N=150 (142 males)	NBSJ N=16 (14 males)	P value*
HPV in Anal Specimen (Positive) N (%)	35/44 (79.5%)	77/90 (85.6%)	133/150 (88.7%)	15/16 (93.8%)	0.1001
Smokers (yes), N (%)	22/44 (50%)	38/90 (42.2%)	65/150 (43.3%)	5/16 (31.3%)	0.6165
Number of Male Partners/year** N (%)					
• 0	23 (52.2%)	37 (41.1%)	44 (29.3%)	3 (18.7%)	0.1595
• 1-2	12 (27.3%)	32 (35.6%)	66 (44.0%)	10 (62.5%)	
• ≥3	9 (20.5%)	21 (23.3%)	40 (26.7%)	3 (18.8%)	
Number of Female *** Partners/year, N (%)					
• 0	29 (85.3%)	73 (85.3%)	130 (91.5%)	12 (85.7%)	0.4687
• ≥1	5 (14.7%)	12 (14.7%)	12 (8.5%)	2 (14.3%)	
History of Unprotected Anal Sex (yes), N (%)	6 (13.6%)	18 (20.0%)	29 (19.3%)	2 (12.5%)	0.7835
History of Unprotected Oral Sex (yes), N (%)	12 (27.3%)	50 (55.6%)	71 (47.3%)	12 (75.0%)	0.0025
History of Unprotected Vaginal Sex**** (yes), N (%)	3 (30.0%)	1 (20.0%)	3 (37.5%)	1 (50.0%)	1.000
History of Anal Pap (yes)**, N (%)	3 (6.8%)	10 (11.1%)	14 (9.3%)	6 (37.5%)	0.0195

*: t test for continuous variables and chi square test for categorical variables
**: Denominator is entire cohort
***: Denominator is number of males
****: Denominator is number of females

The findings from Table 12 showed that there was a significant difference in the rates of reported unprotected oral sex (p=0.0025) and in the rates of anal Pap test (p=0.0195) among the four study sites with their highest rates at the NBSJ (75.0% & 37.5% respectively). The distribution of other risk factors for HPV associate malignancy did not differ significantly across the study sites.

Table 13: Distribution of Risk Factors for HPV Infection by Provinces

RISK FACTOR	NLSJ (St. John's) N=44	NBM (Moncton) N=90	NSH (Halifax) N=150	NBSJ (Saint John) N=16	P value*
HEP B (yes), N (%)	7 (15.9%)	8 (8.9%)	15 (10.0%)	3 (18.8%)	0.4456
HEP C (yes), N (%)	4 (9.1%)	10 (11.1%)	15 (10.0%)	3 (18.8%)	0.7492
ANOGENITAL WARTS (yes), N (%)	18 (40.9%)	33 (36.7%)	53 (35.3%)	6 (37.5%)	0.9508
CHLAMYDIA (yes), N (%)	2 (4.5%)	10 (11.1%)	14 (9.3%)	3 (18.8%)	0.3767
GONORRHEA (yes), N (%)	7 (15.9%)	20 (22.2%)	40 (26.7%)	5 (31.3%)	0.4710
SYPHILIS (yes), N (%)	2 (4.5%)	3 (3.3%)	26 (17.3%)	1 (6.3%)	0.0032
GENITAL HERPES (HHV 2), (yes), N (%)	6 (13.6%)	12 (13.3%)	27 (18.0 %)	2 (12.5%)	0.7487
Total Number of STIs/person Mean (SD), (min, max)	1.1(1.2) 0-4	1.0 (1.1) 0-4	1.3 (1.3) 0-6	1.4 (1.5) 0-4	0.3863

***: t test for continuous variable (# of STIs) and chi square test for all categorical variables**

The findings from Table 13 showed that the distribution of history of STIs among HIV-positive participants did not differ significantly across the study sites. However, the proportion of previously contracted Syphilis was significantly different among the four study sites (p=0.0032) with the highest at the NSH site (17.3%) and the lowest at the NBM site (3.3%).

Table 14: Participants' Knowledge of HPV and HPV-associated Conditions

SURVEY QUESTIONS	NLSJ (St. John's) N=44	NBM (Moncton) N=90	NSH (Halifax) N=150	NBSJ (Saint John) N=16	P value*
HPV doesn't cause Anogenital Warts, N (%)					
• True	8 (18.2%)	14 (15.6%)	9 (6.0%)	2 (12.5%)	0.147
• *False***	20 (45.5%)	37 (41.1%)	65 (43.3%)	8 (50.0%)	
• Don't know	16 (36.4%)	39 (43.3%)	76 (50.7%)	6 (37.5%)	
HPV can cause Cervical Cancer, N (%)					
• *True***	32 (72.7%)	68 (75.6%)	97 (64.7%)	12 (75.0%)	0.522
• False	1 (2.3%)	1 (1.1%)	5 (3.3%)	1 (6.3%)	
• Don't know	11 (25.0%)	21 (23.3%)	48 (32.0%)	3 (18.8%)	
HPV Vaccine can lower risk for Cancer & Warts, N (%)					
• *True***	30 (68.2%)	40 (44.4%)	55 (36.7%)	10 (62.5%)	0.003
• False	0 (0.0%)	9 (10.0%)	9 (6.0%)	1 (6.3%)	
• Don't know	14 (31.8%)	41 (45.6%)	86 (57.3%)	5 (31.3%)	
Importance of PAP test for Women with HPV Vaccination (*yes*), N (%)	44 (100%)	80 (88.9%)	120 (80.0%)	14 (87.5%)	0.069
Importance of Safer Sex for those with HPV Vaccination (*yes*), N (%)	44 (100%)	83 (92.2%)	131 (87.3%)	14 (87.5%)	0.114

***: Chi square test for all categorical variables**
****: Correct answers are *italicised*.**

Our findings from Table 14 showed that the levels of knowledge about HPV and HPV-associated conditions did not differ significantly among the study sites. However, the proportion of the participants who checked True in the questionnaire about HPV vaccine and its impact on the risk of cervical cancer and warts was significantly different among the study sites (p=0.003) with the highest at the NLSJ (68.2%).

3.1.3. Results from the Analyses of the Laboratory Tests

In this prospective cohort study, the annual measurements of CD4 T cell counts and plasma HIV RNA load levels were analyzed from the baseline and three consecutive years of follow up. The 12- and 9-item Clinical Questionnaires (Appendix D & E) included only snap-shot annual measurements of both HIV markers at the time of appointment with a treating physician, and did not provide data related to these parameters' mean, median, nadir, and range measurements during the whole year of observation. For research purposes, the CD4 T cell count was traditionally categorized at the 200 cells/mL being the cut-off. These two categories were: patients with CD4 count < 200 cells/mL and patients with CD4 count ≥200 cells/mL. The following references were used to justify this categorization:

According to the US DHHS, CD4 T count < 200 cells/uL in HIV-positive adults is the AIDS defining illness. Both HIV RNA load > 100,000 copies/mL and CD4 count < 200 cells/uL associated with an increased risk of anal cancer (US Department of Health and Human Services, 2009).

There is evidence from different studies on the incidence of oral opportunistic infections in adults with HIV/AIDS. Studies showed that among those with CD4 cell count less than 200 cells/mL, the incidence of oral opportunistic infections [Kaposi's sarcoma (100%), candidiasis (82.2%), linear gingival erythema (70.0%), hairy leukoplakia (66.3%), and others] was strongly associated with severe immune suppression. The incidence of these infections was found to be significantly correlated to a reduced CD4 cell count, thus serving as a potential clinical marker of HIV viremia and progressive HIV disease (OR (95%CI)=3.1 (1.9-4.9); $p<0.001$) (Bodhade AS, Ganvir SM & Hazarey VK, 2011; Patton LL, 2000).

Plasma HIV RNA viral load was reported by the study sites as categorical data: (0) "Never had test" (1) "Undetectable level of serum RNA at ≤50 copies/mL" (2) "Detectable level at >50 copies/ml" (3) "Unknown." These categories of serum HIV RNA have already been widely used by our co-investigators. In our statistical analyses, serum HIV RNA was always treated as a continuous variable.

Table 15: Important Variables included in the Statistical Analyses

Name and Type of Variable		Statistical Analysis
Age	**Continuous** **Categorical**	**Mean (SD), t-test** **Chi Square**
Gender	**Binomial**	**Chi Square**
CD4 T cell count	**Continuous** **Categorical**	**Mean (SD), Median, Min-Max, t-test** **Chi Square**
Plasma HIV RNA Viral Load	**Continuous**	**Mean (SD), Median, Min-Max, t-test**
Number of HPV Genotypes	**Continuous**	**Mean (SD), Median, Min-Max, t-test**
HPV Genotype Individually	**Binomial**	**Chi Square**
Smoking	**Binomial**	**Chi Square**
Precancers/Cancer	**Binomial**	**Chi Square**
Number of Male Partners	**Continuous** **Categorical**	**Mean (SD), Median (IQR)** **Chi Square**
History of STIs	**Dichotomous**	**Chi Square**
History of Hepatitis C	**Dichotomous**	**Chi Square**
History of Anogenital Warts	**Dichotomous**	**Chi Square**
History of Genital Herpes	**Dichotomous**	**Chi Square**

The association between the outcome variables (the precancerous lesions and incident cancers) and predictors was examined in logistic regression analyses.

The mean, median and min/max values of the laboratory tests were provided. Our findings are shown in Tables 16 & 17 and Linear Graphs 1 & 2.

Table 16: Comparison of the CD4 T cell count Measurements (cells/mL) Through the Study Years

Year in the study & N	Mean CD4 Count	Median CD4 Count	Min CD4 Count	Max CD4 Count
Baseline (300)	**366**	**360**	**3**	**1404**
1st F-up (285)	**549**	**529**	**10**	**1697**
2nd F-up (271)	**587**	**576**	**15**	**1771**
3rd F-up (263)	**1,368**	**583**	**39**	**141,493**

Graph 1

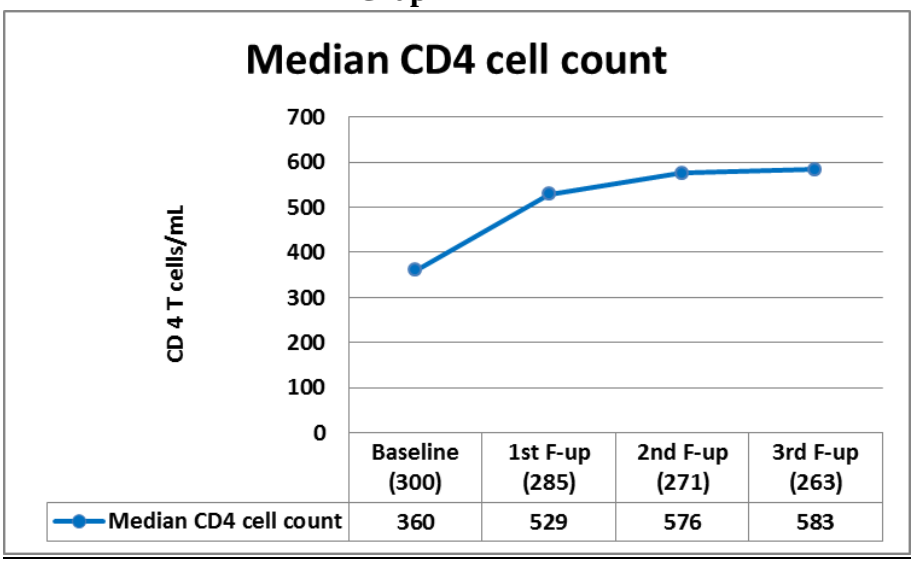
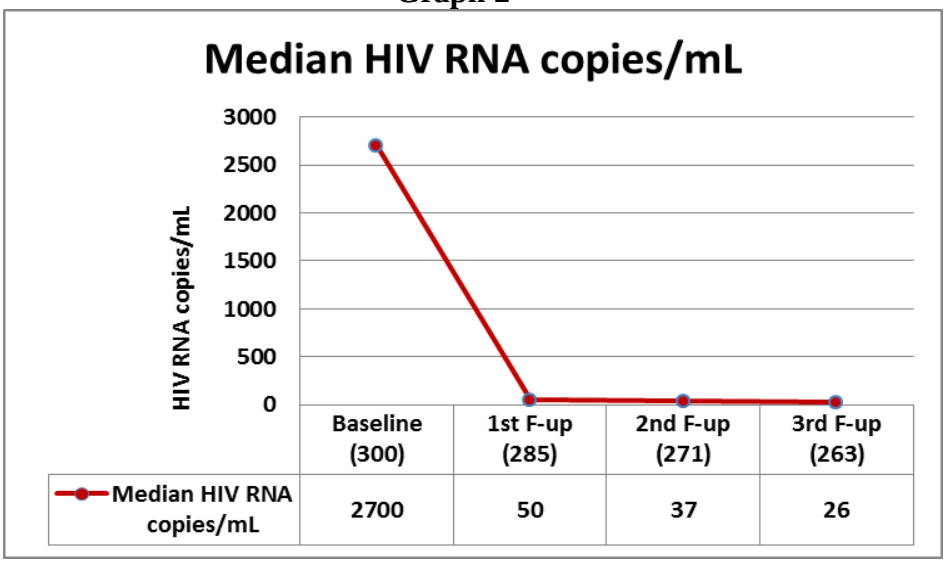


Table 17: Comparison of the Levels of Plasma HIV RNA (copies/mL) Through the Study Years

Year in the study & N	Mean	Median	Min	Max
Baseline (300)	**216,397**	**2,700**	**50**	**7750,000**
1 year F-up (285)	**6,484**	**50**	**40**	**1620,000**
2 year F-up (271)	**2,567**	**<50**	**20**	**180,000**
3 year F-up (263)	**206**	**<50**	**0**	**159,000**

Graph 2



Our results showed an improvement in both CD4 cell counts and serum HIV RNA levels at different levels of measurements of central tendency (mean, median) and variability (range) of the study data.

The 12-item Baseline Clinic Questionnaire (Appendix D) included two questions on HIV antiretroviral status: (1) “Ever on anti-HIV medication?” (2) “Currently (at the time of entry into the current study) on anti-HIV medication?” Analysis of this data showed that of the 300 baseline participants, 271 (90.3%) were on anti-HIV therapy prior to the study. We may assume that at enrolment time, 29 (9.7%) participants were naïve to combined antiretroviral therapy, and that their health conditions were improved after initiation of anti-HIV therapy. Moreover, 40 (13.3%) participants have restarted their ARV treatment at some point during the study years. Another explanation of the improvement in the participants’ average health status might be related to a more responsible intake and better compliance with anti-HIV drugs. The number of participants on anti-HIV therapy varied slightly, from 260 (86.7%) to 232 (88.2%) throughout the 4 years of observation.

Among the 300 participants at the screening year, a total 46 HPV genotypes were detected. Of them, 18 (39%) were high-risk (HR: 16, 18, 31, 33, 35, 39, 43, 45, 50, 51, 52, 53, 56, 58, 59, 68, 73, 82) and 28 (61%) were probable high and low-risk (PHR & LR: 6, 11, 23, 26, 30, 32, 34, 42, 44, 54, 55, 57, 61, 62, 66, 67, 69, 70, 71, 72, 81, 84, 86, 87, 89, 90, CP108, IS39) types. Of 300 patients, 246 (82.0%) were “positive” for HPV infection and of these, 156 (63.4%) were infected with 3 and more HPV types simultaneously. The highest number of HPV genotypes in one anal sample was 15. The number of HPV genotypes in a single specimen was divided into the two large categories: 1) integrating cases with 1 or 2 HPVs and 2) integrating cases with 3 & more HPVs. All HPV genotypes were also divided into Low Risk (LR) and High Risk (integrating HR & PHR HPVs) categories. The 2X2 contingency table is shown below:

Table 18: Number of HPVs in a Single Specimen stratified by Risk Categories*

Number of HPVs in one specimen	Risk category		Total N (%)
	LR N (%)	HR N (%)	
1 or 2 HPVs	42 (46.7)	48 (53.3)	90 (100)
≥ 3 HPVs	59 (37.8)	97 (62.2)	156 (100)
Total	101	145	246 (100)

***: Chi square test, p=0.174**

The interpretation of the findings is:

There was no difference in distribution of HR HPVs between two groups: those patients with 3 or more HPV genotypes in a single specimen and those with 1 or 2 HPVs in a single specimen (62.2% vs. 53.3%, p=0.174).

A total of 625 cytology reports were collected in the screening year from 300 anal, 300 oropharyngeal, and 25 cervical specimens. The cytology analysis of the specimens showed that abnormalities were mostly detected among the anal specimens.

In this study, we analyzed the distribution of High Risk (HR) HPV genotypes. The most frequently observed HR HPV types in all 625 specimens at the screening year were: HPV 16 – 31.3%; HPV 52 – 15%; HPV 45 – 11.7%; HPV 51 – 11.3%, and HPVs 18 & 59 at 9.3% each.

The distribution of these and other HPV genotypes at the baseline by both gender and age (continuous variable) is shown in Table 19. The differences between groups were examined by Chi-Square (χ^2) test for gender and by t-test for age.

Table 19: Distribution of the Frequently Observed HPV Genotypes by Gender and Age among 300 Participants at the Baseline

HPV TYPE	GENDER			χ^2	AGE	t-test
	Males N=275 91.7%	vs.	Females N=25 8.3%	P*	Mean (SD)	P
HPV16 +	89 (32.4%)		5 (20.0%)	0.386	47.1 (8.4)	0.824
-	186 (67.6%)		20 (80.0%)		46.9 (9.8)	
HPV18+	25 (9.1%)		3 (12.0%)	0.716	43.4 (8.4)	0.035
-	250 (90.9%)		22 (88.0%)		47.3 (9.4)	
HPV31+	17 (6.2%)		2 (8.0%)	0.665	48.6 (9.5)	0.421
-	258 (93.8%)		23 (92.0%)		46.9 (9.3)	
HPV35+	20 (7.3%)		3 (12.0%)	0.422	45.4 (8.4)	0.416
-	255 (92.7%)		22 (88.0%)		47.1 (9.4)	
HPV39+	24 (8.7%)		1 (4.0%)	0.707	45.7 (8.2)	0.475
-	251 (91.3%)		24 (96.0%)		47.1 (9.4)	
HPV45+	33 (12.0%)		2 (8.0%)	0.751	45.8 (8.3)	0.447
-	242 (88.0%)		23 (92.0%)		47.1 (9.5)	
HPV51+	31 (11.3%)		3 (12.0%)	1.000	43.8 (7.5)	0.037
-	244 (88.7%)		22 (88.0%)		47.4 (9.5)	
HPV52+	39 (14.2%)		6 (24.0%)	0.236	49.3 (8.2)	0.064
-	236 (85.8%)		19 (76.0%)		46.5 (9.5)	
HPV53+	23 (8.4%)		2 (8.0%)	1.000	47.6 (8.2)	0.738
-	252 (91.6%)		23 (92.0%)		46.9 (9.0)	
HPV56+	17 (6.2%)		2 (8.0%)	0.665	51.6 (10.7)	0.024
-	258 (93.8%)		23 (92.0%)		46.6 (9.2)	
HPV59+	27 (9.8%)		1 (4.0%)		44.0 (8.4)	0.075
-	248 (90.2%)		24 (96.0%)	0.489	47.3 (9.4)	
HPV62+	23 (8.4%)		1 (4.0%)		52.7 (10.8)	0.002
-	252 (91.6%)		24 (96.0%)	0.705	46.5 (9.1)	

***: Fisher Exact p-value when the expected count of cell was less than 5**

The study findings from Table 19 showed that at the baseline, the distribution of HPV-positive genotypes did not differ significantly between males and females. The age distribution between HPV-positive and HPV-negative participants also did not differ significantly. However, the mean age of HPV 18 and HPV 51 positive participants was significantly lower than the mean age of participants negative to those HPVs (43.4 vs. 47.3; p=0.035 & 43.8 vs. 47.4; p=0.037). The mean age of HPV 56 and HPV 62 positive participants was significantly higher than the mean age of participants negative to those HPV types (51.6 vs. 46.6; p=0.024 & 52.7 vs. 46.5; p=0.002).

At the baseline, the mean (SD) age in the study sample was 46.9 (9.3) years, with the following distribution by age categories: 25-39 years of age – 62 (20.7%), 40-59 years of age – 209 (69.7%) and 60 years and older – 29 (9.6%). Over the four years of observation, the study population aged appropriately, skewing towards the middle and older age categories. The mean (SD) age at the study end was 51.3 (9.1), with the following distribution by the age categories: 25-39 years of age – 34 (12.9%), 40-59 years of age – 187 (71.1%) and 60 years and older – 42 (16.0%).

At the study end, the most frequently observed HR HPV genotypes were: HPV 16 – 38.8%; HPV 45 – 19.8%; HPV 52 – 19.4% and HPV 18 – 16.7%. Their distribution was stratified by gender and age and is displayed in Table 20. The Chi Square test was carried out for gender and t-test for age.

Table 20: Distribution of the Frequently Observed HPV Genotypes by Gender and Age among 263 Participants at the Study End

HPV TYPE	GENDER			χ^2	AGE	t-test
	Male N=245 93.2%	vs.	Female N=18 6.8%	P*	Mean (SD)	P
HPV16+	96 (39.2%)		6 (33.3%)	0.803	47.2 (8.6)	0.963
-	149 (60.8%)		12 (66.7%)		47.2 (9.5)	
HPV18+	42 (17.1%)		2 (11.1%)	0.746	44.4 (8.7)	0.023
-	203 (82.9%)		16 (88.9%)		47.8 (9.2)	
HPV31+	21 (8.6%)		2 (11.1%)	0.663	47.4 (8.7)	0.939
-	224 (91.4%)		16 (88.9%)		47.2 (9.2)	
HPV33+	20 (8.2%)		3 (16.7%)	0.200	46.7 (10.3)	0.779
-	225 (91.8%)		15 (83.3%)		47.3 (9.0)	
HPV35+	32 (13.1%)		3 (16.7%)	0.717	47.2 (8.6)	0.979
-	213 (86.9%)		15 (83.3%)		47.2 (9.2)	
HPV39+	29 (11.8%)		3 (16.7%)	0.467	48.4 (8.5)	0.443
-	216 (88.2%)		15 (83.3%)		47.1 (9.2)	
HPV45+	50 (20.4%)		2 (11.1%)	0.540	45.8 (7.9)	0.224
-	195 (79.6%)		16 (88.9%)		47.6 (9.4)	
HPV51+	40 (16.3%)		2 (11.1%)	0.747	43.9 (8.4)	0.009
-	205 (83.7%)		16 (88.9%)		47.9 (9.2)	
HPV52+	49 (20.0%)		2 (11.1%)	0.539	48.7 (8.3)	0.188
-	196 (80.0%)		16 (88.9%)		46.8 (9.3)	
HPV53+	25 (10.2%)		3 (16.7%)	0.420	49.1 (12.5)	0.246
-	220 (89.8%)		15 (83.3%)		47.0 (8.7)	
HPV56+	25 (10.2%)		2 (11.1%)	1.000	50.0 (9.4)	0.090
-	220 (89.8%)		16 (88.9%)		46.9 (9.1)	
HPV59+	32 (13.1%)		1 (5.6%)	0.709	44.9 (8.8)	0.141
-	213 (86.9%)		17 (94.4%)		47.5 (9.2)	
HPV62+	31 (12.7%)		1 (5.6%)	0.707	51.4 (11.5)	0.014
-	214 (87.3%)		17 (94.4%)		46.8 (8.8)	

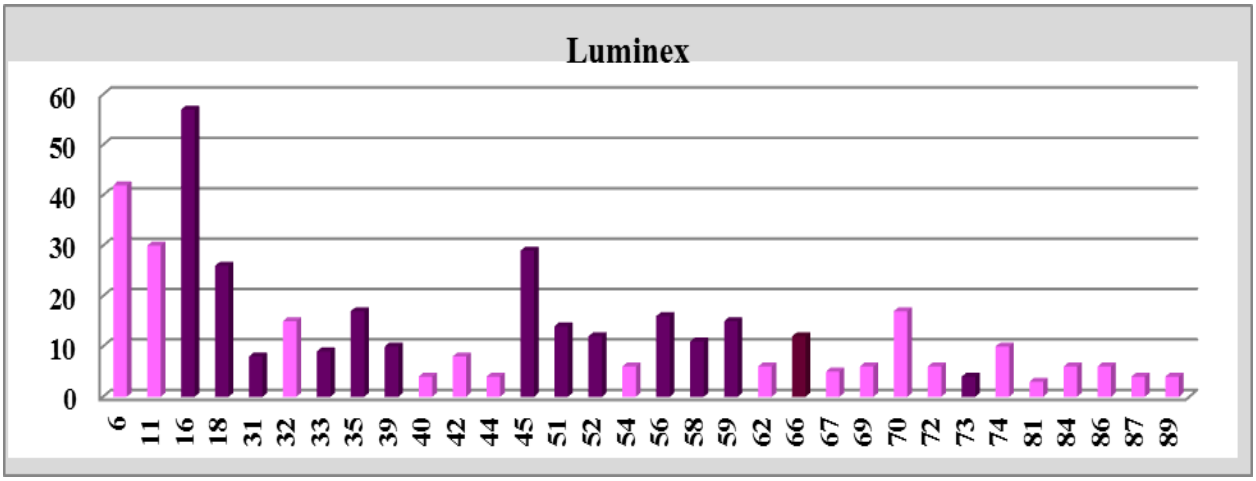
***: Fisher Exact p-value when the expected count of cell was less than 5**

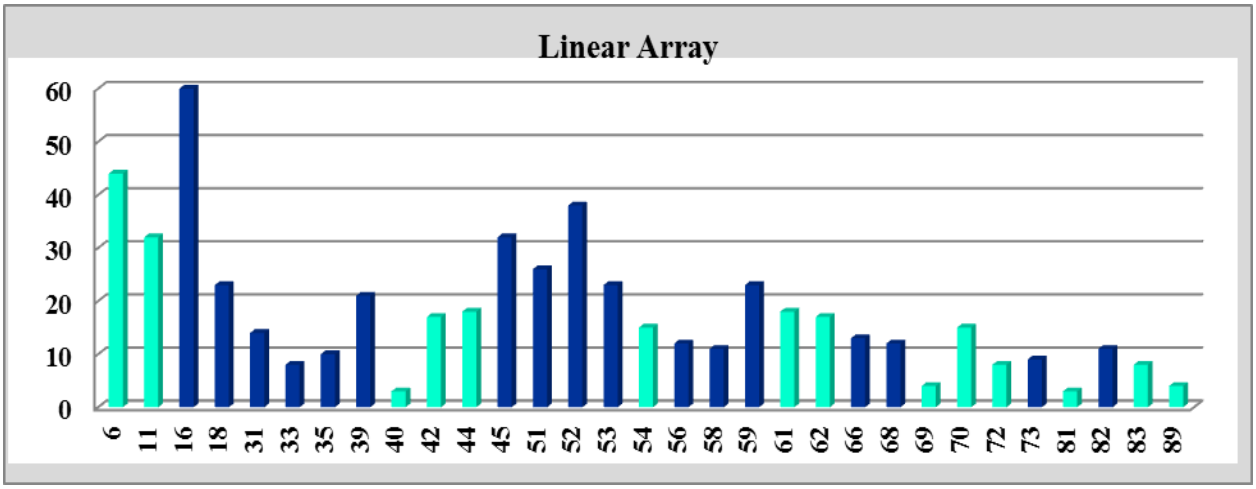
At the study end, the distribution of HPV-positive genotypes did not differ significantly between males and females. Neither did the age distribution between HPV-positive and HPV-negative participants. However, the mean age of HPV 62 positive participants was significantly higher than the mean age of participants negative to HPV 62 genotype (51.4 vs. 46.8; p=0.014). The mean age of HPV 18 positive participants was significantly lower than the mean age of participants negative to HPV 18 genotype (44.4 vs. 47.8; p=0.023).

In summary, the most frequently observed HR HPV genotypes were mainly distributed among participants in the middle-age group from 40 to 59 years of age. Throughout the study, infection with HPV 18 was more frequently observed among participants with lower mean age, while HPV 62 was more frequently observed among participants with higher mean age.

We had an opportunity to compare results from two different PCR assays used during the screening year only. Reliability of data collection is of overall confidence in research study's accuracy. The importance of technologists in a clinical laboratory having a high degree of consistency when evaluating samples is an important factor in the quality of healthcare and clinical research studies (McHugh ML, 2012). The Kappa statistics are used to test interrater reliability. Kappa ranges from -1 to 1. As with all correlation statistics, the k is a standardized value and thus is interpreted the same across multiple studies (Marston L., 2010). It was suggested the kappa results be interpreted as follows: value ≤ 0 – as no agreement; 0.01-0.20 – as none to slight; 0.21-0.40 – as fair; 0.41-0.60 – as moderate; 0.61-0.80 – as substantial; and 0.81-1.00 – as almost perfect agreement. Many texts recommend 80% agreement as the minimum acceptable interrater agreement. The SAS Freq. procedure was used to calculate the k coefficient between two PCR assays: Luminex and Linear Array.

Graphs 3 & 4: HPV Genotypes Detected in the Anal Samples by Luminex vs. Linear Array Assays





The dark columns are HR HPV types
The light columns are LR HPV types

X axis – HPV genotypes
Y axis – Number of HPV+ cases

The Kappa statistics to test interrater agreement in detection of HPV16 and HPV18 in anal specimens showed strong agreement between Luminex and Linear Array PCR assays:

HPV16: k=0.81, 95%CI (0.73-0.89), p<0.001; HPV18: k=0.90, 95%CI (0.81-0.99), p<0.001

The PCR assays are costly and time-consuming. Due to the strong agreement between two tested assays, we decided to use a single PCR - Luminex®-Based Genotyping Assay for the HPV DNA and Genotyping analysis during the follow-up years.

The total number of gathered specimens during the study years was 2416 (including 92 samples from 46 male patients that were originally enrolled at the baseline and were later excluded from the study due to the project's financial issues), and from them, 2324 specimens were tested for abnormalities and included in the final analysis (Table 21).

Table 21: Number of Patients and Specimens throughout the Study

	Baseline	1st Year F-up	2nd Year F-up	3rd Year F-up	Total
Number of Patients	**300**	**285**	**271**	**263**	
Number of Specimens	**625**	**595**	**560**	**544**	**2324**

During the study, 2324 specimens were analysed in total. Of them, 1119 were anal specimens, 1119 oropharyngeal specimens and 86 cervical Pap smears. Of the unsatisfactory or inconclusive specimens reported by the cytopathology laboratory, all were anal Pap smears:

Baseline: 69 unsatisfactory, of them 28 were repeated within 3 months

First follow-up: 14 unsatisfactory, 6 were repeated within 3 months

Second follow-up: 10 unsatisfactory, 7 were repeated within 3 months

Third follow-up: 21 unsatisfactory, 15 were repeated within 3 months

Definition by the TBS, 2001: Unsatisfactory specimen means that the laboratory has been unable to come to a firm conclusion on the basis of the specimen provided (e.g., not all cell divisions are collected, obscuring blood, etc.).

There were 69 anal specimens at baseline that were reported as unsatisfactory for evaluation. A literature review and on-line discussion with the study PI and co-investigators was initiated by author. It was decided to switch from cotton swabs to Dacron swabs (NYS Guidelines recommendations on anal pap smears). As a result, the proportion of inconclusive anal specimens was reduced. No incidence of inconclusive results was reported from the National Microbiology Laboratory about PCR tests on HPV DNA and HPV genotyping.

The type and number of the detected cytological abnormalities varied from year to year, and are presented below in Table 22 with calculated prevalence (%).

Prevalence is the number of individuals identified as cases during a specified period of time, divided by the total number of people in that population (Principals of Epidemiology in Public Health Practice, CDC, 2012).

In our study, no cancer cases were diagnosed at the baseline. All cancer cases were diagnosed during the follow-up years and treated in the final analysis as incident cases.

The case definition of precancerous lesion is based on cytological abnormality. There were precancerous lesions detected at the study baseline and during the follow-up years (Table 22). However, the time of occurrence of any particular abnormality is questionable, and a cut-point in time to differentiate the prevalent and incident cases is not clear. Development of a precancerous lesion is influenced by factors such as a long viral latency, high rate of viral clearance and reinfection, transient manifestation of productive viral infection, and persistent HPV exposure. Thus, we treated all precancerous lesions as prevalent cases.

Table 22: Prevalence of Cytological Abnormalities in the Study Sample by Type, Body Site and Year of Observation

Body Site	Type of Cytological Abnormality	Baseline N=300	1st year F-up N=285	2nd year F-up N=271	3rd year F-up N=263	Prevalence (%)
ANUS	ASC-US	100 (56.1%)	29 (16.3%)	27 (15.2%)	22 (12.4%)	178/300=59.3
(denominator is entire cohort)	LSIL	73 (49.0%)	36 (24.2%)	19 (12.8%)	21 (14.0%)	149/300=49.7
	HSIL	9 (40.9%)	4 (18.2%)	4 (18.2%)	5 (22.7%)	22/300=7.3
MOUTH	ASC-US	4 (66.6%)	-	1 (16.7%)	1 (16.7%)	6/275=2.2
(denominator is number of males)	LSIL	1 (16.7%)	-	-	-	1/275=0.4
CERVIX	ASC-US	1 (25.0%)	2 (50.0%)	1 (25.0%)	-	4/25=16
(denominator is number of females)	LSIL	2 (8.0%)	-	-	-	2/25=8
	HSIL	1 (4.0%)				1/25=4

All cytology reports were sent back to the study co-investigators. They in turn, referred these patients to the specialists (gastroenterology surgeons, gynecologists or ENT specialists) for further investigation and/or appropriate treatment with follow-up observations. The results from the histology readings throughout the years were accumulated and analyzed when the study was completed. Table 23 demonstrates precancerous lesions and cancers among the 263 study patients included in this analysis. The cases with ASC-US (TBS) or Atypia (CIN) were excluded from the analysis, as cytological changes that might have reversed to the cells' normal status.

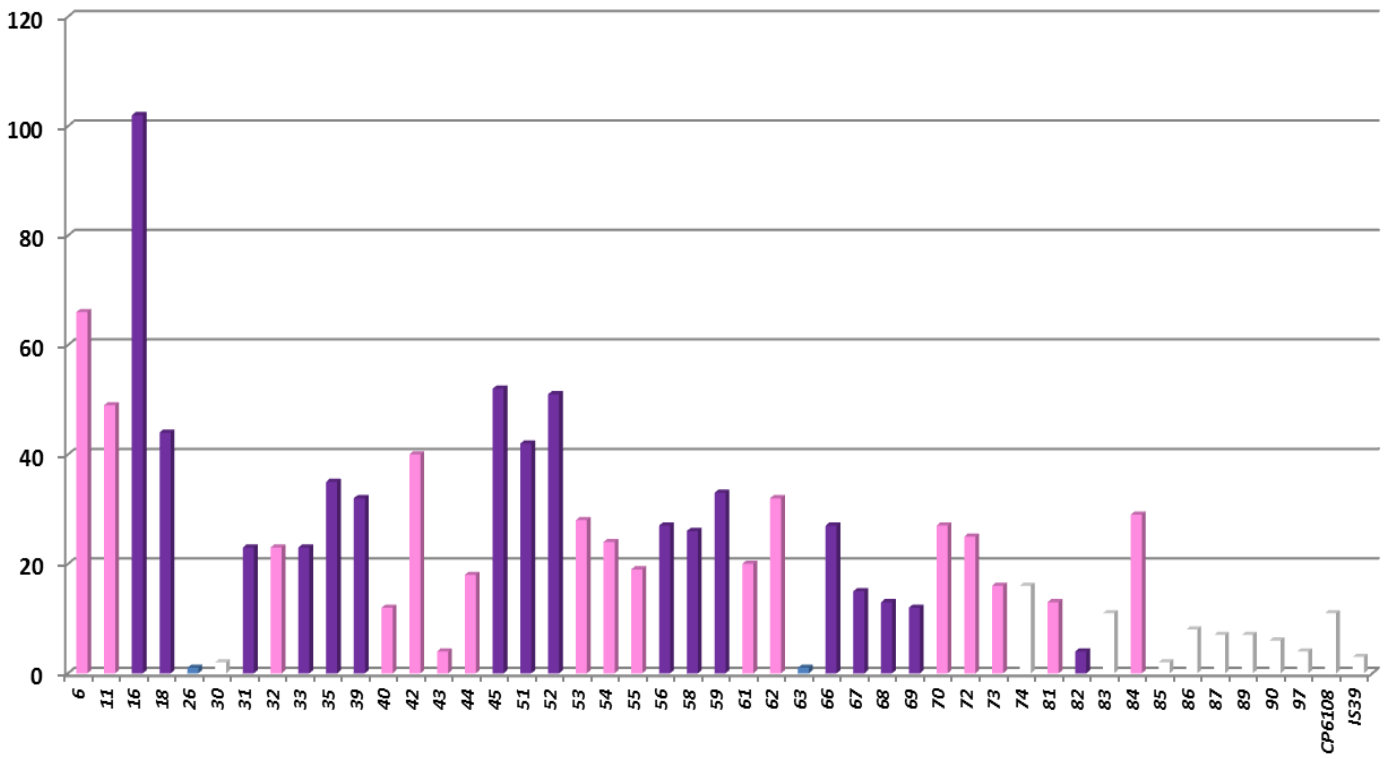
Thirty-one precancerous cases were confirmed by histopathology analyses. Of them, 29 anal precancers were diagnosed among the study males. Eight incident cancer cases were diagnosed and all of them among the study males, including six anal and two oral cancers.

Table 23: Precancerous Lesions and Incident Cancers by Body Site

Body Site	Precancerous Lesions	Cancer
ANUS	**AIN I – 20** **AIN II – 5** **AIN III – 4**	**Adenocarcinoma – 1** **Invasive Cancer – 3** **Rectal SCC *in situ* – 1** **Rectal SCC – 1**
OROPHARYX	**-**	**Throat Cancer – 1** **Tongue Cancer – 1**
CERVIX	**VaIN II (Vaginal HSIL) – 1** **VIN II (Vulvar HSIL) – 1**	**-**

In summary, in the screening year, the prevalence of HPV-positive cases in the study population was 82.0% (246/300); of which, the prevalence of cases with high-risk (HR) HPV genotypes was 45.9% (113/246). During the follow-up years, the number of the detected HPV types varied. At the end of the study, the total number of the detected HPV genotypes among 263 participants increased to 50 (Graph 5), with their following distribution: the total number of patients positive for HPV infection was 227 (86.3%) and of them, 143 (63.0%) were infected by HR HPV genotypes. Of these 227 participants, 200 (88.1%) had HPV infection at one body site (mostly anus) and 27 (11.9%) patients had HPV infection at two body sites simultaneously (anus/cervix or anus/oropharynx). During the study years, the prevalence of HPV-positive patients increased by 4.3% (from 82.0% to 86.3%) and the prevalence of cases with the HR HPV genotypes increased by 17.1% (from 45.9% to 63.0%).

Graph 5: Distribution of the 50 HPV Genotypes among the Study Population (N=263)



The dark columns are HR HPV genotypes
The light columns are LR HPVs
The white columns are Unknown Effect HPVs

X axis – HPV genotypes
Y axis – Number of patients (cases)

Our further analysis showed that from those 50 genotypes, 8 (16%) were significantly associated with the histologically-confirmed 31 (11.8%) cases of precancerous lesions in both males and females, and 8 (3.3%) cases of newly diagnosed cancers among the 245 male participants. These important Low Risk, High Risk and Unknown Effect (UE) HPV genotypes were **6, 11, 16, 18, 45, 52, 69, & 74**. From those 8 important HPV genotypes, 7 HPV genotypes were significantly associated with the precancerous lesions (HPV 39 was at borderline significance with p=0.0564) and 2 HR HPV genotypes (16 & 52) were in significant relationship with the cancer cases in univariate logistic regression analysis (Tables 24 & 25).

Table 24: Seven HPV Genotypes Significantly Associated with Precancerous Lesions (N=263)

HPV Genotype N (%)		Precancerous Cases N=31 Proportion of Precancer Cases within HPV(+) and HPV(-) Cases	Univariate Logistic Regression OR (95%CI), p
HPV6:	**Posit in 58 (22.1%)**	**16/58 (27.6%)**	**4.5 (2.0 – 10.3) , 0.0004**
	Negat in 205 (77.9%)	**15/205 (7.3%)**	**1**
HPV11:	**Posit in 43 (16.3%)**	**14/43 (32.6%)**	**6.1 (2.6 – 14.2), <0.0001**
	Negat in 220 (83.7%)	**17/220 (7.7%)**	**1**
HPV16:	**Posit in 102 (38.8%)**	**28/102 (27.5%)**	**6.3 (2.5 – 15.6), <0.0001**
	Negat in 161 (61.2%)	**3/161 (1.9%)**	**1**
HPV18:	**Posit in 44 (16.7%)**	**10/44 (22.7%)**	**3.4(1.4– 8.3), 0.0073**
	Negat in 219 (83.3%)	**21/219 (9.6%)**	**1**
HPV45:	**Posit in 52 (19.8%)**	**11/52 (21.2%)**	**2.7 (1.1 – 6.3), 0.0227**
	Negat in 211 (80.2%)	**20/211 (9.5%)**	**1**
HPV69:	**Posit in 12 (4.6%)**	**4/12 (33.3%)**	**6.1 (1.6 – 23.4), 0.0077**
	Negat in 251 (95.4%)	**27/251 (10.8%)**	**1**
HPV74:	**Posit in 18 (6.8%)**	**10/18 (55.6%)**	**6.9 (2.0 – 23.4), 0.0021**
	Negat in 245 (93.2%)	**21/245 (8.6%)**	**1**

Table 25: Two HR HPV Genotypes Significantly Associated with Cancer Cases among 245 Study Males

HPV Genotype N (%)		Cancer Cases N=8 Proportion of Cancer Cases within HPV(+) and HPV(-) Cases	Univariate Logistic Regression OR (95%CI), p
HPV16:	**Posit in 96 (39.2%)**	**7/96 (7.3%)**	**11.6 (1.4-96.2), 0.0227**
	Negat in 149 (60.8%)	**1/149 (0.7%)**	**1**
HPV52:	**Posit in 49 (20.0%)**	**6/49 (12.2%)**	**13.5 (2.6-69.4), 0.0018**
	Negat in 196 (80.0%)	**2/196 (1.0%)**	**1**

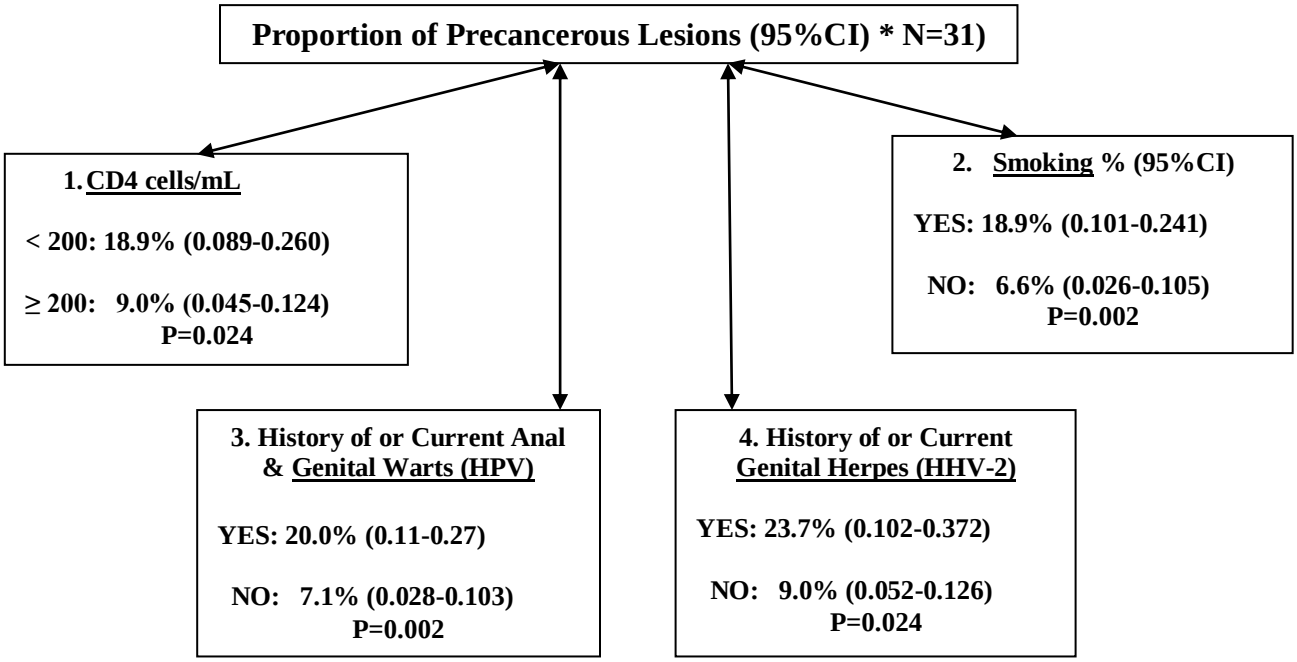
Our findings from Tables 24 & 25 showed that:

The proportions of precancerous lesions were significantly higher among participants who were positive to the following seven HPV genotypes: 6, 11, 16, 18, 45, 69 & 74 than among participants negative to the same HPV genotypes.

The proportions of cancers were significantly higher among patients who were positive to HPV 16 and/or HPV 52 as compared to those who were negative to these two HR HPVs.

As part of additional analyses, both the Chi Square test and t-test were conducted to find out how well the study outcomes and patient's characteristics are related. Analysis of cancer did not show any significance (the sample of 8 cases being too small). The t-test of the association between mean values of the continuous variables such as CD4 T cell count and HIV RNA viral load and the precancers did not show any significance as well. The Chi-Square tests (χ^2) between the categorical variables such as CD4 T cell count categories, smoking status, history of anogenital warts and HHV-2 and precancerous lesions showed significant association (Chart 2).

CHART 2: Association between Precancerous Lesions and Predictor Variables



***: Chi-square (χ^2) test**

The interpretation of these findings is:

The proportion of precancerous lesions among the study participants with CD4 cell count less than 200 cells/mL was more than 2 times higher than proportion of precursor lesions among those with CD4 cell count equal to or greater than 200 cells/mL (18.9% vs. 9.0%, p=0.024).

The proportion of precancerous lesions was almost 3 times higher among smokers than proportion of precancerous lesions among non-smokers (18.9% vs. 6.6%, p=0.002).

The proportion of precancers was 2.8 times higher among patients with a history of ano-genital warts and 2.6 times higher among patients with a history of genital herpes than proportions of precancerous lesions among those without history of these sexually transmitted infections (20.0% vs. 7.1%, p=0.002; and 23.7% vs. 9.0%, p=0.024 respectively).

3.1.4. Results from Logistic Regression Analyses

The study results showed zero cancer cases among the study females. Therefore, the author excluded females from the Logistic Regression analyses and focused only on 245 male subjects.

3.1.4. IA: The Results from Univariate Logistic Regression Analysis of Precancerous Lesions

Univariate analysis was carried out to evaluate association between precancers and each of the risk factors individually. Prevalence of precancers (yes vs. no) was used in the logistic regression models. The following predictor (independent) variables were included in the univariate logistic regression analysis of precancers (Table 26): (1) Age (continuous) (2) Gender (dichotomous) (3) Two education categories with "high school" being a cut-off point (4) Two categories of male partners ("0" partner vs. "≥1" partners) (5) Smoking (yes vs. no) (6) CD4 T cell count categories (<200 vs. ≥200 cells/mL) (7) HIV RNA viral load (continuous) (8) History of unprotected sex: anal, oral (yes vs. no) (9) History of STIs: anogenital warts, genital herpes (yes vs. no).

Table 26: Univariate Analysis of Association between Predictors and Precancerous Lesions among 245 Study Males

Predictor Variable	Mean (SD) values & Proportions of Precancers	OR (95%CI)	ULRM P
Age, mean (SD)	**47.6 (9.1)**	**1.0 (0.96-1.1)**	**0.8947**
Education Level, N (%)			
None/Element/High School Dipl. (74)	**12/74 (16.2%)**	**1.8 (0.8-3.9)**	**0.1666**
Undergrad/Grad/Postgrad (171)	**17/171 (9.9%)**	**1**	
Male Partners, N (%)			
≥1 (145)	**21/145 (14.5%)**	**1.9 (0.8-4.6)**	**0.1277**
0 (100)	**8/100 (8.0%)**	**1**	
Smoking, N (%)			
Yes (103)	**20/103 (19.4%)**	**3.6 (1.5-8.2)**	**0.0028**
No (142)	**9/142 (6.3%)**	**1**	
CD4 T cell count			
<200 cells/mL (65)	**12/65 (18.5%)**	**2.2 (1.0-4.8)**	**0.0580**
≥200 cells/mL (180)	**17/180 (9.4%)**	**1**	
Plasma HIV RNA, mean (SD)	**206 (701.7)**	**1.0 (1.0-1.1)**	**0.6543**
History of Unprotected Anal Sex			
Yes (46)	**7/46 (15.2%)**	**1.4 (0.6-3.6)**	**0.4330**
No (199)	**22/199 (11.1%)**	**1**	
History of Unprotected Oral Sex			
Yes (124)	**15/124 (12.1%)**	**1.1 (0.5-2.3)**	**0.8985**
No (121)	**14/121 (11.6%)**	**1**	
History of Anogenital Warts			
Yes (88)	**17/88 (19.3%)**	**2.9 (1.3-6.4)**	**0.0085**
No (157)	**11/157 (7.6%)**	**1**	
History of Genital Herpes			
Yes (34)	**7/34 (20.6%)**	**2.2 (0.9-5.7)**	**0.0954**
No (211)	**22/211 (10.4%)**	**1**	

The results from Table 26 showed that the study smokers are 3.6 times more likely to have a precancerous lesion than non-smokers (OR=3.6, p=0.0028). The study participants with the history of anogenital warts were 3 times more likely to have a precancerous lesion than those without history of warts (OR=2.9, p=0.0085). Other predictor variables did not show significant association with the precancerous lesions.

Seven HPV genotypes (6, 11, 16, 18, 45, 69 & 74) were significantly associated with the precancers in univariate regression model (Table 24).

3.1.4. IB: The results from Multivariate Logistic Regression Analysis of Precancerous Lesions

Multivariate Logistic Regression model was built to identify significant risk factors for precancerous lesions after adjusting for other factors. A purposeful selection method was used to build our final model. The ensuing analysis answered the following question: Which patient's characteristic and significant HPV genotype were associated with the precancerous lesions after adjusting for other factors? (Table 27)

Table 27: Significant Risk Factors Associated with the Development of Precancerous Lesions

Predictor Variable*		OR (95%CI)	P value
Smoking	**yes**	**4.9 (1.6-15.9)**	**0.0080**
	no	**1**	
CD4 cell count	**<200 cells/mL**	**7.5 (2.1-26.3)**	**0.0018**
	≥200 cells/mL	**1**	
HPV11	**positive**	**3.9 (1.3-12.1)**	**0.0182**
	negative	**1**	
HPV16	**positive**	**34.8 (6.8-177.0)**	**<0.0001**
	negative	**1**	
HPV69	**positive**	**14.6 (1.2-177.0)**	**0.0350**
	negative	**1**	
HPV74	**positive**	**18.7 (4.1-85.4)**	**0.0002**
	negative	**1**	

***: MLR model included age, smoking, CD4 cell count categories, HPVs 11, 16, 69 & 74**

The results from Table 27 showed that from risk factors included in the final multivariate regression model, smoking, CD4 T cell count and four HPV genotypes were significantly associated ($p<0.05$) with the precancerous lesions. Smokers were 5 times more likely to have a precancerous lesion than non-smokers (OR=4.9; p=0.0080) ; Among the study participants those with the CD4 T cell count less than 200 cells/mL were 7.5 times more likely to have a precancerous lesion than participants with CD4 T cell count ≥200 cells/mL (OR=7.5; p=0.0018); Among the study participants those positive to one or more of the four HPV genotypes (11, 16, 69, 74) were significantly more likely to have a precancerous lesion than participants who were negative to these HPV genotypes.

3.1.4. IC: Multivariate Logistic Regression Model of the Interaction between Four Significant HPV Genotypes

The next step in our analysis was to find out: Which combinations of or interactions between those 4 significant HPV genotypes (11, 16, 69, 74) were the most important in their association with the precancerous lesions? The multivariate model included main effects and statistically significant ($p<0.05$) interaction terms.

Table 28: Interaction of 2 HPV Genotypes in Significant Relationship with the Precancerous Lesions

HPV74*HPV16		OR (95%CI)	P value
HPV74 (+)	**HPV16 (+)**	**8.3 (2.2-30.9)**	**0.0016**
	HPV16 (-)	**1**	
HPV74 (-)	**HPV16 (+)**	**0.4 (0.03-5.46)**	**0.4813**
	HPV16 (-)	**1**	

***: Interaction between HPV genotypes**

The interpretation of the findings from the multivariate regression analysis is:

Among the study patients who were HPV 74 positive, those with HPV 16 positive were 8.3 times more likely to have a precancerous lesion than those HPV 16 negative (OR=8.3, p=0.0016). Among the study patients who were HPV74 negative, no such association was observed (OR=0.4, p=0.4813).

We also examined the effect of multiple HPV infections in a single specimen on the risk of the precancerous lesions. The number of HPV genotypes per specimen was treated in the model as a continuous variable (mean [SD] = 3.6 [3.1]).

Table 29: Effect of Multiple HPV Infections on the Risk of Precancerous Lesions (N=245)

Variable*	OR (95%CI)	P value
Multiple HPVs In a single specimen	**1.1(1.08 – 1.13)**	**<0.0001**

***: Variable was treated as continuous in the logistic regression model.**
MLR model included: age, smoking, CD4 T cell count and number of HPV+ types in a specimen.

The interpretation of the finding: The multiplicity of HPV infection in a single specimen is significantly associated with the precancerous lesions. Among the study participants who are infected with multiple HPV genotypes, the risk to have a precancerous lesion increases by 10% with every one more count of HPV genotype in a single specimen (OR=1.1; p<0.0001).

3.1.4. IIA: Univariate Logistic Regression Analysis of Cancers

Univariate analysis was carried out to evaluate the association between each of the risk factors and cancer cases. The incident cancer cases (yes vs. no) were used in the logistic regression models. The univariate analysis showed that (Table 30) none of the demographic and clinical characteristics was significantly associated with the newly developed cancers (8 cases comprise a fairly small sample). However, the study participants with the history of Hepatitis C were almost 6 times more likely to develop cancer than those without history of this blood born viral infection (OR=5.86, p=0.0206).

Table 30: Analysis of Association between Predictor Variables and Cancer (ULRM)

Predictor Variable	Mean (SD) values & Proportions of Cancer	OR (95%CI)	P value*
Age, mean (SD)	**51.3 (9.1)**	**1.1 (1.0-1.13)**	**0.2424**
Education Level, N (%)			
None/Element/High School Dipl.(74)	**4/74 (5.4%)**	**2.4 (0.6-9.8)**	**0.2280**
Undergrad/Grad/Postgrad (171)	**4/171(2.3%)**	**1**	
Male Partners, N (%)			
0 (100)	**4/100 (4.0%)**	**1.5 (0.4-6.0)**	**0.5931**
≥1 (145)	**4/145 (2.8%)**	**1**	
Smoking, N (%)			
Yes (103)	**4/103 (3.9%)**	**1.4 (0.3-5.7)**	**0.6442**
No (142)	**4/142 (2.8%)**	**1**	
CD4 T cell count			
<200 cells/mL (65)	**1/65 (1.5%)**	**0.4 (0.05-3.2)**	**0.3779**
≥200 cells/mL (180)	**7/180 (3.9%)**	**1**	
Plasma HIV RNA, mean (SD)	**205.6 (701.7)**	**1.0 (0.99-1.0)**	**0.4463**
History of Unprotected Anal Sex			
Yes (46)	**0 (0%)**	**NA**	**NA**
No (199)	**8 (4.0%)**		
History of Unprotected Oral Sex			
Yes (124)	**4/124 (3.2%)**	**1.0 (0.2-4.0)**	**0.9719**
No (121)	**4/121 (3.3%)**	**1**	
History of Hepatitis C			
Yes (25)	**3 (12.0%)**	**5.86 (1.31-26.20)**	**0.0206**
No (220)	**5 (2.3%)**	**1**	
History of Anogenital Warts			
Yes (88)	**5 (5.7%)**	**3.09 (0.72-13.26)**	**0.1286**
No (157)	**3 (1.9%)**	**1**	
History of Genital Herpes			
Yes (34)	**1 (2.9%)**	**0.88 (0.11-7.41)**	**0.9088**
No (211)	**7 (3.3%)**	**1**	

***: p-value from univariate logistic regression model**

3.1.4. IIB: Multivariate Logistic Regression Analysis of Cancers

Multivariate Logistic Regression model was developed in order to identify significant predictors of cancer after adjusting for other factors. The incidence of cancer (yes/no) was used in this analysis as a binary dependent variable.

Table 31: Predictor Variables Associated with Cancer in Multivariate Logistic Regression Model adjusted to other Factors

Predictor Variable*		OR (95%CI)	p value
Hepatitis C			
	yes	**5.8 (1.07-31.20)**	**0.0410**
	no	**1**	
HPV16	**positive**	**6.3 (0.68-59.5)**	**0.1059**
	negative	**1**	
HPV52	**positive**	**7.0 (1.20-40.81)**	**0.0310**
	negative	**1**	

***: MLR model included age, history of HCV, HPVs 16 & 52**

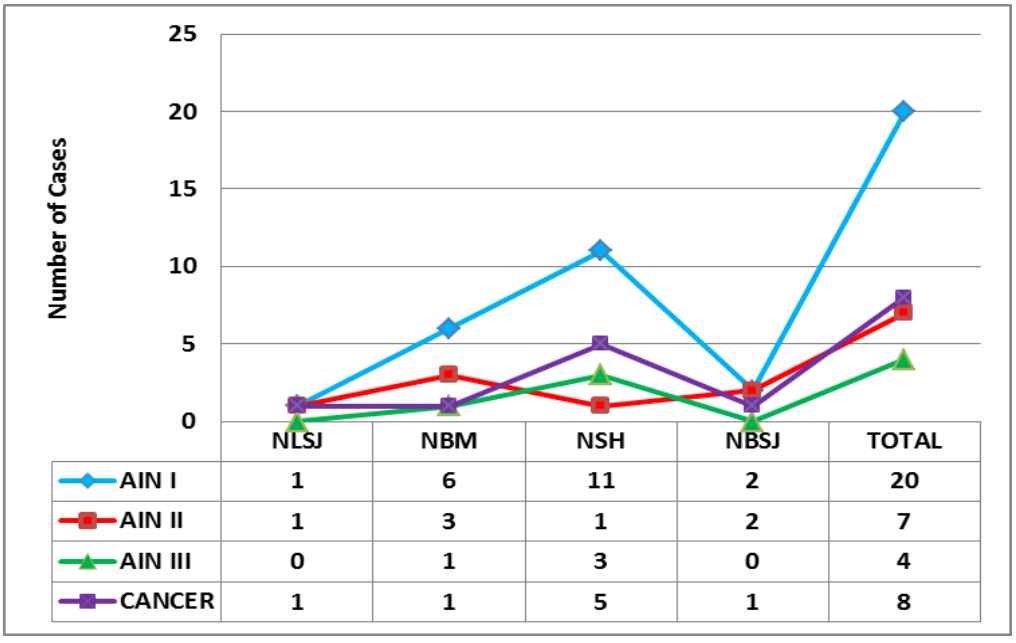
The results from Table 31 showed that the study participants who had a history of Hepatitis C were almost 6 times more likely to have cancer (OR=5.8, p=0.0410) than those without the history of Hepatitis C. Among the study participants those positive to HPV 52 were 7 times more likely to develop cancer than those negative to HPV 52; among the study participants those positive to HPV 16 were 6 times more likely to develop cancer than those negative to HPV 16. However, the result did not reach statistically significant level at $\alpha<0.05$.

3.1.4. IIC: Multivariate Logistic Regression Model of the Interaction between Two Significant HPV Genotypes 16 & 52

Next, we investigated if there was an interaction between those two HPV genotypes. Our results showed that there was no interaction terms between HPV 16 and HPV 52 (p=0.386). Each genotype acted individually and was independently associated with incident cancers.

In our study, the prevalence of precancerous lesions and incidence of malignancy varied among Atlantic Provinces with the highest numbers in Halifax, Nova Scotia with 15 (48.4%) cases of precursor lesions and 5 (62.5%) cancer cases. Two study sites in New Brunswick had total 14 precancerous cases, of them 10 (32.3%) in Moncton and 4 (12.9%) in Saint John. There was one new cancer case per the abovementioned sites in NB (12.5% each). The lowest numbers were observed in St. John's, NL: two (6.5%) precancerous and one (12.5%) cancer cases (Graph 6 and Table 32):

Graph 6: Precancerous Lesions and Cancers stratified by the Study Site



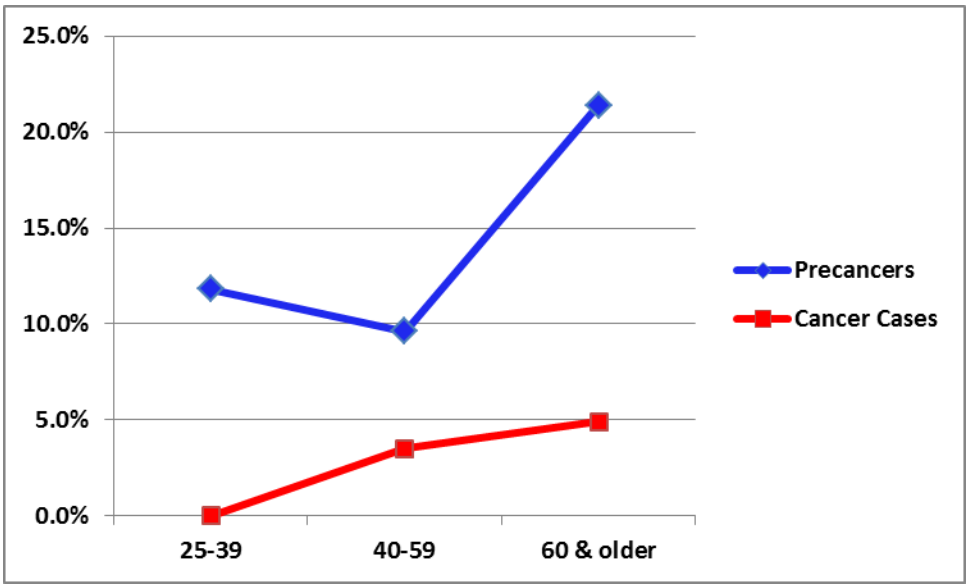
	NLSJ	NBM	NSH	NBSJ	TOTAL
AIN I	1	6	11	2	20
AIN II	1	3	1	2	7
AIN III	0	1	3	0	4
CANCER	1	1	5	1	8

Table 32: Distribution of the Precancerous and Cancer Cases by the Study Site

Study Site, N-%	AINI	AINII	AINIII	CANCER
NLSJ (42 - 16%)	1	1	0	1
NBM (84 - 32%)	6	3	1	1
NSH (121 - 46%)	11	1	3	5
NBSJ (15 - 6%)	2	2	0	1
Total	20	7	4	8

The proportion of precancerous lesions and cancers was also varying within the age categories with their highest numbers (%) among the oldest participants (Graph 7):

Graph 7: Precursor Lesions and Cancers stratified by Age Categories



X axis – Age Categories
Y axis – Precancerous Lesions and Cancers (%)

The prevalence of precancerous lesions in the study cohort was 31/263 (11.8%), of them 29 (93.5%) anal cases were diagnosed in men. The distribution of these lesions was different across the cohort's age groups (Graph 7, blue line): 25 – 39 years of age – 4/34 cases (11.8%); 40 – 59 years of age – 18/187 cases (9.6%); 60 years of age & older – 9/42 cases (21.4%).

The incidence of cancers was 8/245 (3.3%). The distribution of cancer cases was different across the male age categories (Graph 7, red line): 25 – 39 years of age – 0/31 cases (0.0%); 40 – 59 years of age – 6/173 cases (3.5%); 60 years of age & older – 2/41 cases (4.9%).

3.1.5. Calculation of Incidence Rate (Person-time Rate) and ASIR of Cancer

We investigated 300 HIV-infected adults and followed them annually for 3 consecutive years to determine the incidence rate of HPV-associated anal and oropharyngeal cancers.

Incidence Rate (Unadjusted) or Person-time Rate incorporates missing data (Principals of Epidemiology in Public Health Practice, CDC, 2012). Incidence Rate of cancer was calculated using the ratio of the number of cancer cases to the total time the sample population was at risk of HPV-associated cancer:

Number of new cancer cases / Time each person was observed, totaled for all persons

Person-time is an estimate of the actual time-at risk in years that all persons contributed to this study. We assumed that the probability of cancer during the study period was constant (one of person-time assumptions). We also assumed that participants lost to follow-up were in the study for half the year, and thus contribute half of the calendar year to the denominator. The same assumption is made for participants diagnosed with cancer. We assumed that they were disease-free for half the year, and thus contribute to the denominator 1/2 of the calendar year when the event happened. The Person Years (PYs) were calculated among 275 males at the study baseline.

From 39 participants that were lost to follow up, 7 were females and 2 died but reached the study end point and became a case. A total number of males that were lost during the study was 30.

Age Standardized Incidence Ratio (ASIR) is used to determine if the occurrence of cancer in a relatively small population is high or low. To calculate the age-standardized incidence rate, we must first calculate the age-specific incidence rates for each age group by dividing the number of cancer cases by the respective population, and then multiplying the resulting number by 100,000 (Principals of Epidemiology in Public Health Practice, CDC, 2012). We used the Canadian male population as a reference group to account for age distribution (Stat. Canada 2015).

Anal Cancer

		Loss to Follow-up & Cancers			Person Years (PYs)			
Age group	**N at baseline**	**1st year**	**2nd year**	**3rd year**	**1st year**	**2nd year**	**3rd year**	**Total**
25-29	10		**1 L**		10	9.5	9	28.5
30-34	13		**1 L**	**1 L**	13	12.5	11.5	37
35-39	23			**1 L**	23	23	22.5	68.5
40-44	59	**3 L 2 C**	**3 L**	**2 L**	56.5	52.5	50	159
45-49	65	**4 L**	**1 C**	**3 L**	63	60.5	58.5	182
50-54	43	**4 L**	**1 C**	**1 L**	41	38.5	37.5	117
55-59	34		**2 L**	**2 L 1 C**	34	33	30.5	97.5
60-64	17	**1 L**		**1 C**	16.5	16	15.5	48
65-69	8		**1 L**		8	7.5	7	22.5
70-79	3	**12L + 2C**	**8L + 2C**	**10L + 2C**	3	3	3	9
Total	**275**				268	256	245	**769**

L: Number of Participants Lost to Follow-up
C: Number of Incident Anal Cancers

95% CI= Adj. Rate ± 1.96*SE =0.005 ± 1.96*0.0024 = (0.0003-0.0097)

Age group	N of Cases (di)	PYs (pi)	Rate (di/pi)	Standard Pop (wi)	Expected cases (wi*di/pi)	Proportion (wi/sum(wi))	Variance
25 to 29	0	28.5	0	2517100	0	0.095511843	0
30 to 34	0	37	0	2530200	0	0.096008925	0
35 to 39	0	68.5	0	2456100	0	0.093197186	0
40 to 44	2	159	0.01257862	2345400	29501.88679	0.088996653	6.18708E-07
45 to 49	1	182	0.00549451	2415200	13270.32967	0.091645228	2.52165E-07
50 to 54	1	117	0.00854701	2711300	23173.50427	0.102880799	7.666E-07
55 to 59	1	97.5	0.01025641	2653200	27212.30769	0.100676183	1.05528E-06
60 to 64	1	48	0.02083333	2300100	47918.75	0.087277736	3.23729E-06
65 to 69	0	22.5	0	1975700	0	0.074968316	0
70 to 79	0	9	0	4449500	0	0.168837132	0
	6	**769**	0.00780234	26353800	141076.7784	1	5.93004E-06
			0.00780234	Adj. Rate=	**0.005353185**	SE=	0.002435167

pi : the number of PYs in age group i in the study population
Ri: the cancer rate in age group i in the study population
wi: the number of persons in age group i in the standard population

Unadjusted Incidence Rate (per 100,000): 780.234

Adjusted Incidence Rate (per 100,000): 535.319 95%CI (30-970)

Oral Cancer

Age group	N at baseline	Loss to Follow-up & Cancer			Person Years (PYs)			
		1st year	2nd year	3rd year	1st year	2nd year	3rd year	Total
25-29	10		**1 L**		10	9.5	9	28.5
30-34	13		**1 L**	**1 L**	13	12.5	11.5	37
35-39	23			**1 L**	23	23	22.5	68.5
40-44	59	**3 L**	**3 L**	**2 L**	57.5	54.5	52	164
45-49	65	**4 L**		**3 L**	63	61	59.5	183.5
50-54	43	**4 L 1 C**		**1 L**	40.5	38	37.5	116
55-59	34	**1 C**	**2 L**	**2 L**	33.5	32	30	95.5
60-64	17	**1 L**			16.5	16	16	48.5
65-69	8		**1 L**		8	7.5	7	22.5
70-79	3	**12L + 2C**	**8L + 0C**	**10L + 0C**	3	3	3	9
Total	**275**				268	257	248	**773**

L: Number of Participants Lost to Follow-up
C: Number of Incident Oropharyngeal Cancers

95% CI= Adj. Rate ± 1.96*SE =0.005 ± 1.96*0.0024 = (0.0003-0.0097)

Age group	N of Cases (di)	PYs (pi)	Rate Ri=(di/pi)	Standard Pop (wi)	Expect. cases (wi*di/pi)	Proportion (wi/sum(wi))	Variance
25 to 29	0	28.5	0	2517100	0	0.095511843	0
30 to 34	0	37	0	2530200	0	0.096008925	0
35 to 39	0	68.5	0	2456100	0	0.093197186	0
40 to 44	0	164	0	2345400	0	0.088996653	0
45 to 49	0	183.5	0	2415200	0	0.091645228	0
50 to 54	1	116	0.00862069	2711300	23373.27586	0.102880799	7.79817E-07
55 to 59	1	95.5	0.0104712	2653200	27782.19895	0.100676183	1.0997E-06
60 to 64	0	48.5	0	2300100	0	0.087277736	0
65 to 69	0	22.5	0	1975700	0	0.074968316	0
70 to 79	0	9	0	4449500	0	0.168837132	0
	2	**773**	0.00776197	26353800	140344.509	1	5.88095E-06
			0.00776197	Adj. Rate=	**0.005325399**	SE=	0.002425067

pi : the number of PYs in age group i in the study population
Ri: the cancer rate in age group i in the study population
wi: the number of persons in age group i in the standard population

Unadjusted Incidence Rate (per 100,000): 776.197

Adjusted Incidence Rate (per 100,000): 532.540 95%CI (30-970)

The ASIR of Anal Cancer among the study HIV-infected male population is 535.3/100,000 as compared to 1.5/100,000 in the Canadian general male population (PHAC, 1997-2006).
The ASIR of Oropharyngeal Cancer among the study HIV-infected male population is 532.5/100,000 as compared to 5.7/100,000 in the Canadian general male population (PHAC, 1997-2006).

Interpretation of these findings should be exercised with caution due to the short follow-up time for the exploration of the study outcomes.

CHAPTER 4

DISCUSSION

4.1 Summary of Key Findings

Our key findings from this longitudinal cohort study included eight (3.3%) cases of cancer that were diagnosed among 245 male participants. Of them, six patients had anorectal cancer and 2 males were diagnosed with oropharyngeal cancers. Thirty-one (11.8%) cases of precancerous lesions were confirmed among 263 study participants. Of them, two cases of vaginal/vulvar dysplasia belonged to the study females. Fifty HPV genotypes were observed among 263 HIV-positive adults living in Atlantic Canada provinces. We have found that among those 50 HPV genotypes, seven were in significant relationship with the precancerous lesions (6, 11, 16, 18, 45, 69 & 74) and two HR HPV genotypes HPV16 and HPV52 were significantly related to the incident cancer cases. We have also found a significant association between precancerous lesions and the following predictors: CD4 T cell count < 200 cells/mL, smoking, history of anogenital warts and genital herpes (HHV-2). An interaction between HPV 16 and HPV 74 was never reported prior to our study. Among the study patients who were HPV 74 positive, those with HPV 16 positive were 8.3 times more likely to have a precancerous lesion than those who were HPV 16 negative (OR=8.3; p=0.0016). Furthermore, our findings showed that the risk of developing of an anal precancerous lesion increases by 10% with every one more count of HPV genotype in a single anal specimen (OR=1.1, p<0.0001).

Our most significant and clinically important findings supported our main hypothesis about the higher incidence rates of HPV-associated cancers in HIV-infected people compared to the Canadian general population.

The calculated Unadjusted Incidence Rates of anal cancer is 780/100,000 and oropharyngeal cancer is 776/100,000 in the study males.

The ASIR (95%CI) of anal cancer in our HIV-positive males, using the Canadian general male population as a reference population (Census and Statistics Canada, 2015), was 535/100,000 (30-970) as compared to 1.5/100,000 in the Canadian general male population (PHAC, 1997-2006). Our 95% confidence interval is overlapping with the 95%CIs from two USA studies (Silverberg MJ et al in 2012; and Chaturvedi AK et al in 2009). The ASIR (95%CI) of anal SCC in HIV-infected MSM from the first study was 80.3 (42.7-151.1) and in HIV-positive men was 26.7 (11.5-61.7). In the second study, they reported the ASIR (95%CI) of anal cancer being 34.6 (30.8-38.8) among their participants with AIDS (Table 1).

The ASIR (95%CI) of oral cancer among our males was 533/100,000 (30-970) as compared to 5.7/100,000 in the Canadian general male population (PHAC, 1997-2006).

Even though our study was not designed to evaluate the burden of HPV-associated malignancy or to evaluate the feasibility of the anal cancer screening program for HIV-infected populations, we believe that our results contribute to quantifying the burden of anal cancer for HIV-positive males and their need for anal cancer screening program, early treatment of precancerous lesions and appropriate care. Immunosuppression is reported to be an important factor in the development of anal cancer, even though the association between anal cancer and HIV infection is difficult to confirm due to confounders (Van der Zee RP et al, 2013). Since HIV-positive MSM have 80-fold higher risk for anal cancer, an increase in the proportion of HIV-positive MSM in the population will contribute to a higher incidence of anal cancer in the general population (Gras L et al, 2007 and Van Sighem A et al, 2012). Diagnostic and therapeutic guidelines should be implemented for at-risk populations for anal dysplasia/anal cancer, such as HIV-positive men who have sex with men.

The implementation of the anal cancer screening program in Canada is timely important. Anal cancer is increasing in its incidence and is affecting more people across the world every year (http://analcancerfoundation.org/learn/anal-cancer/). The prevalence of the HIV-positive population in Canada is also rising and reached 75,750 in 2015. In addition, there is about 21% of Canadians with HIV who are unaware of their infection (PHAC, Fact Sheets, 2015).

In this study, 50 HPV genotypes were detected. The number of the HPV infected participants increased by 4.3% from 82.0% at the baseline to 86.3% at the study end. The most frequently observed HPV genotype was HPV16 (102 (38.8%) cases), either as a single type in the specimen or in various combinations with other HPV genotypes. More than half of the study population (63.0%) had three and more HPV genotypes in their anal specimens. The number of patients infected with high-risk HPV genotypes increased by 17.1% from 45.9% at the baseline to 63.0% at the study completion. We found that the most important HPV genotypes associated with the precancerous lesions and malignancy among Atlantic Canada PHAs were the following eight HPVs: 6, 11, 16, 18, 45, 52, 69 & 74. The findings from other studies support our findings. Evidence from a large international retrospective cross-sectional study from 38 countries, investigating the incidence of cervical cancer and related HPV genotypes in the general population for a period from 1949 to 2009, showed that the most frequently observed HPV genotypes were 16, 18, 31, 33, 35, 45, 52, & 58. Evidence from another comprehensive study in Spain demonstrated almost the same findings: eight most common HPV types in the development of cervical cancer were: 16, 18, 31, 33, 34, 45, 52 & 58 (de Sanjose et al, 2010).

The most recent study conducted by Mendez-Martinez and colleagues in 2014 (Mexico) was investigating the most prevalent HPV genotypes in the anal canal of HIV-positive MSM. They reported that the most frequently observed HPV genotype was 16 in various combination with 21 other HPV types: 6, 11, 18, 26, 31, 33, 35, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74 & 82. They listed the combination of HPVs 16 and 74 which interaction we found to be important in their association with the precancerous lesions. Two HR HPV genotypes 16 and 52 were considered in our study to be individually important in their association with the development of anal cancers. Two HPV genotypes 69 and 74 were never reported as significant ones prior to our study. We cannot explain the uniqueness of our findings because our study was the first one among these types of investigation in Atlantic Canada HIV-positive adults.

As we expected, the highest rates of both the precancerous lesions and cancers were observed in the older age category (21.4% and 4.9%, respectively). Prior to the study, we hypothesized that an increasing proportion of HIV-positive men in the older age group (60 years and older) would have an anal dysplasia or develop a cancer because of the contribution of the following factors: (a) an absence of the effective anal cancer screening program in Atlantic Canada provinces (b) the increasing life span of HIV-positive people from HAART; their prolonged survival may be associated with increased risk of certain HPV-associated morbidity and cancer (c) the increasing number of people belonging to these risk groups. The age distribution of the pre- and malignant lesions in our study differs from other similar studies. The "EXPLORE" study was conducted to investigate the age-associated prevalence of HPV infection and anal cancer precursors in 1,409 HIV-negative MSM (Chin-Hong PV et al, 2004).

The overall prevalence of HPV infection was 57% and was similar across all age groups. Prevalence of both precancerous lesions LSILs and HSILs were also similar across age groups. The overall prevalence of any dysplasia was 32%, which was similar across age groups. The difference between our findings and findings from the "EXPLORE" study can be explained by the fact that we investigated HIV-positive males vs. HIV-negative MSM in Chin-Hong's project.

Our findings showed a significant effect of multiple HPV types (HPV polymorphism) on the risk of cancer precursors (OR=1.1, $p<0.0001$). From this, it is plausible to confirm findings from the previous studies that infection with multiple HPV genotypes may be a marker of persistent disease and of the progression of LSILs to HSILs. Both the presence of HPV infection and the number of HPV genotypes in the sample were important risk factors for precancerous lesions (Palefsky JM, et al, 1998; Kreuter A. & Wieland U., 2009; Palefsky JM et al, 2000).

The underlying behavioral risk factors that were included in this analysis were: the number of sexual partners (both males and females), smoking status, self-reported history of STIs, and history of unprotected sex. We found a significant association between the precancerous lesions and patients' smoking status ($p=0.0070$), history of anogenital warts and genital herpes ($p=0.0020$ & $p=0.0071$ respectively). Previous studies have also demonstrated that behavioral determinants were strongly associated with the risk of anal squamous intraepithelial lesions (ASILs) (Fairley CK et al, 1994; Burk RD, 1996; and Elam G et al, 2008).
In 2004, Chin-Hong PV et al reported that patients who are knowledgeable about HPV and HIV can and do engage in high-risk sexual behaviors.

We found that the CD4 T cell count less than 200 cells/mL was associated with an increased risk of progression of HPV infection into precancerous lesions (p=0.0341). We did not find a significant association between high levels of plasma HIV RNA and the AINII/AINIII. Our findings partially confirmed the results from two previous Canadian studies. Mougin C. in 2001 and Leece P. in 2010 investigated the correlations between HIV laboratory markers (CD4<200 cells/mL and high levels of plasma HIV RNA) and incidence of anal dysplasia, and found a strong relationship between these parameters.

Most men (82.0%) in our study have never been screened for anal cytology abnormalities prior to the study. We compared our baseline data with the Canadian Human Immunodeficiency and Papillomavirus Research (HIPVIRG, 2011) baseline data. The sample size, median male age, percentage of smokers in the cohorts, percentage of patients who were taking antiretroviral therapy, as well as their baseline CD4 T cell counts were comparable between the cohorts. The HIPVIRG investigators aimed to establish a comprehensive understanding of the risk factors (age, smoking, initiation of anti-HIV treatment, CD4 cell count, and viral load level) for progression of AIN1 to AIN2 and AIN3 in HIV+ MSM. On entry to their study, 19% of patients had NILM (vs. 157 (57.1%) in our study); 50% had LSIL (vs. 36 (12.7%) in our study); AIN2 was confirmed in 17% (vs. 5 (2.0%) in our males), and 13% of their males had AIN3 (vs. 4 (1.7%) among our males). The incidence of AIN2/AIN3 in their HIV-positive male cohort was 23% after two years of observation and 37% after three years of observation. In our study, the incidence of AIN2/AIN3 among the study males was ten times less at 3.7% (9/245) at the end of the three years of follow-ups. The discrepancies in the results can be explained by (1) Significantly higher number of NILM reports among our male population at the enrollment time

(2) The homogenous MSM population in HIPVIRG versus our male sample which included proportions of MSM, MSW and MSMW.

In our study, we detected six ASC-US and one LSIL oropharyngeal lesions. These patients were referred to ENT specialists for further investigation and observation. We did not receive histopathology reports regarding these patients' oral lesions. Therefore, we assumed that by specialist's opinion their lesions did not require a biopsy. Two male patients diagnosed with tongue and throat cancers had NILM in their cytology reports and were never referred to ENT specialists at that time. They were referred to specialists later on because of their clinical signs and symptoms. Both oropharyngeal cancers were associated with HPV 16. The other HPV genotypes detected in these patients' oropharyngeal specimens were: 32, 35, 45, 58, 70 & 72. Men who have sex with men have a higher risk of developing oral HPV infection. A 2009 study conducted by D'Souza et al found that oral HPV acquisition is more positively associated with the number of recent oral sex and open mouth kissing partners than with the number of vaginal sex partners. Additionally, the prevalence of oral condylomas (large warts) has increased dramatically since the introduction of highly active anti-retroviral therapy (HAART) (Rabkin CS, 1998; Bower M et al, 2004; and Palefsky JM et al, 2001), which may be due to immune reconstitution (BCCA Vancouver Centre). HPV infection not only causes oral condylomas but is also strongly associated with oropharyngeal cancers and other oral diseases (Canadas MP et al, 2004; Gillison ML et al, 2008). The incidence of HPV-associated carcinomas of the oropharynx substantially increased from 1973 to 2004 ($p<0.001$), most likely because of a shift in sexual behavior, particularly oral sex in young males (Kreuter A. & Wieland U., 2009).

4.2 Policy Implications

The CFA (Centre for AIDS Information & Advocacy) advocates both locally and nationally for better treatments and better access to care for persons living with HIV/AIDS. No direct evidence exists to support the effectiveness of an anal Pap test screening program to reduce anal cancer mortality and morbidity (Goldie SJ et al, 1999). There are however, strong parallels between cervical pap testing and cervical cancer (D'Souza G et al, 2009). Sexually transmitted HPV viral infection is currently an acknowledged common causative agent for both anal and cervical cancers. Recent anal cancer rates in high-risk populations (HIV+ MSM) exceed those of cervical cancer before the implementation of the cervical cancer screening program (77.8-134/100,000 vs. 40/100,000, respectively) (Machalek DA, et al, 2012). Screening tests for these populations may be effective in reducing incidence, morbidity and mortality rates of anal cancer, as has been documented with cervical cancer. The implementation of cervical cancer screening resulted in a drop in cervical cancer rates from ~40/100,000 to ~8/100,000. Based on the success of the Pap test for cervical cancer screening, use of a similar Pap test for detection and early eradication of anal cancer precursors could potentially prevent their progression to anal cancer. Anal cancer screening may be cost-effective in HIV-infected and HIV-uninfected MSM (Goldie SJ et al, 1999 & 2000; Katz KA et al, 2009; RITA Report, 2013).

The author's opinion is supported by the findings from this study and from previously-conducted studies. In their qualitative study in 2010, Reed AC et al. found that 83% of gay and bisexual men were willing to accept free screening for anal cancer, leading to the conclusion that the screening's cost is a major barrier. Ours was a unique prospective cohort study investigating the prevalence of HPV genotypes in HIV-positive adults in Atlantic Canada provinces and their association with the precancerous lesions and newly diagnosed malignancy.

Our findings might be found important in the context of clinical management and prevention of HPV-associated dysplasia and cancers in Canadians living with HIV/AIDS.

4.3 The Study Limitations

We acknowledge the study's limitations and provide their detailed description.

The 26-item Patient Questionnaire (PQ) was administered to all participants at the enrollment time only. We might have drawn more conclusions about the association between patient's behavior and incidence of primary outcome if the PQ was updated by patients at each year during the follow-up period

There was a probability of systematic bias such as information and particularly recall bias regarding the information provided in the self-reported 26-item PQ, such as patients' history of STIs, number of sexual partners during the year, previously performed anal Pap test, and others. There was also a probability of the sample bias taking into the consideration that all our participants were volunteers.

The information in the 12-item Baseline and 9-item Clinic Follow-up Questionnaires included snap-shot annual values of CD4 T cell count and HIV RNA load. We never had an opportunity to analyze these measurements' means, maximum and minimum values during the year of observation. The snap-shot nature of the measurements has definitely limited the outcome analysis.

Our male participants were not asked to self-identify as MSM, MSMW or MSW in the 26-item PQ, even though the data in majority of questionnaires indicated them as MSM. This data would perhaps allow us to draw stronger conclusions about the prevalence of HPV genotypes and their association with patients' sexual behavior by comparing HIV-positive homosexual men with HIV-positive heterosexual men.

Another possible limitation of the study was the fact that we had one cytopathologist. There was a fair chance of bias in the results reading and their reports. However, any potential under- or over-diagnosis of squamous intraepithelial lesions (SILs) would probably affect only the incidence estimates, and not the estimates of associations with the potential risk factors. Ideally, this study needed to follow all cases out to histology with biopsy to confirm the detected ones and discover potentially undetected cytologic abnormalities.

The PCR assay (Luminex) used in the study for HPV DNA detection had limited sensitivity level. We might assume that a certain proportion of dysplasia was not detected to begin with. It should be considered that detection of HPV DNA usually indicates current infection, while not totally excluding surface contamination. Similarly, failure to detect HPV DNA does not exclude HPV infection as low-level infections or mere sampling errors.

4.4 Future Research

Although this study answered some important questions, other questions related to the subject remain unanswered. One of the questions that need to be answered is the prevalence of HR HPV genotypes in HIV-positive MSM living in Atlantic Canada provinces and their association with anal/oropharyngeal dysplasia and cancer. This question became more focused and feasible because of our findings. We also suggest further investigation clarifying the age influence on the incidence and prevalence of HPV-related precursor lesions observed in this study. Another promising avenue of research would be to continue to investigate the feasibility of anal cancer screening program for HIV-infected adults living in Atlantic region of Canada. More research is needed to better understand the complex relationship between independent predictors and incident cancers/precursor lesions, increased health risk and its association with health service utilization.

CHAPTER 5

CONCLUSIONS

The main outcome of this study was the incidence of HPV related cancers in HIV-positive adults living in Atlantic region of Canada. HIV-infected individuals are living longer and therefore may have the opportunity to acquire more slowly developing HPV-associated malignancies. Based on the data analysis and study findings, the following conclusions have been reached.

The factors predictive of progression of HPV infection to high-grade anal intraepithelial neoplasia (AIN), the immediate precursor of anal cancer in HIV-positive males are (1) HIV seropositivity per se (2) Advanced age (3) Persistence of anal HPV infection with one or more of the seven genotypes 6, 11, 16, 18, 45, 69 & 74 (4) Presence of multiple (≥3) HPV genotypes in a single specimen (5) Blood CD4 T cell count less than 200 cells/mL (6) Smoking and (7) History of anogenital warts and genital herpes. The highest rates of both the precancerous lesions and cancers are found to be among the study PHAs of 60 years of age and older (21.4% and 4.9%, respectively). Two high-risk HPV genotypes 16 & 52 are individually associated with the development of cancer. The ASIRs of anal cancer is 535/100,000 and of oral cancer is 533/100,000. Based on the findings from this study, we developed recommendations that need to be addressed to our knowledge users, policy makers and stakeholders such as Atlantic Canada Health Care Authorities, medical communities, and people living with HIV/AIDS.

5.1 Individual-Based Approach and Intervention

A screening approach is not yet established as the standard of care in HIV clinics (Palefsky JM, 2013 [RITA]). Our recommendations for HIV-infected individuals who practice receptive anal intercourse include use of protection and annual anal Pap and HPV genotyping tests. The Human Papillomavirus test can be the dominant screening method for people living with HIV/AIDS until the implementation of the anal cancer screening program. The combination of DRE, anal HPV and Pap test screening would be beneficial for these populations (Palefsky JM et al, 2011; Franco E., 2016).

We also believe that there is a need for medical communities in Canada, and particularly in Atlantic Canada, to start performing optimal early screening and treatment protocols for these populations, something that has not yet been coordinated. HPV is a common infection and certain interventions might affect the incidence of HPV-associated cancers. Many clinicians are unfamiliar with the procedure and the purpose of anal Pap testing. Appropriate triage and referral for care of anal cytologic abnormalities should ideally be clearly-defined before implementation of anal Pap test screening (Ostoski RA & Kell CS, 2011). Clinicians should be aware of the risk factors for HPV, which could prove useful in identifying patients at high risk for HPV-associated cancers, and modifying patient care to minimize this risk. The investigators believe that patients with a low nadir CD4 cell count might especially benefit from screening for precancerous lesions. In addition, "typing could also be useful as an adjunct to cytological examination in primary screening" (De Pokomandy A. et al, 2011; RITA Report, 2013). Just as it has been previously done with cervical cancer, we believe that widespread practice of effective protocols for early detection and early intervention of HPV-related anal dysplasia and cancer might help prevent many HIV-positive individuals from ever developing anal cancer.

5.2 Challenges and Opportunities to the Individual-Based Approach

One challenge to the individual-based approach is that it is labor intensive and requires coordinated input from many healthcare professionals. A second challenge to this approach is the requirement of increased collaboration between disciplines and the need to establish a team of multidisciplinary professionals in order to deliver the services recommended. The recommended screening and management options for HIV-positive adults may be associated with increased costs in the short-term due to the resources required (e.g., training, equipment, financial support). The hope however is that in the long-term, this approach will be more cost-effective and the health of HIV-positive individuals will improve as the health effects of HPV-associated precancerous lesions and cancers are treated early, managed effectively, and potentially minimized.

Our study was the first study that investigated prevalence of HPV-associated precursor lesions and incidence of cancers in HIV-positive adults living in Atlantic Canada provinces. It was the first study that reported the prevalence of HR HPV genotypes and examined association of those HPVs with the dysplasia and cancers. We also found a unique association of HPV69 & HPV74 with the 29 precancerous lesions among the 245 HIV-positive study males. The interaction between HPV74 and HPV16 in their association with these precancerous lesions was also a unique finding as compare to other studies. The impact of the study findings are their contribution to quantifying the burden of HPV-associated anal cancer for HIV-infected males and their need for an anal cancer screening program, early treatment of precancerous lesions, and appropriate care. The high incidence of anal cancer among HIV-positive individuals must not be ignored, since it may be preventable (Palefsky JM. et al., 2013). The evidence from this and other studies strongly suggest continuing research in this direction to enhance the dissemination and implementation of research findings into clinical management and policy decisions.

APPENDICES

APPENDIX A: ***Study Ad Poster***

HIV-HPV STUDY

If you are HIV-positive male or female & live in Atlantic Canada, we are seeking your participation in an

Atlantic Canada HIV-HPV Study

For more information or to enrol in this study, please contact your HIV clinic nurse or physician

Or, you may contact the study principal investigator:

Dr. Gerry Mugford
Associate Professor of Medicine and Psychiatry
Faculty of Medicine, Memorial University of Newfoundland, St. John's
Telephone: 709 777 7390
Fax: 709 777 7877
Email: gmugford@mun.ca; Pager: 709 570-9090; Secretary: 709 777-7346

APPENDIX B: *Consent Form*

Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial
University of Newfoundland; Eastern Health; Dr. H. Bliss Murphy Cancer Centre

CONSENT TO TAKE PART IN RESEARCH

TITLE
HPV genotype distribution in HIV-positive adults and HPV-related underlying risk factors for oral, anal and genital malignancy: An Atlantic Canada prospective cohort study

SPONSOR
Public Health Agency of Canada, Ottawa

CO-PRINCIPAL INVESTIGATORS
Dr. Gerry Mugford, Faculty of Medicine, Memorial University; Dr. Dan Fontaine, Faculty of Medicine, MUN; Dr. Sam Ratnam, Faculty of Medicine/Public Health Laboratory

CO-INVESTIGATORS
Drs. Tom Wong and Gayatri Jayaraman, Public Health Agency of Canada, Ottawa; Dr. Alberto Severini, National Microbiology Laboratory, Winnipeg; Drs. Gordon Dow and Bill Thompson, The Moncton Hospital, Moncton; Dr. Duncan Webster, Atlantic Health Sciences Centre, Saint John; Drs. Lynn Johnston and Susan Kirkland, Faculty of Medicine, Dalhousie University, Halifax; Dr. Rod Wilson, South End Family Practice, Halifax; Dr. Todd Hatchette, CDHA, Nova Scotia Health, Halifax.

You have been invited to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:
- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

If you decide not to take part or to leave the study, this will not affect your usual health care and treatment.

1. Introduction/Background
Human Papillomavirus (HPV) is the most common sexually transmitted virus. In most people, HPV infection is temporary and does not lead to disease. There are many types of HPV. Some types tend to persist, and these types can cause oral, cervical, or anal cancer. These types may also cause cancer of the vulva, vagina and penis. HPV is also the cause of genital warts. People with HIV or with HIV positive partners are at a higher risk of cancer and genital warts. A number of studies say these people should be tested for HPV-related diseases. As the risk of HPV related cancers is higher with some types of HPV, knowing the types may help care for patients. Also, there is little known about which types of HPV are more common in HIV positive Canadians. This information is useful for public health HPV surveillance and vaccination programs. Therefore, we are doing this study to find out: the types of HPV are found in persons living with HIV in Atlantic Canada, underlying

disease or disease outcome, and the associated risk factors. This study will help us better understand HIV-HPV co-infection.

2. Purpose of Study

This study will assess what types of HPV are found in persons living with HIV in Atlantic Canada. We will also look at the risk of HPV associated cancers and genital warts and the underlying risk factors.

3. Description of Study Procedures and Tests

You will be asked to fill out a confidential questionnaire. The questionnaire will ask about your risk factors and behaviour. Some of the questions are general, and others are more personal about your sex life. We ask these types of questions because we are trying to find behaviours that might increase your risk of HPV related diseases. You will only have to complete the questionnaire once. You can freely refuse to answer any of the questions.

You will be asked by the clinic nurse or doctor to give an anal specimen and throat specimen, and if you are female you will be asked to give a cervical specimen as well, for Pap and HPV tests. Your doctor will receive the results and this may be useful in your care and treatment. You will be asked to provide the specimens once a year for 3 more years. This is to see if you have or developed any HPV related disease. We also ask your permission to get information from your medical file. This is to get information such as your HIV viral load and treatments, etc. We use this information to see if it relates to any HPV related disease you might have or develop.

4. Length of Time

This study will take place during your regular clinic visits. It will take about 20 minutes to complete the questionnaire once at enrolment. The specimens will be collected once a year for 3 more years.

5. Possible Risks and Discomforts

There are usually no serious risks associated with taking an oral, anal or cervical specimen. Some people feel discomfort when specimens are taken. Some people may find giving specimens embarrassing. The specimens will be taken in a standard way and the nurse or physician will try to minimize any discomfort.

There are usually no risks associated with completing the questionnaire. Some people may experience discomfort filling in questionnaires. The stress is usually mild.

6. Benefits

The information from your annual oral, anal and/or cervical Pap smear will be useful in your care and treatment. Knowing the HPV types may also be useful. However, it is not certain this study will benefit you.

7. Liability statement

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. Confidentiality

Your participation in this study and all information about you will be treated as confidential. Only people involved in this study or part of your health care team will have access to your records. The questionnaire will be completed in your privacy and placed in a sealed envelope and given to the clinic nurse. This will be sent to the principal investigators. Throughout this study, your identification will be retained so that we can link Pap and HPV results to see if you have any risk for HPV related diseases. Your name will not appear in any of the study reports. All information

will be held in encrypted computer databases and protected by passwords. All records will be kept in a locked storage and will remain confidential. The research records will be kept for 10 years and your specimens for up to10 years after the completion of the study. After this, the research records and your specimens will be destroyed and not used for any future research studies.

9. Questions

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is: Dr. Gerry Mugford, **709 777 7390**
Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Office of the Human Investigation Committee (HIC) at **709 777 6974**
Email: hic@mun.ca
Health Canada Research Ethics Board at **613 941 5199** (Collect call accepted)

Signature Page

Study Title: HPV genotype distribution in HIV-positive adults and HPV-related underlying risk factors for oral, anal and genital malignancy: An Atlantic Canada prospective cohort study
Investigators: Drs. Gerry Mugford, Dan Fontaine and Sam Ratnam

To be filled out and signed by the participant

	Please check as appropriate:	
I have read the consent [and information sheet].	Yes { }	No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { }	No { }
I have received satisfactory answers to all of my questions.	Yes { }	No { }
I have received enough information about the study.	Yes { }	No { }
I have spoken to Dr. _____ and he/she has answered my questions	Yes { }	No { }
I understand that I am free to withdraw from the study	Yes { }	No { }
• at any time		
• without having to give a reason		
• without affecting my future care [student status, etc.]		
I understand that it is my choice to be in the study and that I may not benefit.	Yes { }	No { }
I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study.	Yes { }	No { }

Future use of tissue/DNA samples (if applicable):

In order to preserve a valuable resource, your specimens will be stored at the end of this research project. It is possible that these samples may be used in future research projects. Any such studies will be carried out as determined by a research ethics committee. Any future research would have to be approved by a Research Ethics Board (REB).

Please tick one of the following options:

	I agree that my specimens can be used for approved research studies without contacting me again, but only if my name and other personal information cannot be linked, in any way, to the specimens.
	Under no circumstances may my specimens be used for future research. My specimens must be destroyed at the end of the present research study.
	I agree that I may be contacted in future to be invited to provide consent for the use of my specimens in any new approved research studies.

If you have agreed that your specimens can be used for future research, they will be stored at the National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, for an undetermined period of time. Your name, MCP number, address etc, cannot be linked to the specimen. If you have indicated that your specimens cannot be used for future research, they will be destroyed at the end of this study.

I agree to take part in this study Yes { } No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

To be signed by the Study Nurse/Doctor

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study, and that he or she has freely chosen to be in the study.

_____ _____

Signature of investigator Date

APPENDIX C: *26-item Patient Baseline Questionnaire*

Site ID: _____ **Today's Date:** ____/____/____
YYYY MM DD

Encrypted Participant ID: _____

H2 STUDY
Patient Questionnaire

You can help us better understand the relationship between HPV and HIV by answering a few questions. These questions are completely confidential. You may refuse to answer any or all of these questions. Your medical care will not be affected in any way if you choose not to take part in this study. Thank you for your time.

First are some general questions about your background:

1. Date of Birth: _____/___/___
YYYY MM DD

2. In what country were you born?
_____

3. If you were not born in Canada, in what year did you come to Canada to live? _____ (YYYY)

4. What are the first three characters of your postal code? _ __ __

5. What is your ethnic/cultural background? (check all that apply)

- ☐ Aboriginal:
  - ☐ First Nations
  - ☐ Metis
  - ☐ Inuit
- ☐ Middle-Eastern/West Asian (ex: Armenian, Egyptian, Iranian, Lebanese, Moroccan, Israeli)
- ☐ Chinese
- ☐ Filipino
- ☐ Japanese
- ☐ Korean
- ☐ Latin American (ex: Central or South American)
- ☐ Black African
- ☐ Black Caribbean
- ☐ South Asian (ex: Indian, Pakistani, Bangladeshi, Sri Lankan)
- ☐ South-East Asian (ex: Indonesian, Thai, Cambodian, Malay)
- ☐ White (Caucasian)
- ☐ Other, please specify_____

6. What is the highest level of schooling you completed?

- ☐ None
- ☐ Elementary school
- ☐ Some high school, but no diploma
- ☐ High school diploma
- ☐ Some college/university
- ☐ College/university diploma or degree
- ☐ Graduate Degree

Next are some questions about your medical history, health and sexual practices:

7. Do you smoke cigarettes?

- ☐ Yes, daily
- ☐ Yes, occasionally
- ☐ I used to smoke, but quit
- ☐ No, I have never smoked cigarettes

8. Are you male or female?

- ☐ Male *(If MALE, please go to question 14)*
- ☐ Female
- ☐ Other, specify: _____

Site ID: _____ **Today's Date:** ____ / ____ / ____
YYYY MM DD

Encrypted Participant ID: _____

9. How many children have you given birth to?

☐ None
☐ 1
☐ 2
☐ 3 or more

10. Have you ever used oral contraceptives?

☐ Yes
☐ No
☐ Don't know

11. Have you ever had a cervical Pap test before today? (*If NO, go to question 13*)

☐ Yes
☐ No
☐ Don't know

12. When was your last cervical Pap test before today?

☐ Less than 6 months ago
☐ 6 months to less than 1 year ago
☐ 1 year to less than 3 years ago
☐ 3 years to less than 5 years ago
☐ 5 or more years ago
☐ Don't know

13. Have you had cancer of the cervix?

☐ Yes
☐ No
☐ Don't know

14. Have you ever had an anal Pap test before today?

☐ Yes
☐ No
☐ Don't know

15. Have you received the new HPV vaccine?

☐ Yes
☐ No
☐ Don't know

16. Have you ever had oral, anal, or vaginal sex or other genital-to-genital contact?

☐ Yes
☐ No
☐ Don't know

17. During the *last year*, how many sexual partners have you had?

_____ male partners _____female partners

18. In the *last year*, have you had unprotected vaginal sex?

☐ Yes
☐ No
☐ Don't know

19. In the *last year*, have you had unprotected anal sex?

☐ Yes
☐ No
☐ Don't know

20. In the *last year*, have you had unprotected oral sex?

☐ Yes
☐ No
☐ Don't know

Site ID: _____ **Today's Date:** ____ / ____ / ____
YYYY MM DD

Encrypted Participant ID: _____

21. Have you ever been told by a doctor or nurse that you had any of the following sexually transmitted infections?

	Yes	No	Don't know		Yes	No	Don't know
Chlamydia	☐	☐	☐	Anal or genital warts	☐	☐	☐
Gonorrhea	☐	☐	☐	HIV	☐	☐	☐
Syphilis	☐	☐	☐	Hepatitis B	☐	☐	☐
Genital herpes	☐	☐	☐	Hepatitis C	☐	☐	☐
Other (please specify)						_____	

Please answer, to the best of your knowledge, whether you think the following three statements are true or false:

22. HPV does not cause anogenital warts

☐ True
☐ False
☐ Don't know

23. HPV can cause cervical cancer

☐ True
☐ False
☐ Don't know

24. There is a vaccine that can lower risk for anogenital warts and abnormal Pap tests

☐ True
☐ False
☐ Don't know

The next two questions ask your <u>opinions</u> about Pap testing, safer sex practices, and HPV vaccination:

25. **<u>In your opinion</u>**, how important is it for women who have the HPV vaccine to have regular Pap tests? (Pap tests are used to check for cervical cancer)

☐ Extremely important
☐ Very important
☐ Somewhat important
☐ Not at all important
☐ Not sure

26. **<u>In your opinion</u>**, how important is it for those who have the HPV vaccine to continue to practise safer sex?

☐ Extremely important
☐ Very important
☐ Somewhat important
☐ Not at all important
☐ Not sure

Thank you!

APPENDIX D: *12-item Clinic Baseline Questionnaire*

H2 STUDY
12-Item Baseline Clinic Questionnaire
To be completed by Physician / Nurse

Patient ID: _____ **Today's Date:** _____/ _____/ _____
YYYY MM DD

1. Specimens collected
   - ☐ Anal
   - ☐ Oropharyngeal
   - ☐ Cervical

2. Date of collection
   _____/ _____/ _____
   YYYY MM DD

3. Has the participant received HPV vaccination?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

   If yes:
   a. Date of 1st HPV vaccine dose
      _____/ _____/ _____
      YYYY MM DD
      ☐ Date unknown

   b. Date of 2nd HPV vaccine dose
      _____/ _____/ _____
      YYYY MM DD
      ☐ Date unknown
      ☐ Did not receive 2nd dose

   c. Date of 3rd HPV vaccine dose
      _____/ _____/ _____
      YYYY MM DD
      ☐ Date unknown
      ☐ Did not receive 3rd dose

4. Date of first positive HIV test
   _____/ _____/ _____
   YYYY MM DD

5. Baseline CD4+ count and date (use the closest date to the first positive HIV test date)
   _____ cells/ml
   _____/ _____/ _____
   YYYY MM DD

6. Baseline HIV plasma viral load and date (use the closest date to the first positive HIV test date)
   - ☐ Undetectable at <50 copies/ml
     _____/ _____/ _____
     YYYY MM DD
   - ☐ Detectable at _____ copies/ml
     _____/ _____/ _____
     YYYY MM DD
   - ☐ Never had viral load test

7. Ever on anti-HIV medication?

- ☐ Yes
- ☐ No
- ☐ Don't know

8. Currently (i.e., at the time of entry into the current study) on anti-HIV medication?

- ☐ Yes
- ☐ No
- ☐ Don't know

9. Current AIDS-defining criteria / event

- ☐ Yes (if yes, please describe below)
- ☐ None
- ☐ Don't know

_____
_____
_____

10. Previous AIDS-defining criteria / event

- ☐ Yes (if yes, please describe below)
- ☐ None
- ☐ Don't know

_____
_____
_____

11. Any previous histology / clinical data relative to oral, cervical, anal, genital pre-cancer lesions, warts etc.

- ☐ Yes (if yes, please describe below)
- ☐ No
- ☐ Don't know

_____
_____
_____

12. Any prior treatment for any of the above

- ☐ Yes (if yes, provide info below)
- ☐ No
- ☐ Don't know

_____
_____

Physician Name _____
(Please print)

Signature _____

APPENDIX E: *9-item Follow-up Clinic Questionnaire*

H2 STUDY
9-Item Clinic Annual Follow up Questionnaire
To be completed by Physician/Nurse at each of the 3 annual FU visits

Patient ID: _____ **Today's Date:** _____/ ____/ ____
YYYY MM DD

1. Specimen type collected
   - ☐ Oropharyngeal
   - ☐ Anal
   - ☐ Cervical

2. Date of collection
   _____/ ____/ ____
   YYYY MM DD

3. Has the participant received HPV vaccination?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

   If yes:
   a. Date of 1st HPV vaccine dose
      _____/ ____/ ____
      YYYY MM DD
      ☐ Date unknown

   b. Date of 2nd HPV vaccine dose
      _____/ ____/ ____
      YYYY MM DD
      ☐ Date unknown
      ☐ Did not receive 2nd dose

   c. Date of 3rd HPV vaccine dose
      _____/ ____/ ____
      YYYY MM DD
      ☐ Date unknown
      ☐ Did not receive 3rd dose

4. CD4+ count and date at last medical assessment
   _____ cells/ml
   _____/ ____/ ____
   YYYY MM DD

5. HIV plasma viral load and date at last medical assessment
   - ☐ Undetectable at <50 copies/ml
     _____/ ____/ ____
     YYYY MM DD
   - ☐ Detectable at _____ copies/ml
     _____/ ____/ ____
     YYYY MM DD
   - ☐ Never had viral load test

6. Currently on anti-HIV medication?
   - ☐ Yes
   - ☐ No
   - ☐ Don't know

7. Current AIDS-defining criteria / event

☐ Yes (if yes, please describe below)
☐ None
☐ Don't know

8. Since enrollment into the study, an histology confirmed or clinical evidence of oral, cervical, anal or genital pre-cancer lesions/warts etc?

☐ Yes (if yes, please describe below)
☐ None
☐ Don't know

9. Any prior treatment for any of the above

☐ Yes (if yes, provide info below)
☐ No
☐ Don't know

Physician Name _____
(Please print)

Signature _____

APPENDIX F: *Patient's Enrolment Card*

H2 STUDY

1. Name: _____ 2. Age: _____ 3. Sex: M ☐ F ☐

4. Mailing Address: _____

5. Telephone #: _____ 6. Cell #: _____ 7. E-Mail: _____

Alternative Contact

8. Name: _____ 9. Telephone #: _____

10. Cell #: _____ 11. E-Mail: _____

Specimens Collected

12. ☐ Anal 13. ☐ Oropharyngeal 14. ☐ Cervical

15. Date of Enrollment: _____ 16. Physician Name: _____

17. **1st Follow up Due Date:** _____

18. ☐ Contacted 19. Date: _____

20. ☐ Recontacted 21. Date: _____

22. ☐ Compliant 23. ☐ Non-Compliant

24. ☐ No Show 25. ☐ Couldn't Contact

26. Specimen Collected: 27. ☐ Anal 28. ☐ Oropharyngeal 29. ☐ Cervical

30. Date Collected: _____

31. **2nd Follow up Due Date:** _____

32. ☐ Contacted 33. Date: _____

34. ☐ Recontacted 35. Date: _____

36. ☐ Compliant 37. ☐ Non-Compliant

38. ☐ No Show 39. ☐ Couldn't Contact

40. Specimen Collected 41. ☐ Anal 42. ☐ Oropharyngeal 43. ☐ Cervical

44. Date Collected: _____

45. **3rd Follow up Due Date:** _____

46. ☐ Contacted 47. Date: _____

48. ☐ Recontacted 49. Date: _____

50. ☐ Compliant 51. ☐ Non-Compliant

52. ☐ No Show 53. ☐ Couldn't Contact

54. Specimen Collected 55. ☐ Anal 56. ☐ Oropharyngeal 57. ☐ Cervical

58. Date Collected: _____

APPENDIX G: *NYS DOH Specimen Collection Guidelines*

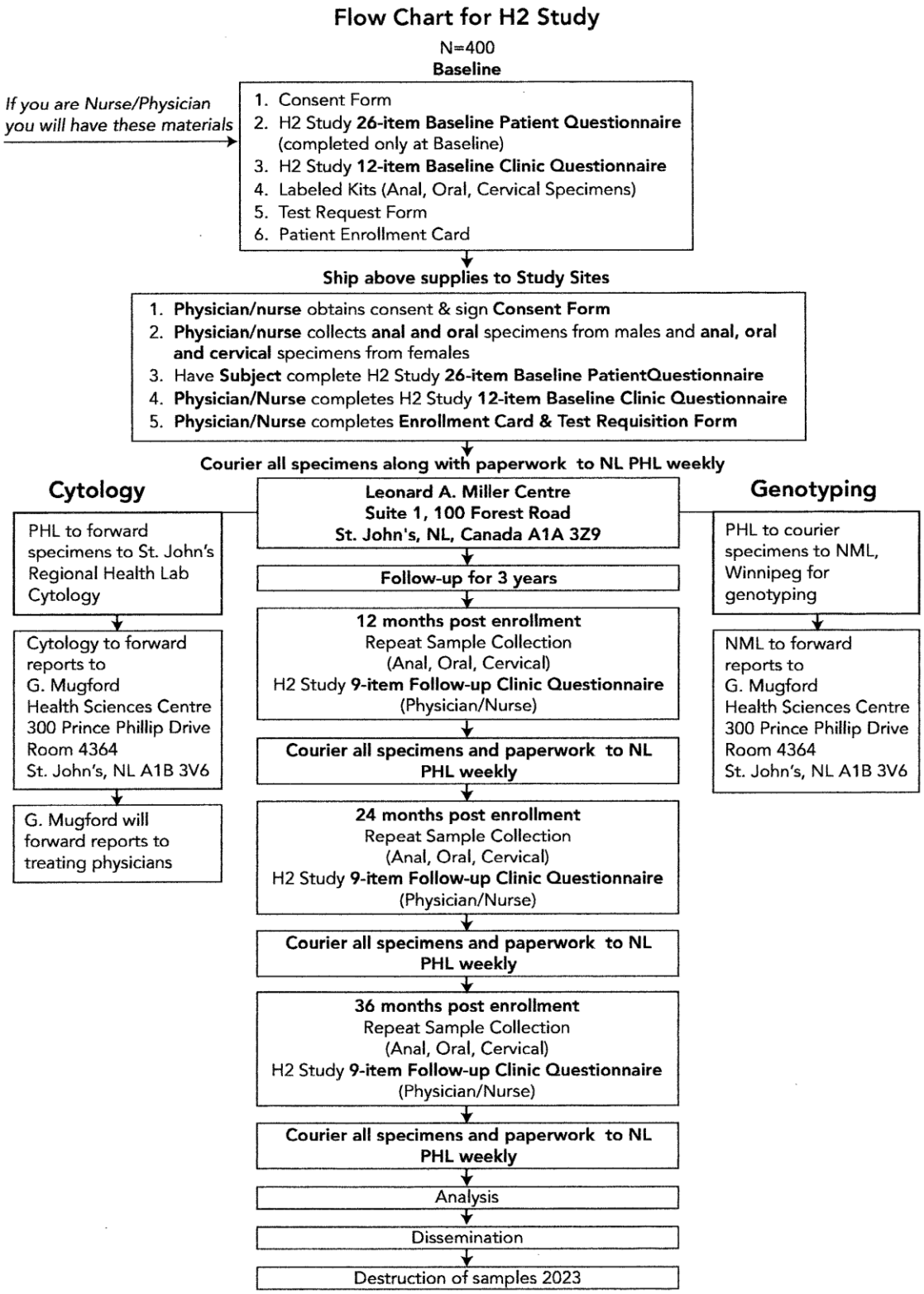
Technique: (NYS Guidelines recommendations on anal pap smears, NYS DOH AIDS Institute's HIV quality-related website - http://hivguidelines.org/Content.aspx).

There is no preparation necessary before obtaining anal cytology. If the digital rectal examination is performed in conjunction with anal cytology and/or HRA, the cytology must be obtained first, before lubrication is introduced into the anal canal. Patients should not have received an enema or engaged in receptive anal sex within 24 hours before sampling because these activities can adversely affect specimen quality.

The standard technique used in obtaining anal cytology is as follows: a Dacron swab (a cotton swab will not yield accurate results) is moistened with sterile or non-sterile water. The anus is spread with the index and thumb of the non-dominant hand so that the anoderm pouts out. The swab is then gently inserted into the anal canal as far as it will go, until it hits the wall of the rectum. If the swab does not go in easily, the angle of insertion should be adjusted. The presence of external hemorrhoids may cause resistance; in this case, different insertion points should be tried until the anal canal is easily accessed. The swab must be inserted above the squamocolumnar transition zone, which is approximately 2 cm (1 inch) from the anal verge.

The swab is then slowly moved in and out without completely withdrawing it, while rotating it in a spiral motion and applying mild pressure to the anal wall. After several rotations, the swab should be withdrawn and immediately immersed in methanol-based preservative-transport solution. Feces or traces of blood on the swab will not affect the result. The swab should be agitated in the solution for 60 seconds to transfer cells from the swab to the medium.

APPENDIX H: *Flow Chart*



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