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

Cross-sectional study of the screening year

RANA ASLANOVA

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A thesis submitted to the
School of Graduate Studies
In partial fulfillment of the
Requirements for the degree of
MASTER OF SCIENCE in MEDICINE

Clinical Epidemiology Department
Faculty of Medicine
Memorial University of Newfoundland and Labrador

March 2012

St. John's, Newfoundland and Labrador, Canada

ABSTRACT

BACKGROUND:

Human Papillomavirus (HPV) is the most common sexually transmitted agent. These small DNA viruses target the basal cells of the epithelium. While the HPV family is comprised of more than 100 genotypes, only about 40 or so types are associated with human anogenital infections. Infections with oncogenic HPV (probably 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) are causally linked to the development of cervical cancer (HPV16 & 18 are related to about 99% of cervical malignancy) as well as a proportion of anal, oropharyngeal, vulvar, vaginal and penile cancers and their associated precancerous lesions. HPV is also the cause of genital warts (low-risk HPV types 06,11,34,40,42,44,53, & others). However, in most people HPV infection is transient and does not lead to disease. Immune suppression increases the likelihood of HPV-related diseases, and people with human immunodeficiency virus (HIV) infection or with HIV-positive partners are at a higher risk of pre-cancer lesions and cancers, as well as genital warts. The literature suggests HIV-HPV co-infection in women increases the risk of anal carcinoma by 30 times and the risk of HPV associated anal cancer is 163-fold greater in young men with HIV. A number of studies have recommended that all HIV-infected individuals be routinely screened for HPV-related disorders to facilitate early detection and treatment because of the heightened risk of persistent HPV infection with the risk of malignant transformation.

OBJECTIVES:

Primary: To determine the prevalence and distribution of high risk (HR) oncogenic HPV-types in HIV-positive adults in Atlantic Canada.

Secondary: To correlate the prevalence of HR HPV genotypes with underlying pre-malignant lesions and malignancy through a cross-sectional study.

To correlate pre-malignant lesions and malignancy with patients' demographics and underlying risk factors.

METHODS:

This thesis is part of a larger prospective cohort study designed to follow consenting persons with HIV infection in Atlantic Canada over a 3-year period subsequent to baseline screening in year one. The data in this thesis are limited to that obtained at baseline in all four Atlantic Provinces during the first year of the study. All HIV-positive adults treated through the participating infectious disease clinics were approached by the clinic physicians or nurses to request participation in the study. Atlantic Canada has approximately 800 routinely followed HIV-positive adults and we expected to enroll approximately 400 of them. This study was approved by ethic committees of all participating institutions as well as the Public Health Agency of Canada, the funding agency for the study.

Recruitment commenced in June, 2009. All procedures were performed after obtaining informed consent. Consenting participants were required to complete a confidential questionnaire to obtain demographic and risk factor data. Participants' identifiers were retained to permit questionnaire data to be correlated with HPV related disease outcomes. SurePath (Becton Dickinson) liquid cytology medium was used in the collection of oropharyngeal and anal swab specimens from all males and females, and an additional cervical specimen was obtained from females. All specimens were tested for cytologic abnormalities, HPV DNA and genotyping. The study cohort will be followed up with the above protocol for a period of 3 years. These results will be subsequently used to assess any HPV related disease outcome and genotypic specific information. Consent also gives access to patients' medical files for information on viral load, $CD4^+$ T cell count and treatment regimens in order to correlate these factors with disease outcome.

RESULTS:

The study analysis is based on a total of 300 patients; of these 91.7% were males. The population and gender distribution among the provinces were: Nova Scotia, Halifax (NSH) – total 150 patients, of these 142 (94.7%) were males; New Brunswick, Moncton (NBM) – total 90 patients, of these 85 (94.4%) were males; Newfoundland and Labrador, St. John's (NLSJ) – total 44 patients, of these 34 (77.3%) were males; and New Brunswick, Saint John (NBSJ) – total 16 patients, of these 14 (87.4%) were males.

The mean (SD) age of the study population was 46.9 (9.36) years and its distribution by age categories was: 62 (20.7%) aged 25 to 39 years; 209 (69.7%) aged 40 to 59 years; and 29 (9.6%) aged 60 years and older.

There was no significant difference in the association of cytologic results with CD4 cells count and plasma viral load levels.

A total of 232 (77.3%) of the participants tested positive for any HPV infection with 125 (54.0%) showing multiple HPV types. Up to 46 HPV genotypes were detected, of which 39% were HR oncogenic types and 61% of low-risk (LR) types. The most frequently detected HR HPV types among all specimens were: HPV16 11.8%; HPV52 7.2%; HPV45 5.6%; HPV51 5.4% & HPV18/HPV59 4.6% each. Six HR HPV types: 16 ($p<0.001$); 45 ($p=0.044$); 51 ($p=0.014$); 52 ($p<0.001$); 53 ($p=0.045$); and 59 ($p=0.006$) were more frequently associated with anal lesions; from them atypical squamous cells of undetermined significance (ASC-US) were detected in 37 (12.3%) patients; atypical squamous cells of undetermined significance cannot exclude high-grade intraepithelial lesion (ASC-H) in 3 (0.5%) patients; low-grade squamous intraepithelial lesions (LSILs) were detected in 35 (11.6%) participants; and high-grade intraepithelial lesions (HSILs) in 3 (0.5%) of them. HPV51 was detected in one oropharyngeal specimen with ASC-US cytologic changes; and HPV35 was more frequently associated with cervical lesions (ASC-US in one female; LSIL in one patients also; and LSIL cannot exclude high-grade lesion (LSIL/HSIL) in one female participant). The highest number of cytologic abnormalities was reported in anal specimens (26%) as compared to cervical and oropharyngeal specimens (12% & 0.3%, respectively). Cytologic changes were significantly associated with patients' high risk behaviour such as unprotected anal and vaginal sex. All patients with detected lesions were referred to an appropriate specialist for further investigation.

The overall prevalence rate of the cases with the high-risk (HR) HPV genotypes was 46.6% (108/232). The overall prevalence of the cytologic abnormalities caused by HR HPV types in the study population was 27.3% (82/300) during the screening year.

CONCLUSION:

In this study, 74% of 300 patients studied had normal anal cytology, with 26% having abnormal anal cytology, and with 15.3% (46/300) of patients having unsatisfactory anal specimens for evaluation. Abnormal cytology was reported mostly as ASC-US (12.3%) and LSIL (11.7%), with fewer samples showing HSIL (1.0%). All participants with detected anal, cervical and oropharyngeal precancerous lesions were referred to specialists to undergo a high-resolution anoscopy (HRA) and colposcopy with biopsy (the results of these tests will be analyzed as a part of the larger study). The results of the data presented here are comparable with the results from other studies. Follow-up analysis of anal, cervical and oropharyngeal biopsy results are required to draw conclusions about real prevalence of cytologic abnormalities in the study population; cytologic outcomes have to be correlated with histologic outcomes.

KEY WORDS:

Human papillomavirus infection, anal cancer, cervical cancer, head and neck cancer, HPV genotyping, HPV prevalence and incidence, HPV and malignancy, HPV risk factors, HIV-HPV co-infection.

ACKNOWLEDGEMENTS

I wish to thank many individuals who have helped in the preparation of this thesis. I appreciate the commitment shown by the physicians and nurses from Halifax, Moncton, Saint John and St. John's from the beginning of the study until now. Without their input my thesis would not have prevailed.

It has been a pleasure to be associated with the Clinical Epidemiology Department, Public Health Laboratory and St. Clare's Mercy Hospital staffs. Their friendship and advice were invaluable. Thank you to Dr. Sam Ratnam, Dr. Gerry Mugford and Dr. Dan Fontaine for serving on my supervisory committee and providing encouragement and sound advice over the years.

My sincere thanks go to my supervisor Dr. Gerry Mugford and physician Dr. Ian Bowmer for their excellent teachings and assistance as well as their confidence in my work. It was an enjoyment working with them and getting to know them throughout my Master's program.

Most importantly I wish to thank my family and especially my son Orkhan for their ongoing patience, understanding, unrelenting help, support and encouragement. For your love and support I will be forever grateful.

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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

EUROGIN European Research Organization on Genital Infection and Neoplasia

E6/E7 Oncogene lineages of Human Papillomavirus type 16 E6, E7 in preinvasive 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

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
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
MSM	Men who have Sex with Men
NBM	New Brunswick, Moncton
NBSJ	New Brunswick, Saint John
NCI	National Cancer Institute
NL	Newfoundland and Labrador
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's (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, St. John's, NL
PGMY/GP	Primers that were used in Luminex-Based Assay
PR	Prevalence Rate
RR	Relative Risk
SIR	Standardized Incidence Ratio
STI	Sexually Transmitted Infection
TBS	The Bethesda System
TZ	Transformation Zone
VL	Plasma viral load

CHAPTER 1

INTRODUCTION

1.1 Introduction to the Thesis

Human Papillomavirus (HPV) infection is estimated 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, 2009) but this viral infection usually clears up by itself and causes no signs or symptoms.

The greatest risk factors for infection with HPV in the general population are gender, young age and sexual activity. Besides these factors, common risk factors for HPV infection and clinical sequelae of infection include high number of sexual partners and co-infection with *Chlamydia trachomatis* or *Herpes Simplex Virus* (HSV). Most HPV infections are non consequential, being cleared by the immune system and do not results in clinical complications.

While HPV infection is mostly transient in the majority of individuals and does not lead to disease, immune suppression increases significantly HPV related diseases and those with human immunodeficiency viral (HIV) infection or with HIV-positive partners are at a higher risk of pre-cancer 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 and are 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; Sebitoane, 2005).

The risk of HPV associated malignancies is genotype-dependent. However, from the standpoint of HPV epidemiology, there is a paucity of information on HPV genotype distribution and the risk of anal, oropharyngeal and cervical cancer among those living with HIV in Canada. This is a prospective cohort study involving HIV-positive persons (PHAs) treated at the Infectious Diseases clinics in Canada's four Atlantic Provinces: Newfoundland and Labrador (NL), New Brunswick (NB), Nova Scotia (NS), and Prince Edward Island (PEI). While the HPV prevalence is likely to be quite high in the target population, testing for the high risk (HR) HPV genotypes and associated cytologic abnormalities should identify those at increased risk of malignancy. Moreover, determining the HPV genotype will be beneficial in assessing the relative risk of earlier developing malignancy and will also be quite useful as part of ongoing patient management.

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 clearly indicate 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 had multiple sexual partners; 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 should increase the risk of acquisition and progression of HPV. 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) compared to HIV-seronegative men.

It also was reported that, compared to men who have sex with men seronegative for HIV, those who are seropositive have a 2-fold higher risk of anal cancer (Goedert et al., 1998). Furthermore, the incidence of anal cancer in seropositive for HIV men has increased significantly since the introduction of highly active antiretroviral therapies (HAART) (Bower et al., 2006; Hessol et al., 2007; D'Souza et al., 2008; Piketty et al., 2008). Lampinen et al. in 2006 reported that the increased risk of anal cancer among HIV-positive men who have sex with men (MSM), can be as high as 140-fold 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). Therefore, 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 serotypes (Uronis & Bendell, 2007).

Uterine 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 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).

HPV16 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 men who have sex with men (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 (99%) related to HPV, other sexually transmitted cancers are associated with HPV to a varying extent: penis 40%, anus 90%, vulva/vagina 40% and oropharynx 12% (Munoz N. et al, 2006). The current estimated worldwide burden of cancer cases caused by HPV and by HPV types 16/18 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 100 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 and anus. Additionally, HPV may infect the glandular epithelium of the endocervix, resulting in neoplasms, such as adenocarcinoma *in situ* or invasive carcinoma.

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, while infections with probably 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.

1.4 Natural History and Pathogenesis of HPV infection

The overwhelming majority of ano-genital cancer patients show serological, histopathological or molecular evidence of prior infection with HPV, and viral DNA sequences can be detected in tumor tissue. The tumors and cell-lines derived from them continue to express the viral E6 and E7 HPV proteins, suggesting that these two proteins are required for the continued growth of the cells (Schwarz et al., 1985; Smotkin and Wettstein, 1986; Androphy et al., 1987; Banks et al., 1987). The "E" designation indicates that these two primary oncogenes are expressed early in the HPV life cycle.

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 HPV16 and HPV18 are capable to bind 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, E6-associated protein – 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 arrest 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.

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. High-risk HPV types also interfere with cell cycle regulation via the E7 proteins by modulation of the induction of DNA synthesis (Sato et al., 1989; Banks et al., 1990).

The E7 protein has a similar effect on host-cell metabolism by binding to retinoblastoma protein and inhibiting its function (Scheffner M, et al 1993). Both E6 and E7 proteins may cause chromosomal destabilization, and inhibit cyclin-dependent kinase inhibitors and host interferons (Shindoh M, et al 1995).

The integration of HPV DNA into the host DNA leads to an increase in cellular proliferation and the likelihood of malignant transformation (Stanley M, 2003).

1.5 Tumorigenic Potential of HPV

Human Papillomavirus (HPV) is a significant source of morbidity and mortality worldwide. Currently there are no effective treatments for individuals with genital warts or malignant lesions; 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, 16 and 18, and the second quadrivalent vaccine (Gardasil) targeting HPV 16 and 18, and the two most common low-risk types, HPV 6 and 11 has recently become available. 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 (16). The Society of Obstetricians and Gynecologists of Canada estimates that 10% to 30% of the Canadian 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/). There are approximately 6.2 million new HPV infections occur every year in the United States, and approximately 20 million individuals are currently 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 ongoing Study to Understand the Natural History of HIV/AIDS (SUN) in HIV-positive adults by Vellozzi C et al in 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 genital tract. HPV exposure of either anatomical site can result in tracking and infection of the other site.

1.5.1 HNSCC (Head & Neck Squamous Cell Carcinoma)

The involvement of HPV in oral and oropharyngeal carcinogenesis was first proposed in 1983 by Syrianen et al. 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 suggest 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 with increasing 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 mucosa was 10% (95%CI=6.1-14.6), in benign leukoplakia was 22% (95%CI=15.7-29.9), in intra-epithelial neoplasia 26.2% (95%CI=19.6-33.6%), in verrucous carcinoma 29.5% (95%CI=23.0-36.8), and, finally, in oral squamous cell carcinoma 46.5% (95%CI=37.6-55.5). HPV 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, and between head and neck anatomical sides (Kreimer et al., 2005); multiple HPV detection methods (polymerase chain-reaction [PCR], *in situ* hybridization [ISH], and others).

Two large studies have recently strengthened the correlation between HPV-associated ano-genital cancers and HNSCC. The first study (Frisch and Biggar, 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 increased. Patients with cancers unrelated to HPV had a relative risk (RR) closed to 1. The second study (Hemminki et al., 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 or *in situ* cervical carcinoma, as well as the occurrence of first primary cancers among their husbands was 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.

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 and HIV

Anal cancer is very similar to cervical cancer biologically, including a causative association with human papillomavirus (Hoots, Palefsky et al., 2009). Furthermore, within the anal canal the squamocolumnar junction (TZ) is anatomically very similar to the squamocolumnar junction (TZ) on the cervix; at this junction there are typically areas of squamous metaplasia, as on the cervix, which are especially susceptible to the oncogenic effects of HPV. It is in these areas 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 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).

The incidence of HPV-associated anal cancer is high among HIV-positive men who have sex with men (MSM), and possibly in HIV-positive women (Kreuter A. et al., 2008). 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 recent studies have shown 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 registry in France of 75/100,000 person-years among HIV-positive MSM since 1999 (French Hospital Database). Patel et al. 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. And, finally, D'Souza et al. 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 prevalence of anal HPV infection in 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 this (EXPLORE) large cohort of MSM of all ages suggest that individuals might be susceptible to reinfection, at least transiently, following re-exposure, so 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 absence of high-risk HPV at a single time point and from a single 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 may not always be picking up the infection; perhaps individuals with HIV have a lower clinical threshold for infection than do general population. If this is true, the method of HPV detection becomes critical as the current assays include clinical thresholds which may not be applicable to this population.

HIV-positive patients other than MSM can have AIN even where there has been no history of receptive anal intercourse; but the risk for these other groups appears to be much lower (Wilkin et al., 2004). This study 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 with 52%. There is also an interpretive factor that needs to be accounted for in both the histologic and cytologic interpretations.

It is also well established that the risk of both prevalent and incident high-grade AIN increases as CD4 cell count falls (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

Cancer of the cervix (CC) is the second most common cancer among women worldwide 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 declined with the introduction of cervical cytology screening to identify and treat women with cervical cancer precursor lesions (high-grade CIN, or CIN 2-3, and particularly CIN 3). 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 cytologic 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. Mougin C. et al in 2001 found that there was a long latency period between primary infection and cancer which led the authors to suggest that additional risk factors are involved in the process of tumor development. These risk factors may include (Bell MC. et al in 2011) younger age, lower education, nutritional status, more sexual partners, younger age at first sexual experience and first pregnancy, and more pregnancies ($p<0.003$). Also associated were recreational drug use, current smoking and history of sexually transmitted diseases. So, although from 10% to 40% of

women 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, HPV16 was the most common type (11.8%) followed by HPV52 (5.6%) and HPV51 (5.4% of positive subjects). Each pre-cancerous lesion tested 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 increased risk for human papillomavirus infection, cervical cancer, and precancerous lesions compared with uninfected 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 cell count (<200 cells/μL vs. ≥200 cells/μL) and having 1 or more abnormal test result (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+ women might receive less screening than general population (Ontario Cervical Screening Program, Cancer Care Ontario: www.cancercare.on.ca/documents/OntarioBethesda2001.pdf - 2006, Jun)

CHAPTER 2

STUDY METHODS

2.1 Study Design and Study Centres

This thesis is part of a larger prospective cohort study which was designed for a screening year followed by three consecutive years of observation and surveillance of the participants. Study centres are located in St. John's (NL), Saint John (NB), Moncton (NB) and Halifax (NS). Patients from Prince Edward Island (PEI) are seen in Halifax and Moncton. The study poster with the contact info was distributed at the local HIV clinics (Appendix A). The screening year recruitment process started in June 2009 and is still ongoing. All HIV-positive patients seen through the Newfoundland & Labrador (NL), New Brunswick (NB), Nova Scotia (NS) and Prince Edward Island (PEI) 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 the written consent. Consent was obtained using ethics board approved consent forms with the clear understanding that patients' unique identification numbers (IDs) will be retained in order to conduct future patients follow-ups (Appendix B). The consent was also obtained to access the patient's medical file information such as HIV viral load, CD4 cells count, and history of sexually transmitted infections in order to correlate these factors with disease outcome. All consenting participants were enrolled during a two-year period and will be followed up at the scheduled intervals as per standard practice for up to three years. During initial interviews, participants were administered a confidential patient's questionnaire to obtain demographic and risk factor data (Appendix C).

At the time of enrolment the clinic physician/nurse filled out the 12-item clinic questionnaire with the earliest tests results related to the HIV markers and patient health status (Appendix D). They also filled out the patient's enrolment card in order to register the dates of the specimen collection at baseline and during the three follow-ups (Appendix E). During the follow-up visits, the 9-item clinic questionnaire has to be obtained from the patient's medical file with the latest data for the year of observation.

2.2 Study Population

All HIV-positive adults $\geq$ 18 years of age treated at the various research sites were invited to participate in the study. The exclusion criteria include only patient's unwillingness to participate in the study. Atlantic Canada has approximately 800 HIV-positive cases and we expected to enroll approximately half of them (400 participants). However, at the date of conducted analysis for the screening year of the study 300 patients had been enrolled. The recruitment process will be continued until the expected number of participants will be reached.

2.3 Specimen Collection/Centralization of the Data

The labels for each study site as well as consent forms, enrolment cards and questionnaires were designed and printed prior to the study beginning. Each site has its particular label color: New Brunswick-red labels, Newfoundland-blue and Nova Scotia-green labels. Each label (patient's ID in the research) included the site name, patient's personal number, and the type of specimens collected (A, O, C), e.g. NLSJ 001 and checked A & O squares on the label indicates that this patient was a male (only anal and oropharyngeal specimens were obtained), his number 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 centre.

The personal kit for the screening year included: labeled paper-work (consent form, 21-item patient's questionnaire, 12-item clinical questionnaire, and laboratory requisition form with the enrolment card), Pap vials with liquid media, and packs with Dacron and cotton swabs (for anal and oropharyngeal specimens, respectively), cervical brush, paper and plastic biohazard bags.

In order to standardize collection among the study sites, the detailed guidelines for specimen collection (NYS Guidelines recommendations on anal pap smears -Appendix F) were sent to the research sites at the beginning of the screening year in June 2009. Trained personnel collected an oropharyngeal and anal swab specimen from all consenting males and females, females were asked to provide an additional cervical specimen. In our study we used SurePath™ Liquid-Based Pap test (BD Diagnostics) supplies for cytologic analysis: SurePath™ vials with 10 ml of ethanol-based media and blue cervical brushes with the detachable end (http://www.bd.com/tripath/physicians/) were used for collection. The cervical transformation zone (TZ) is the site of origin of most cervical neoplastic lesions, and, as in sampling for cervical cytology, were targeted in the study for exfoliated cell collection. The standard technique was used for obtaining anal cytology by rotating a water-moistened Dacron swab in the anal canal without direct visualization (blind or non-guided method) above the squamocolumnar transition zone (TZ), which is approximately 2 cm from the anal verge. (NYS Guidelines recommendations on anal pap smears, NYS DOH AIDS Institute's HIV quality-related website - http://hivguidelines.org/Content.aspx). The oropharyngeal specimen was collected by using sterile cotton swab from the back side of the patient's throat.

The collection devices were then each placed in a SurePath collection vial. The sample handling for all three specimens was similar. The resulting solution was stored at the room temperature in the PHL and later used for the preparation of thin-layer slides for cytologic analysis.

All specimens and completed paper-work were sent to the Public Health Laboratory (PHL), St. John's. The specimens were shipped under conditions that protected sample integrity for testing and, equally important, protected the safety of all who come in contact with the shipment (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: the first one-third to the Eastern Health Regional Cytology Laboratory, St John's and the second two-thirds to the National Microbiology Laboratory (NML), Public Health Agency of Canada (PHAC) in Winnipeg for HPV DNA testing and genotyping. The cytology test results were forwarded to the study physicians through the lead principal investigator who coordinated the study logistics with a secure database and, in collaboration with other study investigators, analyzed and prepared reports to the PHAC in Ottawa. The detailed study Flow Chart is showed in the Appendix G.

2.4 Purpose of the Study

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 Canadian HIV-HPV Surveillance Network", which could provide valuable information and potentially serve as a model to the rest of Canada.

The study data and results will also be quite useful and included as a part of ongoing HIV patient care and management. It can potentially initiate some changes in primary care policies with follow-up recommendations for the annual screening of all HIV-infected adults, regardless of age, to perform both a visual inspection of the perianal region and a digital rectal examination with anal specimen collection for the cytology evaluation and HPV genotyping.

Further, determining HPV genotype prevalence will be beneficial in assessing the relative risk (RR) of acquired malignancy and will provide useful information in the era of genotype-specific HPV vaccination.

Finally, this study should contribute to the national data on genotype distribution and add to the existing of the published knowledge on HIV-HPV co-infection.

2.5 Study Goals

The main goal of this study was to determine the prevalence of high risk (HR) oncogenic HPV types in HIV-positive adults both males and females in Atlantic Canada and correlate them with underlying pre-malignant lesions and malignancy through cross-sectional study of the screening year data.

The other goals of the study included:

- Correlation of the detected pre-malignant lesions and malignancy with patients' demographics, behavioral risk factors such as smoking, multiple sexual partners, unprotected sex, and their history of sexually transmitted infections.

- Correlation of the detected pre-malignant lesions and malignancy with patients' HIV markers and health status (baseline CD4 cells count, viral load level, history of anogenital warts/malignancy).

2.6 Research Questions

The primary research question

What were the distribution and prevalence of high-risk oncogenic HPV genotypes in the HIV-positive population in Atlantic Canada?

The secondary research questions

Were the high-risk oncogenic HPV genotypes correlated with underlying anal, oropharyngeal and cervical pre-malignant lesions and malignancy?

Were the detected pre-malignant lesions and malignancy correlated with patients' demographics and behaviors as well as with patients' health status?

CHAPTER 3

DIAGNOSES ESTABLISHING METHODS

3.1 Brief Description of the Existing Tests to Detect HPV Infection (WHO HPV Laboratory Network Data, July 2010)

There are two tests available to detect the presence of HPV viral DNA: 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 the subtypes of the high-risk virus. Its advantages are that it is quick and less expensive compared to PCR tests. The PCR test can detect the type of HPV present, but 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 combination: episomal or extrachromosomal HPV particles and 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 diffuse and present throughout the nucleus and represents episomal HPV virus. A type 2 signal is punctuate 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, 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:

- Those that rely on signal amplification to detect the targets
- 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 used are detailed in Table 16. Briefly, in this study, the Roche Linear Array 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; plus, if it also does not give more than 1 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 HPV vaccine (6 & 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 validate the assays for both qualitatively and quantitatively to determine their: Sensitivity/Limit of Detection, Specificity, Accuracy, Reproducibility, Robustness, Linearity, and Analytic Range (International Committee on Harmonization. ICH Validation of analytical procedures: text and methodology) http://www.emea.europa.eu/pdfs/human/ich/038195en.pdf

3.2 HPV Genotyping Tests used in this Study

The National Microbiology Laboratory, PHAC in Winnipeg, MB conducted HPV DNA and genotyping analysis for our study. Two HPV assay types were used (#1 and #18 in the Table 16) for this purpose: The Linear Array Genotyping Test (#1) and The Luminex®-Based Genotyping Assay (#18 – In-house PCR Luminex L1 (PGMY-GP). They used supplies from 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 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 anogenital 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 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 of:
    1. Standardized, quality-controlled reagents

2. Primer concentrations that minimize competition due to coamplification.

(Coutlee F et al, 2006).

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; Department of Medical Microbiology, University of Manitoba, Winnipeg, MB. This assay can type simultaneously 45 mucosal HPV genotypes and was evaluated with the Roche Linear Array (LA) test. In this study, amplified single stranded HPV DNA carrying a biotin tag was generated using primers PGMY (Gravitt PE et al, 1998) and GP5+/GP6+ (Husman AM et al, 1995) in a nested PCR reaction; a set of 45 Luminex microspheres coupled with 45 unique HPV probes was used 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 without vs. with 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, respectively, 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 (8 vs. 18).

In conclusion, the tested Luminex assay, while comparable to the Linear Array (LA) test, offers more flexibility, lower cost and less hands-on time (Goleski VA et al, 2008).

3.3 Cytopathology

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 sites such as the throat, 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 (specimen rejected or specimen processed and examined, but 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 as the diameter of the sample is 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 an 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 (LSIL) and high-grade (HSIL) categories. Low-grade lesions encompass the cellular changes variously termed "HPV cytopathic effect" (koilocytosis) and mild dysplasia or Cervical Intraepithelial Neoplasia (CIN) 1. High-grade lesions encompass moderate dysplasia, severe dysplasia, and Carcinoma *in situ* or CIN 2, 3.

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

Epithelial cell abnormalities require further investigation such as colposcopy or high resolution anoscopy (HRA) with biopsy. These abnormality categories along with the recommended management are demonstrated in **Table 16**. 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 in our study for follow-up and management for all detected cytologic abnormalities.

CHAPTER 4

STUDY RESULTS

4.1 Patients' Demographics

In this cross-sectional study the baseline measurements of CD4 cell count and viral load were analyzed. The 12-item clinical questionnaire (Appendix D) included only the primary data (at the baseline visit) and did not include data related to these parameters mean, median, nadir, and range measurements. The collected data was categorized. CD4 cell counts were divided on two categories: CD4 count < 200 cells/ml and CD4 count ≥200 cells/ml. This was done based on evidence from different studies of oral opportunistic infections in adults with HIV/AIDS. Oral opportunistic infections such as Kaposi's sarcoma (100%; p=0.035), candidiasis (82.2%; p< 0.001), linear gingival erythema (70.0%; p=0.015), hairy leukoplakia (66.3%; p<0.001), and others were strongly associated with immune suppression and were found to be significantly correlated to a reduced CD4 cell count below 200 cells/ml, thus serving as potential clinical markers of HIV viremia and progressive HIV disease (p<0.001; OR=3.1; 95%CI 1.9-4.9) (Bodhade AS, Ganvir SM & Hazarey VK, 2011; and Patton LL, 2000).

Plasma viral load was categorized according to four levels: 0 – "Never had test"; 1 – "Undetectable level at ≤50 copies/ml"; 2 – "Detectable level at >50 copies/ml"; and 3 – "Unknown". These categories are widely used by medical practitioners working with HIV-positive adults and participating in the study as co-investigators. IBM® SPSS 19.0 was used for the statistical analyses. All tests were two-sided with the significance at $\alpha<0.05$. The following analyses were conducted:

1. Frequency counts of the patient's demographic information

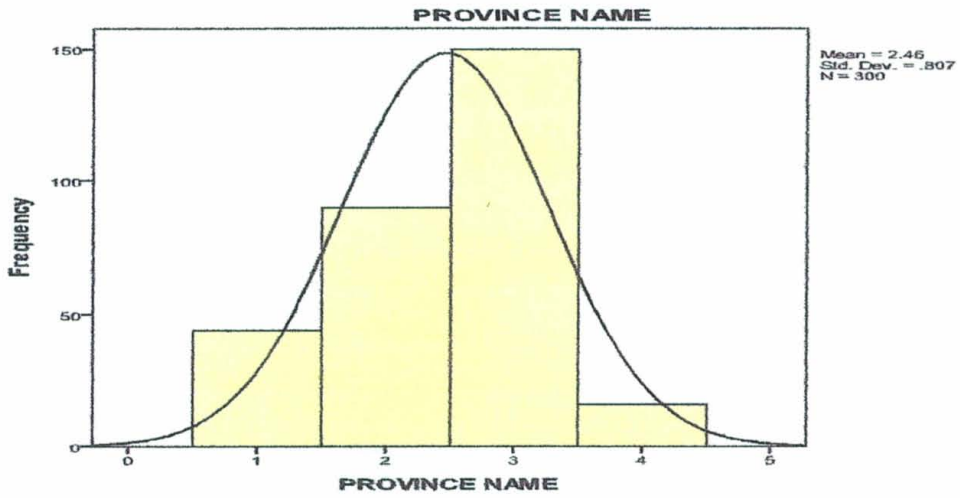
2. Frequency counts of the different presentation of HPV genotypes
3. Frequency counts of oncogenic disease activity (pre-cancer lesions and cancers of the oropharynx, cervix, anus, vulva, vagina, and penis)
4. Calculation of the association between disease expression and genotype presentation (Chi-Square, Logistic Regression)
5. Calculation of the association between the underlying risk factors and oncogenic activity (Chi-Square, Logistic Regression)
6. Calculation of the prevalence rates of HPV genotypes and oncogenic activity

The study population was normally distributed by provinces and participants' age (**Histograms 1 & 2**) and was skewed to the right by gender because of the prevalent male population. Of 300 patients included in the analysis 91.7% were males. The population and gender distribution among the provinces were: NSH – total 150 patients, of these 142 (94.7%) were males; NBM – total 90 patients, of these 85 (94.4%) were males; NLSJ – total 44 patients, of these 34 (87.3%) were males; and NBSJ – total 16 patients, of these 14 (87.4%) were males. The overall mean (SD) age of the participants was 46.9 (9.36) years and the distribution of the study population was: 62 (20.7%) aged 25 to 39 years; 209 (69.7%) aged 40 to 59 years; and 29 (9.6%) aged 60 years and older (**Table 1**).

Of 300 patients, 90.3% vs. 9.7% were on anti-HIV treatment previous to the study (p=0.006); and 86.7% vs. 13.3% were on anti-HIV medication at the screening year of the study with p=0.044. The majority of participants (73.0% vs. 27.0%) did not have an AIDS-defining event previous to the study (p=0.660); while 97.0% of them vs. 3.0% did not have any AIDS-defining event at the screening time (p=0.738).

Histogram 1

Distribution of the Population by Provinces



X-axis: 1. NLSJ; 2. NBM; 3. NSH; 4.NBSJ

Y-axis: Number of Participants

Histogram 2

Distribution of the Population by Age (in years)

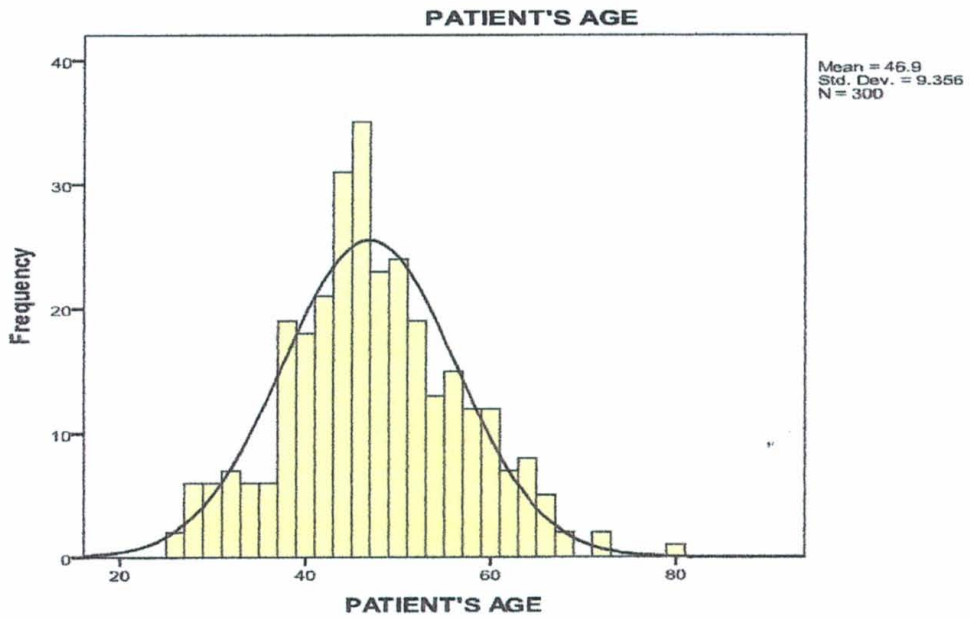


TABLE 1

Distribution of the Study Population by Gender, Age Categories & by Provinces

	Provinces					Age Categories			
GENDER	**NLSJ**	**NBM**	**NSH**	**NBSJ**	**TOTAL**	**25 – 39 y.o**	**40 – 59 y.o.**	**60 & > y.o.**	**P-Value**
MALE	**34 (12.4%)* (77.3%)****	**85 (30.9%) (94.4%)**	**142 (51.6%) (94.7%)**	**14 (5.1%) (87.5%)**	**275 (100%)* (91.7%)**	**50 (80.6%)*****	**200 (95.7%)**	**25 (86.2%)**	**< 0.001**
FEMALE	**10 (40.0%)* (22.7%)****	**5 (20.0%) (5.6%)**	**8 (32.0%) (5.3%)**	**2 (8.0%) (12.5%)**	**25 (100%)* (8.3%)**	**12 (19.4%)*****	**9 (4.3%)**	**4 (13.8%)**	
TOTAL	**44 (100%)****	**90 (100%)**	**150 (100%)**	**16 (100%)**	**300 (100%)**	**62 (100%)*****	**209 (100%)**	**29 (100%)**	

* **Percentage of Total Study Population Genders.**

** **Percentage of Genders in each Province**

*** **Percentage of Genders in each Age Category.**

None of the study participants had been vaccinated against HPV infection. Of the 300 participants, 283 (94.3%) were born in Canada; 6 (2.0%) were from the United States of America (USA); 3 (1.0%) – from the United Kingdom (UK); 1 (0.3%) from New Zealand; 1 (0.3%) from Germany and 6 (2.0%) patients were from African countries. Of the 300 patients, 288 (96.0%) were white Caucasians; 3 (1.0%) were black Africans and 3 patients had aboriginal background; the remaining 6 (2%) patients were each from other ethnic backgrounds. About half of the study population (49%) had a high level of education: 110/300 (36.7%) patients had college/university education and 37/300 (12.3%) had graduate degrees. The remaining 51% of the study population had an education level from elementary school to high school diploma.

According to smoking status, the study population was distributed as follow: 132 (44.0%) patients smoked daily or occasionally; 83 (27.7%) used to smoke but quit; and 85 (28.3%) never smoked prior to the study.

Of the 300 patients, 245 (81.7%) never had an anal Pap test; 33 (11.0%) patients had it at some point of their life but their results were known neither to the patients nor to their current physicians; and 22 (7.3%) patients were not sure. On the question whether they ever had sex, 292 (97.3%) participants answered "yes"; 7 (2.3%) answered "no"; and 1 (0.3%) answered "don't know". The declared number of male sexual partners during the last year varied from "none" (116 (38.7%)) to 50 (1 (0.3%)). The majority of the participants had one male partner during the year (100 (33.3%)). The declared number of female sexual partners varied from "none" (266 (88.7%)) to 10 (1 (0.3%)). The majority of the study participants (245 (81.7%)) had anal sex with condoms during the last year. The number of the patients who had oral sex with condoms (or did not practice it at all) and who did not use condoms for oral sex was almost equal (146 (48.7%) vs. 154 (51.3%), respectively).

All 25 female participants had been screened previously for cervical cancer within different time intervals from a last test. Of the 25 females, 7 (28%) were in each of the following categories: "less than 6 months ago" and "from 1 year to less than 3 years ago". Four (16%) females had the test within the last year; three (12%) women had it "from 3 years to less than 5 years"; one (4%) woman was last screened "5 and more years ago"; and one of them did not remember. Of the 25 women, 18 (72%) used or were still using oral contraceptives (OC). Four females (16%) had a history of precancerous lesions and cervical cancer prior to the study (there was a lack of information about the types of lesions). Of these, two females had a hysterectomy and the specimens for their Pap test were collected from their vaginal vaults

for potential vaginal lesions. The patients' self-reported history of sexually transmitted infections (STIs) was also studied.

Sexually transmitted infection is an infection that can be transferred from one person to another through sexual contact. In this context, sexual contact is more than just sexual intercourse (vaginal and anal) and also includes kissing, oral-genital contact, and the use of sexual "toys," such as vibrators (http://www.medterms.com/).

Of 300 HIV-positive participants, 107 (35.7%) never had STIs other than HIV; 94 (31.3%) patients had a history of one STI; 46 (15.3%) had a history of two STIs; 23 (7.7%) mentioned three STIs; 16 (5.3%) had a history of four STIs; 2 (0.7%) had five STIs, and one patients had six of them; 12 (4.0%) patients were not aware of their history of sexually transmitted infections. The most common STI was ano-genital warts: 106 (35.3%) patients currently have or had previously had them. According to the data from the patient's questionnaire: 32 (10.7%) had Hepatitis B and 31 (10.3%) – Hepatitis C. The history of other STIs among the patients was distributed as follow: 28 (9.3%) had Chlamydia; 70 (23.3%) – Gonorrhea; 32 (10.7%) – Syphilis; and 46 (15.3%) patients had a history of Genital Herpes.

A few questions were related to the patient's awareness about HPV infection and its causative relationship with genital warts and/or cervical cancer. Of 300 patients, 232 (74%) indicated they knew nothing about it at all; and 41 (13.7%) patients were sure that HPV does not cause warts and/or cervical cancer. Of all participants, 147 (49%) had never heard about the HPV vaccine, and 19 (6.3%) participants checked "false" when responding to the statement "HPV vaccine can lower risk for warts/cancer". Finally, 245 (81.7%) patients believed that the Pap test is still extremely/very important for women vaccinated against HPV infection; and 263 (87.7%) patients believed in the importance of the practicing safe sex for those who have received the HPV vaccine.

4.2 Study Outcomes

Among the 300 participants, a total 46 HPV genotypes were detected. Of them, 18 (39%) were high-risk (HR) types and 28 (61%) low-risk (LR) types. The total number of detected HPV infections was 801. Of 300 patients, 232 (77.3%) were "positive" for HPV infection and of these, 125 (54%) were infected with ≥3 HPV types: 98 (78.4%) of these patients had from 3 to 6 HPV genotypes; 24 (19.2%) from 7 to 10 HPV types; and 3 (2.4%) from 11 to 14 HPV genotypes. The highest number of HPV genotypes in one case was 14 (**Table 2 & Chart 1**).

TABLE 2

Distribution of HPV Genotypes in the Study Population

HPV Type	1 HPV	2 HPV	3-6 HPV	7-10 HPV	11-14 HPV	Total
Low-Risk (LR) HPV	41	52	173	92	17	375*** (46.8%)
Probably High-Risk (PHR) HPV	5	6	36	14	3	64*** (8.0%)
High-Risk (HR) HPV	12	40	208	86	16	362*** (45.2%)
Total # of Infections	58	98	417	192	36	801**** (100%)
Total # of Cases	58* (25.0%)	49* (21.1%)	98* (42.2%)	24* (10.3%)	3* (1.4%)	232** (100%)

* **Number of Cases of the detected HPV Types.**

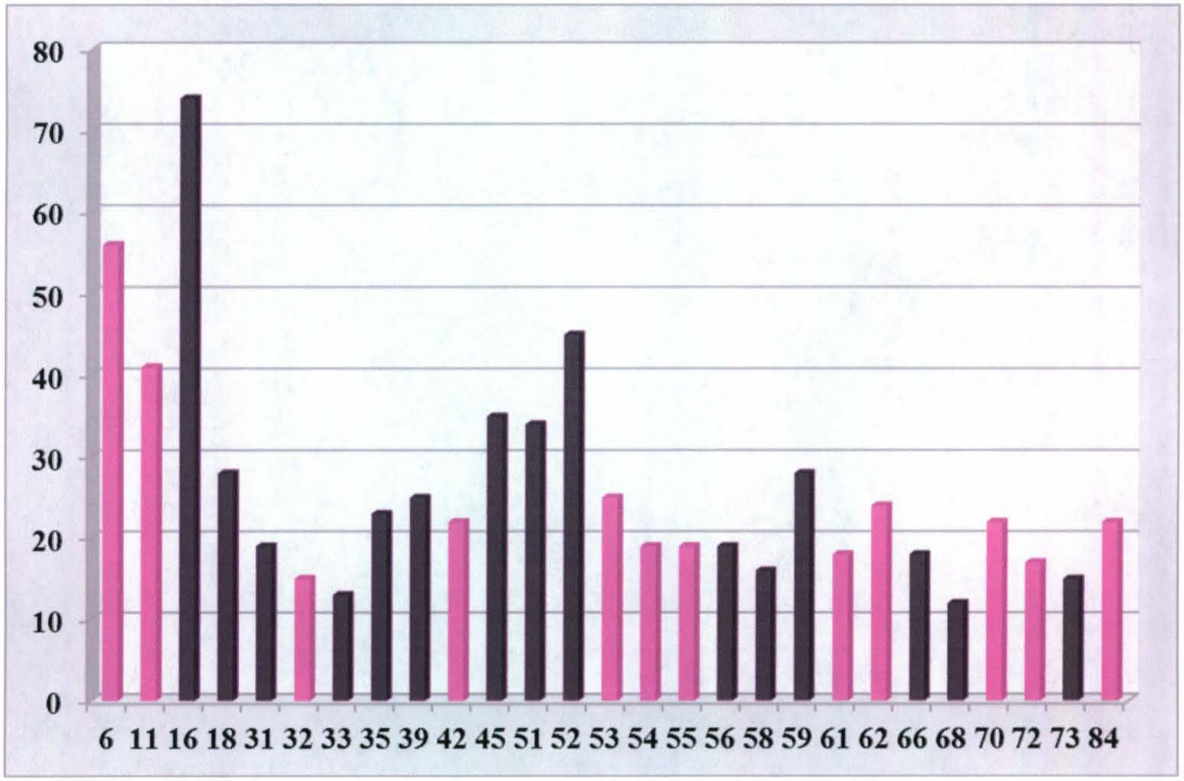
** **Total Number of HPV-Positive Cases.**

*** **Number of HPV Infections (LR, PHR & HR).**

**** **Total Number of the detected HPV Infections.**

Chart 1

Distribution of HPV Genotypes in the Study Cohort



Y axis – number of cases with particular HPV type; **X axis** - detected HPV types.

LR HPV genotypes are light-colored

HR HPV genotypes are dark-colored

In this study we analyzed only HR HPV genotypes and the most frequently detected HR HPV types were: HPV16 11.8% (74/625); HPV52 7.2% (45/625); HPV45 5.6% (35/625); HPV51 5.4% (34/625) & HPV18/HPV59 4.55% each (28/625). The distribution of the HR HPV genotypes by both gender and age categories is showed in the **Table3.**

TABLE 3

Distribution of High-Risk HPV Genotypes

By Gender & Age Categories

HPV TYPE	TOTAL # of CASES	GENDER		Fisher's Exact Test	AGE CATEGORIES			Pearson χ^2
		Male	Female	P-Value	25 - 39 y.o.	40 - 59 y.o.	60 & > y.o.	P-Value
HPV16	74	69 (25.1%)	5 (20.1%)	0.386	16 (25.8%)	52 (24.9%)	6 (20.7%)	0.863
HPV18	28	25 (9.1%)	3 (12.0%)	0.716	10 (16.1%)	17 (8.1%)	1 (3.4%)	0.085
HPV31	19	17 (6.2%)	2 (8.0%)	0.665	4 (6.5%)	15 (7.2%)	0 (0.0%)	0.331
HPV33	13	13 (4.7%)	0 (0.0%)	0.611	5 (8.1%)	7 (3.3%)	1 (3.4%)	0.269
HPV35	23	20 (7.3%)	3 (12.0%)	0.422	4 (6.5%)	17 (8.1%)	2 (6.9%)	0.897
NPV39	25	24 (8.7%)	1 (4.0%)	0.707	7 (11.3%)	16 (7.7%)	2 (6.9%)	0.633
HPV45	35	33 (12.0%)	2 (8.0%)	0.751	8 (12.9%)	24 (11.5%)	3 (10.3%)	0.929
HPV51	34	31 (11.3%)	3 (12.0%)	1	12 (19.4%)	22 (10.5%)	0 (0.0%)	0.02
HPV52	45	39 (14.2%)	6 (24.0%)	0.236	8 (12.9%)	32 (15.3%)	5 (17.2%)	0.842
HPV53	25	23 (8.4%)	2 (8.0%)	1	5 (8.1%)	17 (8.1%)	3 (10.3%)	0.918
HPV56	19	17 (6.2%)	2 (8.0%)	0.665	4 (6.5%)	14 (6.7%)	1 (3.4%)	0.796
HPV58	16	14 (5.1%)	2 (8.0%)	0.632	2 (3.2%)	11 (5.3%)	3 (10.3%)	0.37
HPV59	28	27 (9.8%)	1 (4.0%)	0.489	8 (12.9%)	19 (9.1%)	1 (3.4%)	0.344
HPV62	24	23 (8.4%)	1 (4.0%)	0.705	9 (14.5%)	9 (4.3%)	6 (20.7%)	0.001

Significant differences were detected only in association with HPV51 and the first age category: 12 (19.4%) from 62 patients had this genotype (p=0.020); and between HPV62 and the third age category: 6 (20.7%) from 29 patients had this genotype (p=0.001). The analysis of the association of the HR HPV genotypes with the Anal Cytopathology demonstrated significance (Pearson χ^2) in the prevalence of HPV16 (p<0.001), HPV45 (p=0.044), HPV51 (p=0.014), HPV52 (p<0.001), HPV53 (p=0.045), and HPV59 (p=0.006). The Logistic Regression analysis showed that the following genotypes were less likely to be associated with anal precancerous lesions: HPV59 (**OR=0.363**; p=0.044; 95%CI 0.135-0.974); HPV52 (**OR=0.112**; p<0.001; 95%CI 0.042-0.313), HPV53 (**OR=0.206**; p=0.004; 95%CI 0.070-0.611); while **HPV35** genotype was strongly associated with the Anal LSIL cytologic changes (**OR=10.180**; p=0.011; 95%CI 1.705-60.795). There was no significant difference in the association of HPV genotypes with the Anal HSIL cytologic changes due to the small sample size (**Table 4**).

TABLE 4

Association of High-Risk HPV Genotypes with Anal Cytology

HPV TYPE (PREVALENCE)	ASC-US	ASC-H	LSIL	HSIL	NILM	UNSAT	Pearson χ^2 P-Value
HPV16 (74)	15 (40.5%)	1 (33.3%)	16 (45.7%)	3 (100%)	32 (18.3%)	7 (15.25)	< 0.001
HPV18 (28)	6 (16.2%)	0 (0.0%)	7 (20.0%)	0 (0.0%)	12 (6.9%)	3 (6.5%)	0.163
HPV31 (19)	5 (13.5%)	1 (33.3%)	2 (5.7%)	0 (0.0%)	8 (4.6%)	3 (6.5%)	0.23
HPV33 (13)	3 (8.1%)	0 (0.0%)	1 (2.9%)	1 (33.3%)	7 (4.0%)	1 (2.2%)	0.218
HPV35 (23)	6 (16.2%)	1 (33.3%)	2 (5.7%)	0 (0.0%)	13 (7.4%)	1 (2.2%)	0.168
HPV39 (25)	3 (8.1%)	0 (0.0%)	6 (17.1%)	1 (33.3%)	13 (7.4%)	2 (4.3%)	0.275
HPV45 (35)	8 (21.6%)	0 (0.0%)	7 (20.0%)	0 (0.0%)	20 (11.4%)	0 (0.0%)	0.044
HPV51 (34)	7 (18.9%)	0 (0.0%)	5 (14.3%)	1 (33.3%)	19 (10.9%)	1 (2.2%)	0.014
HPV52 (45)	9 (24.3%)	1 (33.3%)	14 (40.0%)	0 (0.0%)	17 (9.7%)	4 (8.7%)	< 0.001
HPV53 (25)	1 (2.7%)	0 (0.0%)	7 (20.0%)	1 (33.3%)	15 (8.6%)	1 (2.2%)	0.045
HPV56 (19)	4 (10.8%)	0 (0.0%)	5 (14.3%)	0 (0.0%)	9 (5.1%)	1 (2.2%)	0.301
HPV58 (16)	4 (10.8%)	1 (33.3%)	3 (8.6%)	0 (0.0%)	6 (3.4%)	2 (4.3%)	0.165
HPV59 (28)	8 (21.6%)	1 (33.3%)	6 (17.1%)	1 (33.3%)	11 (6.3%)	1 (2.2%)	0.006

NILM–Negative for Intraepithelial lesion
ASC-US/ASC-H–Atypical Squamous Cells Undetermined Significance/Cannot Exclude High-grade lesions
LSIL–Low-grade Squamous Intraepithelial lesion
HSIL–High-grade Squamous Intraepithelial lesion
UNSAT–Unsatisfactory for evaluation specimen

A significant association of the HR HPV genotypes with the Oropharyngeal Cytopathology (ASC-US) was detected (Pearson χ^2) with HPV51 (p=0.042). The analysis of the association of the HR HPV genotypes with the Cervical Cytopathology (LSIL/HSIL) demonstrated significance with HPV35 (p=0.029). The Logistic Regression analysis did not show significance for the same associations.

The comparative analysis of the Sensitivity of the two PCR Assays (Luminex®-Based Genotyping Assay vs. Linear Array Assay (LA)) was presented by column charts and displayed below. The study results confirmed the assays characteristics provided by the WHO HPV LabNet Database. The Luminex®-Based Assay generally detected more cases in the presence of 1 or 2 HPV genotypes compared to LA (46.3% (139) vs. 28.7% (86), respectively), while LA detected comparatively more HPV genotypes in the presence of multiple infections (35% vs. 23%) among the anal samples. (**Tables 5, 6 & 7 with Column-Charts 2 & 3; 4 & 5; and 6 & 7**)

A total of 625 cytology reports were collected in the screening year from 300 A (anal), 300 O (oropharyngeal), & 25 C (cervical) specimens. The cytology analysis of the specimens showed that abnormalities were mostly detected among the anal specimens (78 (26%)). All abnormal reports have been categorized by the specimen type and detected changes (percentage for totals within the total specimens' number (625); percentage in a particular specimen type within this specimen), one cervical L-HSIL case was classified as HSIL:

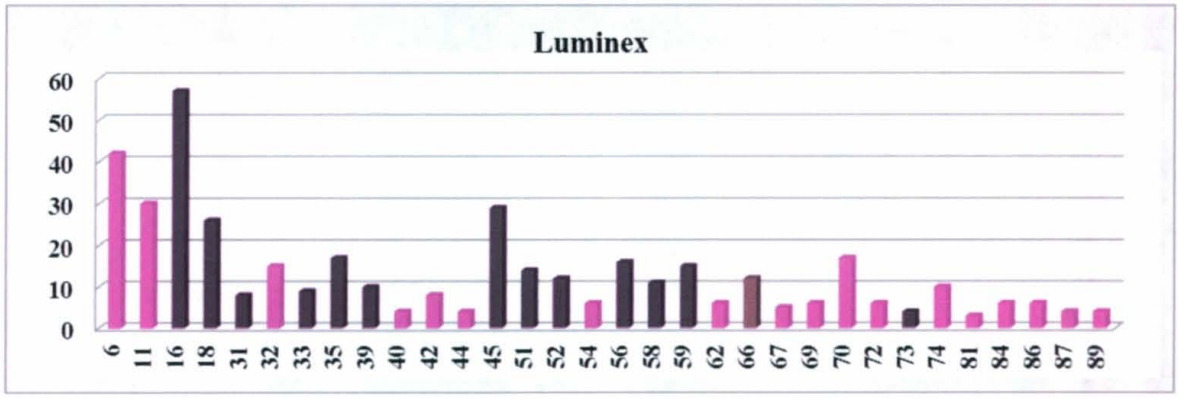
- ASC-US: total number was 39 (6.2%): A-37 (12.3%); O-1 (0.3%); C-1 (4.0%)
- ASC-H: total number was 3 (0.5%) and all 3 (1.0%) were among anal specimens
- LSIL: total number was 36 (5.8%), from them: A-35 (11.6%) and C-1 (4.0%)
- HSIL: total number was 4 from 625 (0.6%), from them A-3 (1.0%) and C- 1 (4.0%)

TABLE 5

Distribution of the HPV Genotypes in Anal Specimens

Number of Cases	*Luminex*	*Linear Array*
Total	*300*	*300*
Positive for HPV infection	*208 (69.3%)*	*211(70.3%)*
Negative for HPV infection	*92*	*89*
1 HPV type	*79*	*53*
2 HPV types	*60*	*43*
3 & > HPV types	*69 (23%)*	*104(35%)*
Max Detected HPV Types in 1 Case	*8*	*12*
Inconclusive	*0*	*11*

Charts 2 & 3: ***HPV Genotypes detected in Anal Samples by Luminex vs. Linear Array***



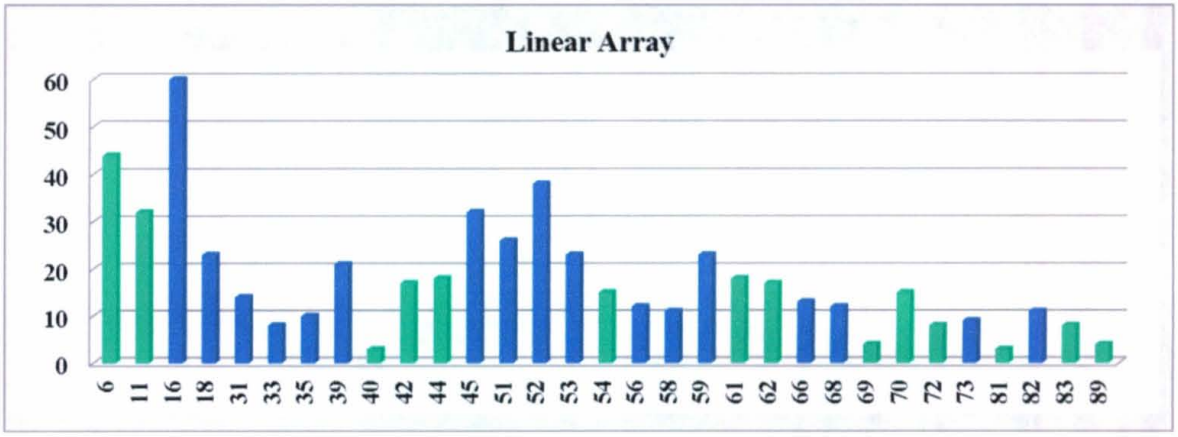


TABLE 6

Distribution of the HPV Genotypes in Oropharyngeal Specimens

Number of Cases	*Luminex*	*Linear Array*
Total	*300*	*300*
Positive for HPV infection	*21*	*12*
Negative for HPV infection	*279*	*277*
1 HPV type	*17*	*8*
2 HPV types	*4*	*4*
3 & > HPV types	*0*	*0*
Max Detected HPV Types in 1 Case	*2*	*2*
Inconclusive	*0*	*11*

Charts 4 & 5: ***HPV Genotypes detected in Oral Samples by Luminex vs. Linear Array***

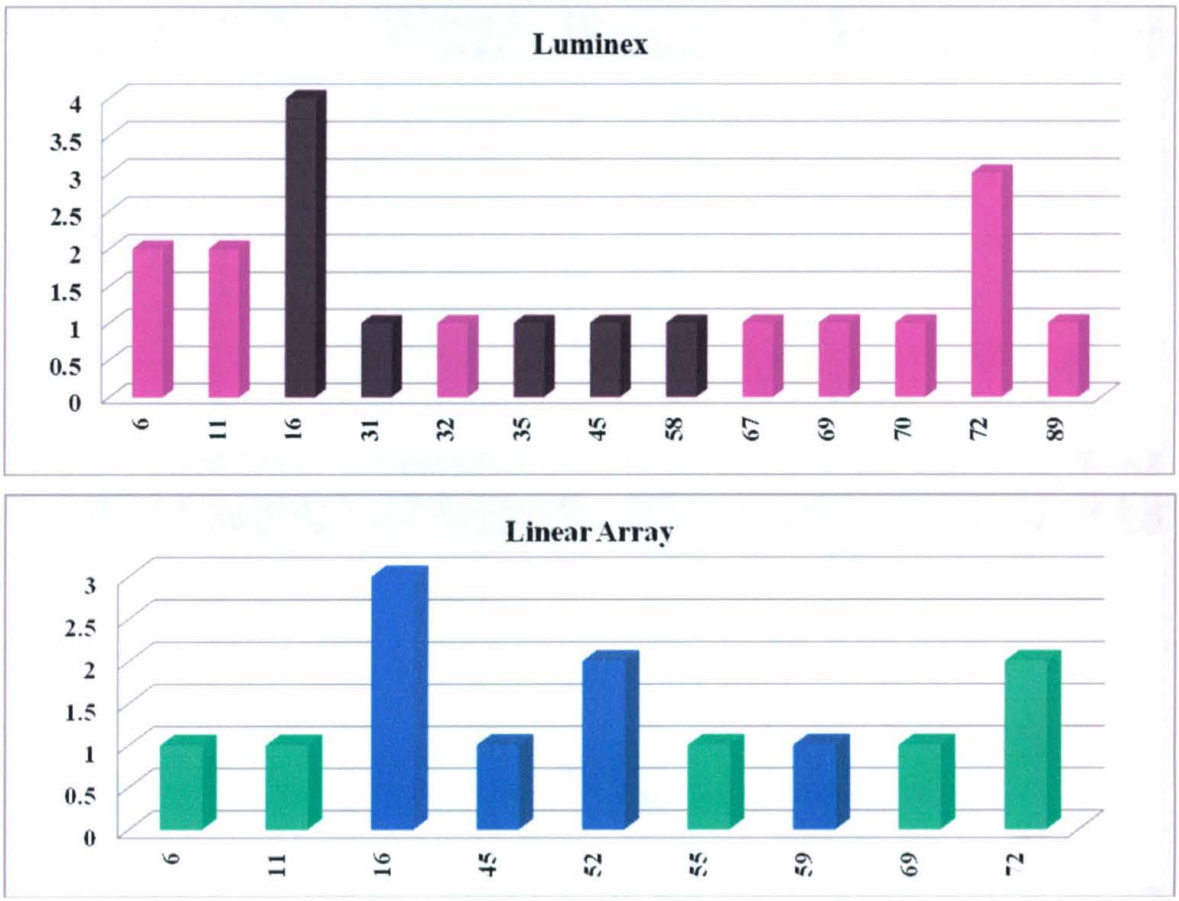
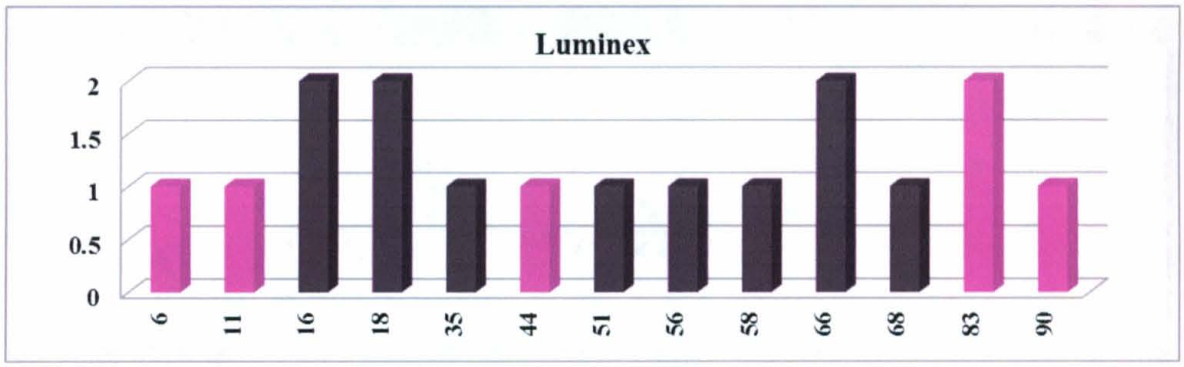


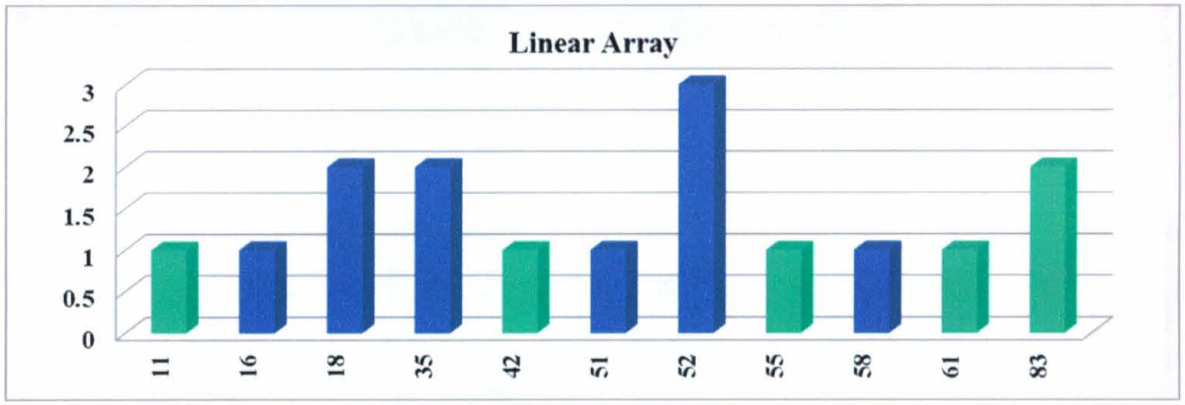
TABLE 7

Distribution of the HPV Genotypes in Cervical Specimens

Number of Cases	*Luminex*	*Linear Array*
Total	*25*	*25*
Positive for HPV infection	*14*	*8*
Negative for HPV infection	*11*	*16*
1 HPV type	*10*	*3*
2 HPV types	*4*	*4*
3 & > HPV types	*0*	*1*
Max Detected HPV Types in 1 Case	*2*	*5*
Inconclusive	*0*	*1*

Charts 6 & 7: ***HPV Genotypes detected in Cervical Samples by Luminex vs. Linear Array***





From 625 specimens, 497 (79.5%) were negative (NILM) for intraepithelial lesions, of these: A-176 (35.4%); O-299 (60.2%); & C-22 (4.4%). Seventy three (24.3%) anal specimens were primarily unsatisfactory for the cytology evaluation. During the year, 27 of them have been recollected with the following final reports: NILM – 18, ASC-US – 4, LSIL – 3, and 2 of them were repeatedly reported as unsatisfactory for evaluation. The remaining 46 unsatisfactory (15.3%) anal samples were not evaluated in the screening year (**Table 8 & Chart 8**).

TABLE 8

Cytology Results in the Study Cohort

	Type of Specimen			
Cytology Result	**ANAL**	**ORAL**	**CERVICAL**	**TOTAL**
ASC-US	**37 (12.3%)***	**1 (0.3%)***	**1 (4.0%)***	**39 (6.2%)****
ASC-H	**3 (1.0%)**	**-**	**-**	**3 (0.5%)**
LSIL	**35 (11.7%)**	**-**	**1 (4.0%)**	**36 (5.8%)**
HSIL	**3 (1.0%)**	**-**	**1 (4.0%)**	**4 (0.6%)**
NILM	**176 (58.6%)**	**299 (99.7%)**	**22 (88.0%)**	**497 (79.5%)**
UNSAT	**46 (15.3%)**	**-**	**-**	**46 (7.4%)**
TOTAL	**300 (100%)***	**300 (100%)**	**25 (100%)**	**625 (100%)****

*** Percentage of the total number of each Specimen**

**** Percentage of the total number of the Cytology Reports**

Chart 8

Cytology Results by Specimen Type

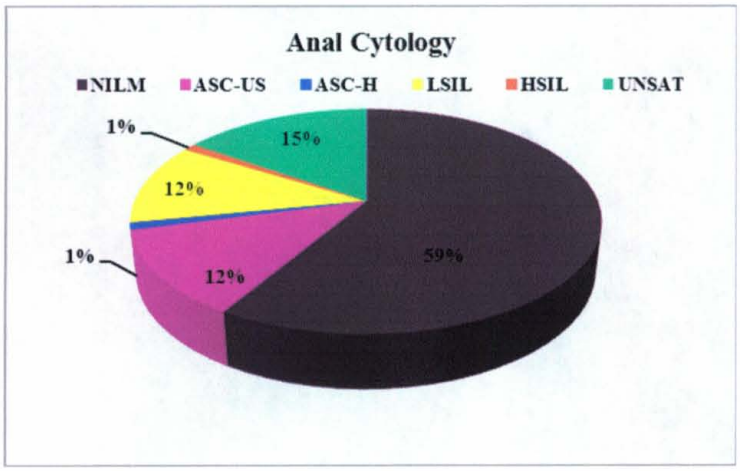


Chart 8A

Chart 8B

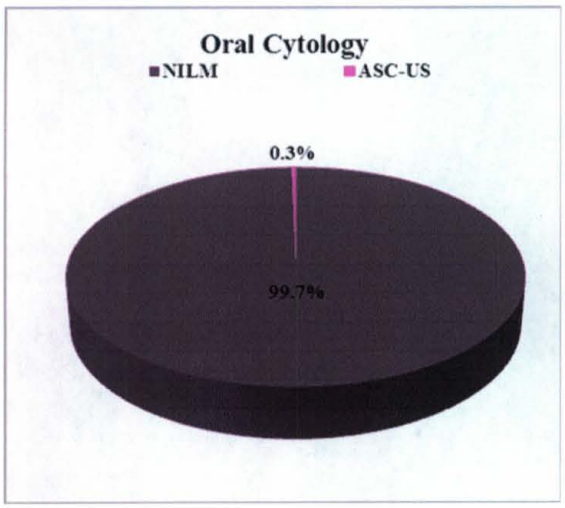
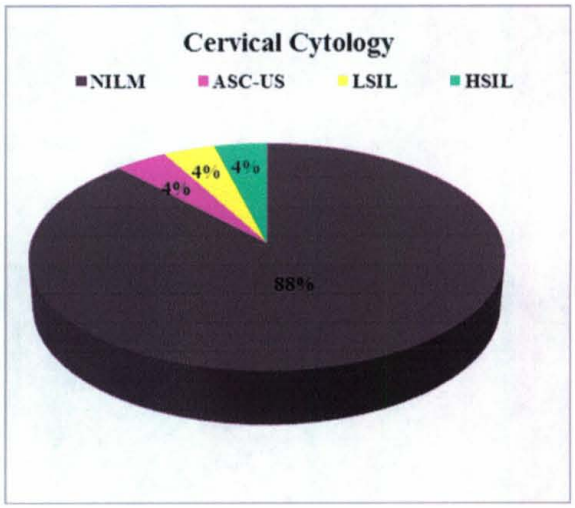


Chart 8C



The analysis of the association between cytology reports and age categories showed no significance except for cervical ASC-US changes in the age category from 25 to 39 years with p=0.010 **(Table 9).**

TABLE 9

Cytology Results (Number & Percentage) within Patients' Age Categories

Cytology Result	ANAL			P	ORAL			P	CERVICAL			P
	25 - 39	40 - 59	60 & >		25 - 39	40 - 59	60 & >		25 - 39	40 - 59	60 & >	
ASC-US	7 (11.3%)	27 (12.9%)	3 (10.3%)	0.930	1 (1.6%)	0 (0.0%)	0 (0.0%)	0.489	1 (8.3%)	0 (0.0%)	0 (0.0%)	0.010
ASC-H	0 (0.0%)	3 (1.4%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	
LSIL	5 (8.1%)	27 (12.9%)	3 (10.3%)		0 (0.0%)	0 (0.0%)	0 (0.0%)		0 (0.0%)	1 (12.5%)	0 (0.0%)	
HSIL	0 (0.0%)	3 (1.4%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	0 (0.0%)		1 (8.3%)	0 (0.0%)	0 (0.0%)	
NILM	41 (66.1%)	118 (56.5%)	17 (58.6%)		61 (98.4%)	209 (100%)	29 (100%)		10 (83.4%)	7 (87.5%)	5 (100%)	
UNSAT	9 (14.5%)	31 (14.8%)	6 (20.7%)		-	-	-		-	-	-	
TOTAL	62 (100%)	209 (100%)	29 (100%)		62 (100%)	209 (100%)	29 (100%)		12 (100%)	8 (100%)	5 (100%)	

Among 300 oropharyngeal samples only one (female patient) was read as ASC-US, all others were negative (NILM) for intraepithelial lesions.

TABLE 10

Cytology Results (Number & Percentage) within Patients' Gender

CYTOLOGY RESULT	ANAL		P	ORAL		P	CERVICAL	P
	MALE	FEMALE		MALE	FEMALE			
ASC-US	35 (12.7%)	2 (8.0%)	0.482	0 (0.0%)	1 (4.0%)	0.010	1 (4.0%)	< 0.001
ASC-H	3 (1.1%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		0 (0.0%)	
LSIL	35 (12.7%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		1 (4.0%)	
HSIL	3 (1.1%)	0 (0.0%)		0 (0.0%)	0 (0.0%)		1 (4.0%)	
NILM	158 (57.5%)	18 (72.0%)		275 (100%)	24 (96.0%)		22 (88.0%)	
UNSAT	41 (14.9%)	5 (20.0%)		-	-		-	
TOTAL	275 (100%)	25 (100%)		275 (100%)	25 (100%)		25 (100%)	

The association of the anal cytologic abnormalities with the underlying risk factors (smoking, number of sexual partners during the last year, practicing in unprotected sex) was not significant; while the association of those with a history of sexually transmitted infections was almost-significant for ano-genital warts compared to other STIs (p=0.054) (**Table 11).**

TABLE 11

Association of Anal Cytology Results with Underlying Risk Factors

Table 11A: *Smoking Status*

Anal Cytologic Abnormalities	Smoking Daily	Smoking Occasionally	Used to Smoke but Quit	Not Smoking	P-Value
ASC-US	19 (51.4%)	2 (5.4%)	6 (16.2%)	10 (27.0%)	0.277
ASC-H	1 (33.3%)	1 (33.3%)	1 (33.3%)	0 (0.0%)	
LSIL	18 (51.4%)	4 (11.4%)	6 (17.1%)	7 (20.0%)	
HSIL	2 (66.7%)	0 (0.0%)	1 (33.3%)	0 (0.0%)	

Table 11B: *History of Sexually Transmitted Infections (STIs)*

Ano-Genital Warts

Anal Cytologic Abnormalities	Neither had nor have warts	Had previously or have currently	P-Value
ASC-US	16 (43.2%)	21 (56.8%)	0.054
ASC-H	3 (100%)	0 (0.0%)	
LSIL	17 (48.6%)	18 (51.4%)	
HSIL	2 (66.7%)	1 (33.3%)	

The association between cytologic abnormalities and a history of unprotected sex was also evaluated. A trend toward a significant difference was detected within Anal specimens: 25 (67.7%) of ASC-US changes were related to patients who practiced safe anal sex (p=0.055), and 33 (89.2%) of all ASC-US changes were detected in patients who had safe vaginal sex or did not practice this kind of sex at all (p<0.001). The majority of NILM results associated with condom use in any kind of sexual intercourse (**Table 12**).

TABLE 12

Association of Cytology Results with the Patients' History of Unprotected (Anal, Oral & Vaginal) Sex

		ANAL			ORAL			VAGINAL		
Type of Specimen	**TYPE of REPORT**	**NO CONDOM**	**CONDOM**	**P**	**NO CONDOM**	**CONDOM**	**P**	**NO CONDOM**	**CONDOM**	**P**
Anal Cytology	ASC-US	12 (32.3%)	25 (67.7%)	0.055	19 (51.4%)	18 (48.6%)	0.548	3 (8.1%)	33 (89.2%)	0.766
	ASC-H	-	3 (100%)		-	3 (100%)		-	3 (100%)	
	LSIL	7 (20.0%)	28 (80.0%)		18 (51.4%)	17 (48.6%)		-	35 (100%)	
	HSIL	0 (0.0%)	3 (100%)		1 (33.3%)	2 (66.7%)		-	3 (100%)	
	NILM	28(16.1%)	146(83.9%)		11(6.3%)	164(93.7%)		88(50.3%)	87(49.7%)	
Oropharyngeal Cytology	ASC-US	1 (100%)	-	0.181	-	1 (100%)	0.512	-	-	0.946
	ASC-H	-	-		-	-		-	-	
	LSIL	-	-		-	-		-	-	
	HSIL	-	-		-	-		-	-	
	NILM	53(17.8%)	244(82.2%)		16(5.4%)	283(94.6%)		146(49.0%)	152(51.0%)	
Cervical Cytology	ASC-US	1 (100%)	-	0.164	-	1 (100%)	0.171	-	-	< 0.001
	ASC-H	-	-		-	-		-	-	
	LSIL	-	1 (100%)		-	1 (100%)		-	1 (100%)	
	HSIL	-	1 (100%)		-	1 (100%)		-	1 (100%)	
	NILM	52(19.0%)	222(81.0%)		140(50.9%)	135(49.1%)		8(2.9%)	267(97.1%)	

Number of both male and female sexual partners during the last year was not significantly associated with cytologic changes. **Table 13A** displays the correlation for the number of male partners. Finally, for males no significant differences were detected in the association of cytologic abnormalities either with patient's CD4 cells count (p=0.192) (**Table 13B**) or with their plasma viral load levels (Anal: p=0.493; Oral: p=0.998; & Cervical: p=0.747).

TABLE 13

Association of Anal Cytology Results with:

Table 13A: ***The Number of Male Partners during the last year***

# of Male Partners	ASC-US	ASC-H	LSIL	HSIL	NILM	P-VALUE
None	13 (35.1%)	1 (33.3%)	13 (37.1%)	1 (33.3%)	61 (34.9%)	0.734
1	14 (37.8%)	1 (33.3%)	12 (34.3%)	1 (33.3%)	60 (34.3%)	
2	1 (2.7%)	-	3 (8.6%)	-	15 (8.6%)	
3 – 5	5 (13.5%)	1 (33.3%)	5 (14.3%)	-	23 (13.1%)	
6 – 10	3 (8.1%)	-	2 (5.7%)	1 (33.3%)	7 (4.0%)	
> 10	1 (2.7%)	-	0 (0.0%)	-	9 (5.1%)	
TOTAL	37 (100%)	3 (100%)	35 (100%)	3 (100%)	175 (100%)	

Table 13B: ***CD4 cell count***

CD4 cells count Categories	ASC-US	ASC-H	LSIL	HSIL	NILM	P-VALUE
CD < 200 cells/ml	11 (29.7%)	1 (33.3%)	14 (40.0%)	0 (0.0%)	41 (23.4%)	0.192
CD ≥ 200 cells/ml	26 (70.3%)	2 (66.7%)	21 (60.0%)	3 (100%)	134 (76.6%)	
Total	37 (100%)	3 (100%)	35 (100%)	3 (100%)	175 (100%)	

The prevalence rate of HPV-positive cases in the study population was 77.3% (232/300), from them the prevalence rate of cases with the high-risk (HR) HPV genotypes was 46.6% (108/232). The overall prevalence rate of cytologic abnormalities caused by HR HPV types in the study population was 27.3% (82/300) during the screening year, from them the prevalence of ASC-US cytologic changes was 47.6% (39/82) and ASC-H was 3.6% (3/82); LSILs - 43.9% (36/82); and HSILs - 4.9% (4/82).

CHAPTER 5

DISCUSSION

5.1 Discussion of the Study Results

In this study we investigated the prevalence of HPV genotypes among HIV-positive adults living in Atlantic Canada. This population is at an increased risk for the development of pre-cancerous and cancerous lesions compared to the general population. Integration of high-risk HPV genotypes into the cellular genome is considered an important event in the pathogenesis of cancer related to the progression from pre-malignant lesions to invasive cancer. It was found that the target population over a wide age-range had a high rate of the cancer precursor lesions as determined by cytologic analysis. Given that the previous study conducted by Palefsky JM in 2000 showed that anal cytology has only 50% sensitivity for diagnosing HSIL compared with the gold standard anal biopsy, the true prevalence of HSILs is likely to be even higher than what is reported here (Panther LA et al, 2004; de Ruiter A et al, 1994). Cytology is well recognized to miss cases of HSIL, but some of the cases of LSIL and ASC/ASC-H could potentially be HSIL on follow up. The overall prevalence of HSILs by cytology for anus, cervix and oropharynx reports was 1.3% (4/300); LSILs – 12% (36/300); and ASCs – 14% (42/300). Most HPV infections are thought to resolve spontaneously; however, we expect that proportions' of our participants will have a persistent HPV infection that may lead to pre-cancerous and cancerous lesions (CSILs), the incidence of which typically peaks 2-3 years after the initiation of sexual activity and then drops substantially among older women (A Global Review, 2008) while continuing to have the same prevalence among older MSM (Burk RD et al, 1996; Herrero et al, 2000; and Szarka K et al, 2009).

In large studies of HIV-positive males, sensitivity of anal the Pap test varied from 46% (95% CI, 36%–56%) to 69% (95% CI, 60%–78%), with specificity ranging from 59% (95% CI, 53%– 65%) to 81% (95% CI, 76%–85%). In the only study of HIV-negative males (EXPLORE), the sensitivity of the anal Pap test ranged from 26% (95% CI, 5%- 47%) to 47% (95% CI, 26%–68%), with specificity ranging from 81% (95% CI, 76%–85%) to 92% (95% CI, 89%–95%) (Walboomers et al, 1999; IARC Monographs, 2004; Mathews WC et al, 2004; Palefsky JM & Holly EA, 1997; and Salit I et al, 2006). Estimates of anal Pap test sensitivity and specificity are highly variable, and depend on the varying prevalence of cytologic abnormalities and differential thresholds for abnormality by both cytology and histopathology (Pantanowitz L & Gezube BJ, 2010; Highleyman L, 2010; Friedlander MA et al, 2004; Panther LA et al, 2004; de Ruiter A et al, 1994). The test is also subject to interpretive variability.

There are very few studies of HPV incidence in Canada, and all have been conducted for women (http://www.phac-aspc.gc.ca/publicat/). HPV prevalence estimates for women in countries around the world range from 2% to 44%, depending on the geographic region, population sampled and testing methodology (Bosch FX, de Sanjose S, 2003). A peak prevalence of HPV infection in women < 25 years of age has been demonstrated consistently, with a decreasing prevalence with age thereafter (Baseman JG & Koutsky LA, 2005). In comparison, cervical Pap testing has also been evaluated mainly in settings where there is a high prevalence of the disease, and estimates of sensitivity and specificity were also low and highly variable (Fahey MT et al, 1995; Nanda K et al, 2000). In a systematic review involving cervical Pap testing, sensitivity ranged from 30% to 87% (mean, 47%) and specificity from 86% to 100% with mean 95% (Elam G et al, 2008; Chin-Hong PV et al, 2009);

No direct evidence exists to support the effectiveness of an anal Pap test screening program to reduce anal cancer mortality or morbidity (Goldie SJ et al, 1999). There are, however, strong parallels with cervical pap testing for 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 cancer. Anal cancer rates in high-risk populations exceed those of cervical cancer before the implementation of Pap testing, and screening tests for these populations may be effective in reducing incidence and mortality rates as has been documented with Pap testing.

In the cross-sectional study conducted by Canadas HP et al in 2010, the overall prevalence of HPV infection in their HIV-positive female cohort was 53% (133/251); the most prevalent genotypes were HPV16 (27%), HPV33 (15%), HPV52 (8%) and HPV 58 (8%).

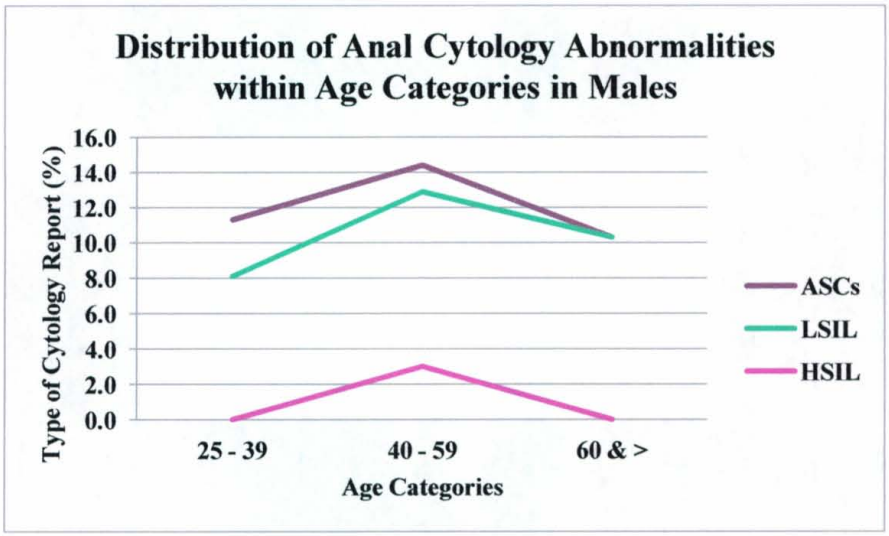
In this study the overall prevalence of HPV infection among our female population was higher compared to the study above: 68% (17/25), while the most prevalent genotype was the same HPV16 – 8% (2/25). The differences may be attributable to the small study sample. All other HR HPV genotypes (18, 35, 51 & 58) were distributed at an equal proportion 1/25 (4% each genotype). The prevalence of abnormal cervical cytology in the Canadas study was 33% (83/251) vs. 12% (3/25) in our study. Several studies have shown that the detection of cervical cytology abnormalities (i.e., LSILs & HSILs) is strongly associated with age (A Global Review, 2008). Using general population-based sampling, Herrero et al in 1997 and 2000 demonstrated that the prevalence of LSILs in the cervix peaked at 5.2% among women younger than 25 years, decreased sharply to 2.7% by ages 25-34 years, and then continued to decrease to 0.3% among women older than 65 years. Herrero R. found that among women less than 25 years of age, oncogenic HPV types predominated, whereas in women older than 55 years, non-oncogenic and uncharacterized types were the most common.

A second peak in HPV prevalence among older women has also been found in some studies (Young TK, McNicol P & Beauvais J, 1997; Koutsky L, 1997), but this has not been seen consistently. In our study the mean (SD) age of the female patients was 43.60 (10.17) years. We detected one cervical LSIL in the second age category from 40 to 59 years (4%), and one cervical LSIL/HSIL in the first age category from 25 to 39 years (4%). These discrepancies can be attributed to a small number of female participants in our study cohort as well as to the fact that four females had been previously diagnosed with cervical pre-cancerous lesions and cancer and treated (two women had a hysterectomy and the Pap smear in the study was collected from their vaginal vaults) prior to their enrollment into the study.

It was hypothesized an increasing proportion of men would develop ASILs in the older age groups because of the absence of the screening for anal cancer and subsequent treatment of disease previously to their enrolment to the study.

The "EXPLORE" study was conducted by Chin-Hong PV et al. in 2004 to investigate the age-associated prevalence of HPV-related anal cytologic abnormalities in MSM. The study reported that there is no age-associated prevalence of ASILs (Anal Squamous Intraepithelial Lesions) in MSM. These facts suggest that many of the lesions that develop after HPV infection are transient and are distributed similarly among all age groups. The overall prevalence of anal abnormal cytology (ASC-US & ASC-H, LSILS, & HSILs) in this study at baseline was 12.3% and 1.0%; 11.7% and 1.0%, respectively and was almost similar for all age categories. The test of trends is demonstrated on Graph # 1.

Graph 1



Further investigation is necessary to make conclusion about the age influence on the incidence and prevalence rates of HPV-related cytologic abnormalities observed in the study.

According to the evidence from the study conducted by Chin-Hong et al. HPV infection by itself might be an independent risk factor for the subsequent development of HIV infection (Chin-Hong PV et al, 2009). Men infected with HPV serve as vectors for the spread of the virus to both men and women (Ferris DG, et al, 2008). Higher HIV infection rates are seen among patients already infected with HPV (Piketty C et al, 2004). This association is believed to occur through two different pathways. One way is that STIs such as HPV disrupt normal mucosal anatomic barriers and may allow HIV-infected body fluids direct access to open and /or bleeding lesions.

Another proposed way is that CD4+ T cells and macrophages are recruited in higher numbers to skin surfaces infected with HPV, allowing closer potential contact between HIV-infected fluids and host CD4+ T cells (Elam G et al, 2008).

Men who have sex with men (MSM) have a high risk of developing oral HPV infection. A 2009 study conducted by D'Souza et al (Chin-Hong et al, 2009), 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 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 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 behaviors, particularly oral sex in young males (Kreuter A & Wieland U, 2009). In our study we detected only one ASC-US cytologic change among all oropharyngeal specimens.

The conducted literature search and review discovered opposite opinions among investigators regarding the impact of anti-HIV treatment on the incidence of HPV-associated anal pre-cancerous lesions and cancer. Cranston RD et al in 2007 found that in contrast to most AIDS-defining cancers, the incidence of HPV-associated anal cancer is increasing since the advent of HAART (Cranston RD et al, 2007). The reasons underlying this trend are still unknown and were not clarified by the investigators, but we can speculate that treatment of HIV-positive men with HAART prolongs their life and subsequently prolongs the exposure of their

anal canals to the high-risk HPV genotypes (Palefsky JM et al, 2001); Bower M et al in 2004 & Palefsky JM et al in 2001 showed that HAART decreased the rate of AIDS-related opportunistic infections and neoplasia, even though anal cancer is not an AIDS-defining malignancy. This evidence was also confirmed by HIPVIRG study results. Most men in our study (mean (SD) age was 47.20 (9.24)) have never been screened for anal abnormalities prior to the study. We compared our baseline data with the Canadian Human Immunodeficiency and Papilloma Virus Research (HIPVIRG, 2011) baseline data except for the two last columns (OR and 95% CI) in the **Table** below: their final results vs. our baseline results (Logistic regression). The sample size, median male age, percentage of smokers in the cohorts, percentage of patients who were taking or about to initiate antiretroviral therapy, as well as their baseline CD4 cell counts are comparable between the cohorts. In our study we had a significantly less number of men with undetectable viral blood level (<than 50 copies/ml) at the enrolment into the study (8.7% vs. 54%).

STUDY	SAMPLE SIZE	MEDIAN AGE	SMOKERS	HAART	CD4 (Median)	VL (undetectable)	OR	95% CI
HIPVIRG (2002 - 2005)	**247**	**43 y.o.**	**38%**	**90%**	**380 cells/mm³**	**54%**	**3.09 (40 - 49 y.o.)** **4.78 (50 & > y.o.)**	**1.12 - 8.52** **1.29 - 17.73**
H2 STUDY (2009 - Ongoing)	**275**	**45 y.o.**	**44%**	**90%**	**363 cells/mm³**	**11.1%**	**0.76 (40 - 49 y.o.)** **1.45 (50 & > y.o.)**	**0.17 - 3.42** **0.41 - 5.10**

The HIPVIRG investigators wanted 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 to AIN2 and 3. On entry to their study, 19% of patients had NILM (vs. 57.1% in our study); 50% had LSIL (vs. 12.7%); AIN2 was present in 17%, and 13% of their males had AIN3 (vs. 1% HSIL in our study). The cumulative incidence of AIN2 and 3 in their HIV-positive male cohort was 23% after two years of observation and 37% after three years of observation.

The follow-up results from our study are required to analyze both the incidence rate of anal dysplasia and its correlation with HAART and other underlying risk factors.

As has been seen in previously conducted studies (Kreuter A & Wieland U, 2009; Palefsky JM et al, 2000), both the presence of HPV infection and the number of HPV genotypes in the sample were important risk factors for pre-cancerous lesions. This study investigated the association between cytologic abnormalities at multiple sites (anal, oropharyngeal, and cervical) and HPV genotypes detected at these sites for both genders separately. From 25 women in the study, 10 had multiple HPV infections (≥3) in anal samples without local cytologic abnormalities. The **Table** below demonstrated HPV genotype distribution in the specimens from four females with detected cytologic abnormalities:

Sample Site	***Anal***	***Oral***	***Cervical***
Cytology Report & HPV Genotypes	**ASC-US 54, 83**	**ASC-US NEGATIVE**	**ASC-US 51, 83**
	UNSAT NEGATIVE	**NILM NEGATIVE**	**LSIL-HSIL 35, 52**
	ASC-US 42, 51	**NILM NEGATIVE**	**NILM NEGATIVE**
	NILM 43, 67	**NILM NEGATIVE**	**LSIL 43**

All 275 men in the study had only anal cytologic abnormalities and of these, 115 (42%) had multiple (3 or more) HPV genotypes. The results demonstrated **(Table 14) a** significant association between multiple HPV types and anal lesions.

TABLE 14

Association of Anal Lesions with the Number of HPV Genotypes in Male Population

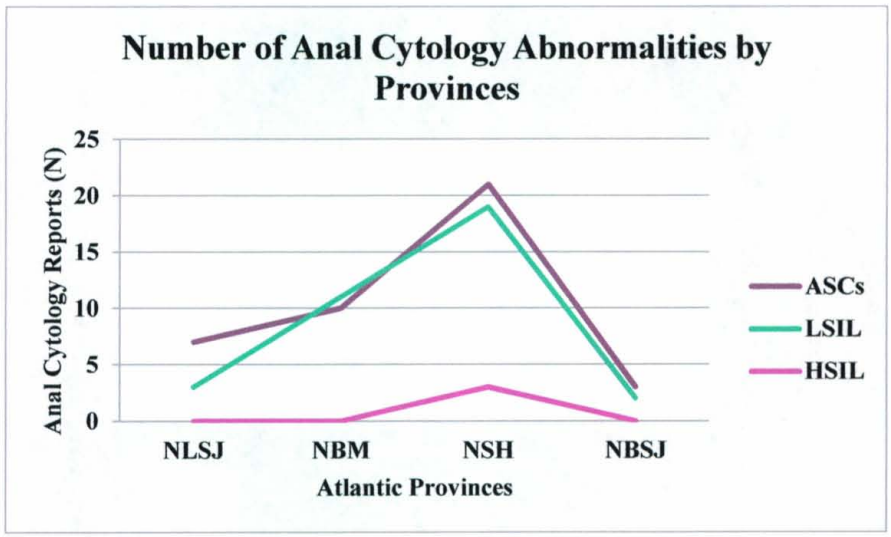
Anal Cytology Results	1 HPV	2 HPV	≥3 HPV	NILM	P
ASCs	5 (12.2%)	5 (12.2%)	28 (68.3%)	3 (7.3%)	0.002
LSIL	0 (0.0%)	8 (22.9%)	26 (74.3%)	1 (2.9%)	< 0.001
HSIL	0 (0.0%)	1 (33.3%)	2 (66.7%)	0 (0.0%)	0.520

From this, it is plausible that infection with multiple HPV genotypes may be a marker of persistent disease and of the progression of LSILs to HSILs (Palefsky JM, et al, 1998).

Previous studies have demonstrated that behavioral determinants were strongly associated with the risk of ASILs (Fairley CK et al, 1994; Burk RD, 1996; and Elam G et al, 2008). Even patients who are knowledgeable about HPV and HIV can and do engage in high-risk sexual behaviors (Chin-Hong PV et al, 2004). Even though most HPV infections are transient, MSM have more sexual partners, more new sexual partners, and therefore more new exposures to HPV infection after age 30 years than most women (Burk RD, 1996; and Elam G et al, 2008).

The underlying behavioral risk factors that were included in this analysis: the number of sex partners (both males and females) (**Table 13A** shows the association related to the number of male partners in a last year), smoking status (**Table 11A**), and history of unprotected sex (**Table 12**). There was no significance observed in the association of these parameters with the cytologic abnormalities. There was no significant difference in the overall prevalence of ASILs by geographic location also, where the highest peak of the prevalence reflects the highest number of participants and consequently the highest number of abnormal cases in Halifax (**Graph 2**).

Graph 2



Finally, the association of cytologic abnormalities with baseline CD4 cell counts and plasma viral load levels was analyzed. Two Canadian researchers Mougin C in 2001 and Leece P in 2010 investigated the correlations between HIV infection laboratory markers (CD4<200 cells/ml and high level of plasma viral load) and incidence of anal dysplasia, and found a strong relationship between these parameters. In this study no significance was observed between low CD4 cell counts (**Table 13B**) and the detected dysplasia in all types of specimen.

All four viral load levels (Never Done; Undetectable; Detectable; Unknown) have been analyzed in their association with cytologic changes among the three types of specimen: Anal with $p = 0.493$; Oral with $p = 0.998$; and Cervical with $p = 0.747$. No significance was detected.

Although no formal programs for anal cancer screening have been implemented in Canada or elsewhere, guidelines on anal dysplasia screening have recently been developed by agencies in the United States, by the United States Public Health Service, Centers for Disease Control and Prevention, and the New York State Department of Health AIDS Institute. In 2004, the United States Public Health Service mentioned anal screening in their guidelines for the prevention of HIV-associated opportunistic infections among HIV-infected MSM (Adelstein DJ et al, 2009). The guideline states "...anal cytological screening of HIV-infected men who have sex with men has not yet become standard of care but is now being done for high-risk persons in some health care centres and may become a useful preventive measure in the near future." They also stated that "...additional studies of screening and treatment programs for anal high-grade SILs need to be carried out."

In March 2007, the New York State Department of Health AIDS Institute (Aberg JA et al, 2004) updated its primary care approach for the HIV-infected patient and released guidelines, which recommended routine anal Pap testing (at baseline and annually) in several high-risk groups. These were MSM, patients with a history of anogenital condylomas, and women with abnormal cervical/vulvar histology. It was further recommended that patients with abnormal anal Pap test findings be referred for high-resolution anoscopy and/or examination with biopsy.

One of the purposes of this study is initiation of an anal cancer screening program for the populations at risk. The evidence from the Canadian HIPVIRG study strongly suggests continuing research in this direction. The investigators believed that patients with a low nadir CD4 cell count might especially benefit from screening for pre-cancerous lesions. In addition "typing could also be useful as an adjunct to cytological examination in primary screening" (de Pokomandy A et al, 2011).

5.2 Analysis of the Study Strengths and Limitations

The Strengths of our study included: the large sample size (N=300), geographic diversity (four Atlantic Provinces), and wide age spectrum of the participants (from 25 to 70 years of age).

There have been a number of Obstacles and Limitations to the study. The Obstacles to overcome included:

- Meeting the requirement of a number of different ethics board

The application for the whole study approval was submitted to the ethics board in Ottawa (HREA). Additionally, each study site (province) submitted the application to their local HREA. The process was a time-consuming and delayed the beginning of the study.

- Inadequate and conflicting literature related to obtaining specimens

Anal Pap smear screening involves the technique of blind insertion of a swab into the anal canal. The swab is inserted into the anus and vigorously rotated to scrape cells from the anal lining. The collection device was then placed in SurePath media. This is different than the majority of studies which have used ThinPrep for evaluation. Using the Liquid-based platform results in better presentation of cellular material as multiple layers of cell material or obscuring factors such as blood or inflammatory cells are removed. Samples taken from the anal canal have the additional disadvantage of potential fecal contamination. During the initiation of the study we received 50 unsatisfactory anal samples for evaluation. A literature search review with the critical appraisal of the sources were conducted and widely discussed with the all research sites. As a result of this process, it was decided to use NYS Guidelines recommendations on anal pap smears for the anal sample collection (NYS Guidelines).

The Guidelines were distributed at all research sites in order to standardize collection in an attempt to reduce the number of unsatisfactory specimens. Furthermore, based on the review of evidence from different studies, it was decided to use Dacron swabs instead of the cotton ones used initially (Lampinen TM et al, 2006; Fox PA, 2006; Piketty C et al, 2003; Chin-Hong et al, 2005). Both these implementations dramatically improved the quality of the collected anal samples and reduced the number of unsatisfactory samples. It was also advised that co-investigators recollect anal specimens from those who provided unsatisfactory samples at the first time. Consequently, only 46 (15.3%) anal specimens remained unsatisfactory for interpretation.

- Relocation of key study personnel

During the first year of the study there was no physician at the St. John's site and research relied only on the help of physician's locum services. This significantly delayed the recruitment process at this site.

- Size of the geographic region
- Varying degrees of buy-in from the study sites
- Small sample size for women (n=25)

Limitations are listed below:

- The major limitation was the study design; prospective cohort is an observational study which is strong for correlations but weak for the investigation of the causative relationship between risk factors and disease outcomes.

- Another possible limitation of the study was the fact that only one pathologist read all of the cytology results. Moreover, any potential under- or overdiagnosis of ASILs would probably affect only the prevalence estimates, not the estimates of associations with the potential risk factors. The study needs to follow all cases out to biopsy to confirm cytologic interpretation.
- Finally, colposcopy and HRA with biopsy as gold standards were applied to all participants with abnormal cytology reports of varying degree but their results were not analyzed at the time of this cross-sectional study.

5.3 Conclusion

In this study, 74% (222) of patients had normal anal cytology, 26% (78) had abnormal anal cytology. Abnormal cytology was reported mostly as ASCs (ASC-US (12.3%) and ASC-H (1.0%)); LSILs (11.7%), with fewer samples showing HSILs (1.0%). That 26% of abnormal anal specimens showed us the real burden of HPV-associated diseases and the need of screening program implication for the population at the higher risk. According to the studies conducted by Cranston ED et al in 2004 and in 2007, more than half of low-grade dysplasia in the cytological evaluation had high-grade dysplasia on the biopsy. Anal cytology has a high sensitivity (95%) for detection of dysplasia (ASIL) but a low specificity (50%) for predicting the severity of the abnormality on subsequent biopsy. Even patients with cytologic diagnoses of ASC-US and LSIL have significant risk (46% to 56%) of being diagnosed with HSIL (AIN 2, 3) at biopsy (Lytwyn A et al, 2005). These numbers suggest that all HIV-positive patients should undergo HRA annually during their regular visit. All participants with the detected anal and cervical ASCs, LSILs and HSILs were referred to the specialists to undergo a high-resolution anoscopy (HRA).

Previous studies have demonstrated that HPV type (i.e., high-risk versus low-risk) is associated with the type of lesions that develop later (Atkins D et al, 2004; Bosch FX et al, 2002).

In this study, 77.3% of the participants were HPV-positive, of these 42% had more than 3 HPV types. A total of 46 HPV genotypes were detected with HPV 16, 18, 45, 51 and 52 most common at anus; HPV 16 and 52 at oropharynx; and HPV 16, 18, 35 and 52 most common at cervix. The different distribution of HPV types at different anatomic locations may suggest the further development of HPV vaccine against 35, 45, 51 and 52 genotypes. In this study, it was also observed that infection with both high-risk and low-risk HPV types was more strongly associated with LSILs or HSILs rather than infection with either high-risk or low-risk HPV types only. This is consistent with the study observation that an increasing number of HPV types are an important risk factor for ASILs. Given that earlier studies reported that only high-risk HPV types are closely linked with the development of invasive cervical and anal lesions, it was concluded that the participants have been at risk for invasive lesions and will need to be followed appropriately.

A cross-sectional study was conducted to examine the temporal association between type-specific HPV infection and progression of ASILs to invasive lesions and clarifies the association between HPV infection with specific HPV types and the risk of HSILs. The comparative analysis of cytologic reports with histologic outcomes is required to draw conclusion about the real prevalence of cytologic changes in participants. The analysis of the follow-up years will also allow us to conduct a comparative analysis in order to determine Incidence Rate (IR), Prevalence Rate (PR) and Relative Risk (RR) in acquiring pre-cancerous and cancerous lesions in HIV-positive people in Atlantic Canada.

Table 15

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)

Table 16

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.

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: *Patient's 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 Clinical 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: *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 F: *Specimen Collection Guidelines*

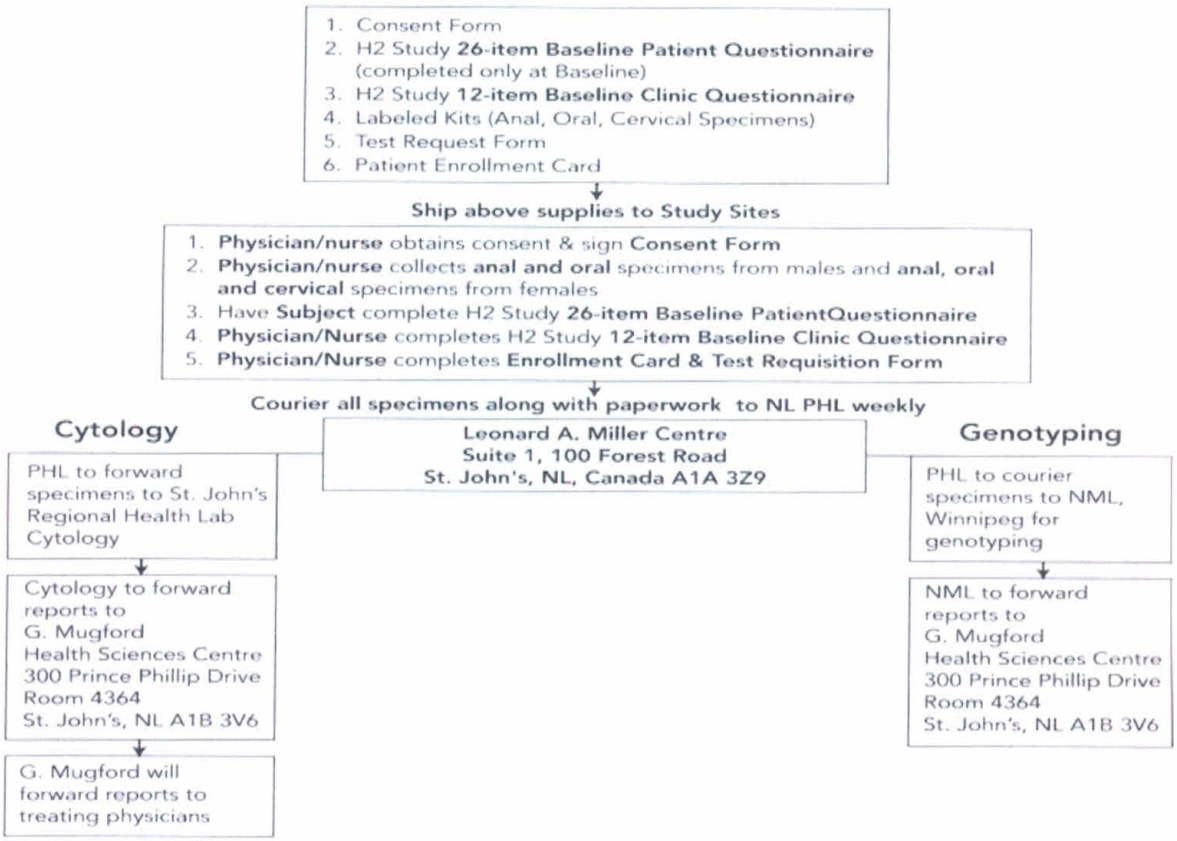
Technique: (NYS Guidelines recommendations on anal pap smears, NYS DOH AIDS Institute's HIV quality-related website - http://hivguidelines.org/Content.aspx).

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 G: *Flow Chart (screening year)*



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