A Tale of Two Viruses:

HPV infection and associated anal dysplasia among Hawaii HIV-seropositive patients and related HIV seroconversion risk in a Thai population

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Eleanore Chuang

Dissertation Committee:

Bruce Shiramizu, Chairperson
Dominic Chow
Brenda Hernandez
F. DeWolfe Miller
Randal Wada

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ABSTRACT

Human immunodeficiency virus (HIV) and human papillomavirus (HPV) are both sexually transmitted pathogens. As the most prevalent sexually transmitted infection in the world, HPV affects both men and women across many demographic categories. Although HPV often clears without treatment, persistent infection can cause dysplasia which can progress to cancer. HPV vaccines, licensed in the US since 2006, hold promise for reducing not only infection rates but also incidence of HPV-associated cancers. Albeit less prevalent than HPV, HIV still represents a considerable health burden worldwide even in an era of effective combination anti-retroviral therapy (cART). In the US, the majority of new HIV cases arise among gay and bisexual men. Despite billions of dollars and years devoted to research, an effective HIV vaccine remains elusive.

Since HIV-positive individuals are more susceptible to other infections even while on anti-retroviral therapy, they are also prone to HPV infection and associated anal dysplasia. Additionally, a growing body of evidence suggests that HPV also impacts the acquisition of HIV. The axes of interactions between these two viral infections are not yet fully understood.

The overall objective of this study was to further elucidate the relationship between HIV and HPV infection in anal dysplasia/cancer by assessing HPV infection in the context of HIV and vice versa. The central hypotheses were that HIV-positive individuals present more frequently with HPV infection and associated anal dysplasia and that HIV seroconversion may occur subsequent to HPV infection.

First, the study demonstrated that HPV at non-anal sites may be associated with anal dysplasia/cancer among HIV-positive males. The presence of HPV and the number of HPV

genotypes at anatomical sites other than the anus trended toward higher odds of ASIL. Presence of HPV and the number of HPV genotypes at the penile shaft conferred the highest, statistically significant odds of ASIL – greater odds than conferred by presence of HPV and the number of HPV genotypes at the anus itself.

Second, the study showed the potential of HPV quantitation for enhanced screening and diagnosis of anal dysplasia/cancer in the context of HIV infection. All participants with HGAIN had HPV-16 E6 DNA levels above 10 copies per cell. Participants with LGAIN or Negative biopsy results and low HPV-16 E6 copy could potentially have avoided invasive HRA if HPV quantitation were used as a supplemental diagnostic marker for anal dysplasia.

Third, the study explored the effect of prior HPV infection on acquisition of HIV. Among MSM and TG women in the Thai Test & Treat cohort, HPV acquisition appeared to increase the risk of subsequent HIV seroconversion. Despite remaining ambiguity over HPV's role in HIV acquisition, some researchers are advocating for study of HPV vaccination as a means for reducing HIV incidence.

This study has contributed to the body of knowledge in the field by identifying new diagnostic indicators of HPV-associated anal dysplasia and by providing additional evidence for HPV's influence on HIV acquisition. Further investigation will permit validation of these novel diagnostic markers and innovative strategy for reducing the global incidence of HIV.

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ABBREVIATIONS

95% CI	95% confidence interval
AIDS	acquired immunodeficiency syndrome
ART	anti-retroviral therapy
ASC-H	atypical squamous cells, cannot exclude HSIL
ASCUS	atypical squamous cells of undetermined significance
ASIL	anal squamous intraepithelial lesions
cART	combination anti-retroviral therapy
CLIA	Clinical Laboratory Improvement Amendments
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
HGAIN	high-grade anal intraepithelial neoplasia
HHIVL	Hawaii HIV Laboratory
HIV	human immunodeficiency virus
HPV	human papillomavirus
hr	high-risk
HR	high-risk
HRA	high-resolution anoscopy
HSIL	high-grade squamous intraepithelial lesions
HSPGs	heparan sulfate proteoglycans
IRB	Institutional Review Board
LCI	lower 95% confidence interval
LGAIN	low-grade anal intraepithelial neoplasia

LSIL	low-grade squamous intraepithelial lesions
lr	low-risk
mRNA	messenger ribonucleic acid
MSM	men who have sex with men
NHANES	National Health and Nutrition Examination Survey
NIH	National Institutes of Health
NIMHD	National Institute on Minority Health and Health Disparities
OR	odds ratio
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
RCMI	Research Centers in Minority Institutions
RMATRIX	RCMI Multidisciplinary and Translational Research Infrastructure Expansion
RNA	ribonucleic acid
SD	standard deviation
T1	timepoint1
T2	timepoint2
TG	transgender
TRCARC	Thai Red Cross AIDS Research Centre
UCI	upper 95% confidence interval
UH	University of Hawaii
VCT	voluntary counseling and testing

CHAPTER 1

INTRODUCTION

PROJECT SUMMARY

Human immunodeficiency virus (HIV) and human papillomavirus (HPV) are both sexually transmitted pathogens. As the most prevalent sexually transmitted infection in the world, HPV affects both men and women across many demographic categories. Although HPV often clears without treatment, persistent infection can cause dysplasia which can progress to cancer. HPV vaccines, licensed in the US since 2006, hold promise for reducing not only infection rates but also incidence of HPV-associated cancers. Albeit less prevalent than HPV, HIV still represents a considerable health burden worldwide even in an era of effective combination anti-retroviral therapy (cART). In the US, the majority of new HIV cases arise among gay and bisexual men. Despite billions of dollars and years devoted to research, an effective HIV vaccine remains elusive.

Since HIV-positive individuals are more susceptible to other infections even while on anti-retroviral therapy, they are also prone to HPV infection and associated anal dysplasia. Additionally, a growing body of evidence suggests that HPV also impacts the acquisition of HIV. The axes of interactions between these two viral infections are not yet fully understood.

The overall objective of this study was to further elucidate the relationship between HIV and HPV infection in anal dysplasia/cancer by assessing HPV infection in the context of HIV and vice versa. The central hypotheses were that HIV-positive individuals present more frequently with HPV infection and associated anal dysplasia and that HIV seroconversion may occur subsequent to HPV infection.

To better understand the interaction between these two viruses, specimens from multiple anatomical sites of HIV patients were tested for HPV and association with anal dysplasia.

Furthermore, HPV-16 E6 DNA in anal cytology specimens of HIV patients was quantitated to analyze correspondence to anal cytology or anal biopsy grade. Finally, HPV infection was assessed as a precursor to HIV seroconversion. Results from this study may provide additional insight for diagnosis of HPV-associated anal dysplasia among HIV-positive patients, and clarification of HPV's role in HIV acquisition may suggest another avenue for reducing HIV transmission in high-risk populations.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus (HIV) is a member of the *Retroviridae* virus family. Its structure includes a spherical envelope and conical nucelocapsid that is faintly discernible in Figure 1 [1]. As a retrovirus, its genome consists of two positive-sense single-stranded ribonucleic acid (RNA) sequences that must be reverse transcribed into deoxyribonucleic acid (DNA) before viral replication can occur. HIV's hallmark is its ability to infect immune cells by binding to CD4 receptors on T cells and macrophage-lineage cells. By infecting the host's immune cells, it not only causes disease – acquired immunodeficiency syndrome (AIDS) – but also inhibits the immune system's ability to respond to the infection. Primary modes of transmission are through blood or sexual contact although vertical transmission from mother to child is also possible during childbirth and breastfeeding [2].

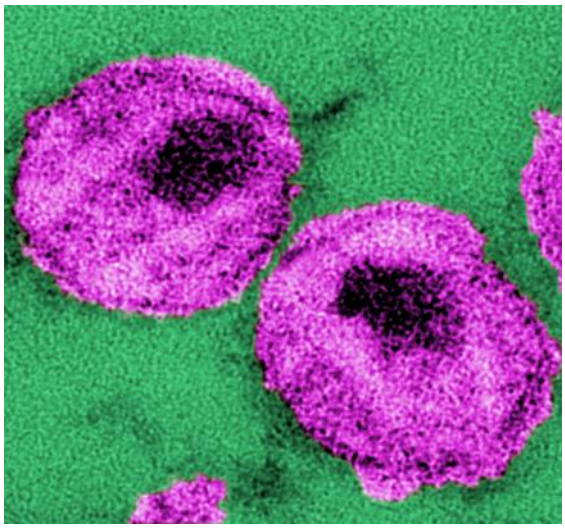

Figure 1. Micrograph of HIV showing spherical envelope and conical nucleocapsid [1]

In 2014, there were an estimated 36.9 million people living with HIV worldwide, 2.0 million new HIV infections, and 1.2 million deaths attibuted to AIDS as shown in Figure 2 [3]. The overwhelming burden of HIV/AIDS lies in sub-Saharan Africa followed by Asia and the Pacific region.

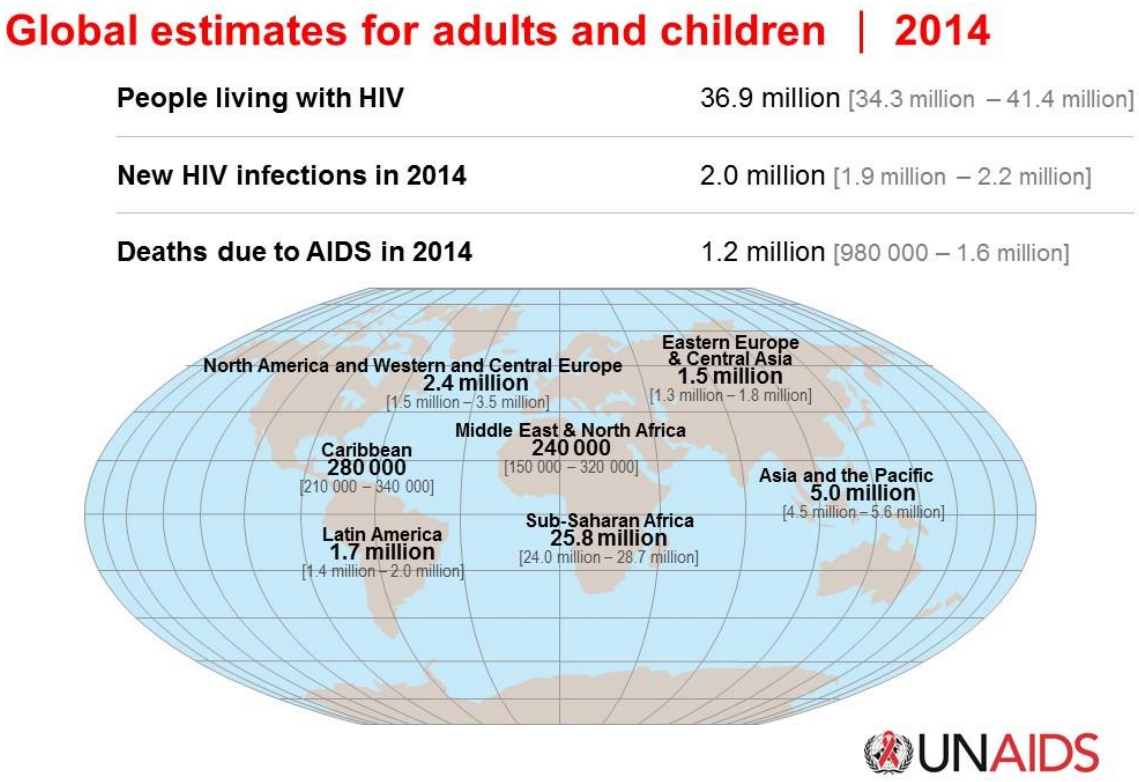

Figure 2. Estimated global burden of HIV in 2014 [3]

In the same year, there were 44,609 new HIV infections reported among adults and adolescents in the United States (US) for a total incidence rate of 16.6 new HIV infections per 100,000 people as shown in Figure 3 [4]. Higher incidence rates (new HIV infections per 100,000 people)

were common among southern US states – Louisiana (36.6), Florida (31.3), Georgia (27.0), Texas (22.1), Mississippi (21.0), and South Carolina (20.7) – and in some densely populated states – Maryland (27.7), New York (22.8), and New Jersey) (20.4) – and cities like Washington, DC (66.9). Relatively high incidence rates also occurred in the territories of the US Virgin Islands (27.4) and Puerto Rico (22.7). In Hawaii, HIV incidence actually fell below the national average at 9.2 new HIV infections per 100,000 people.

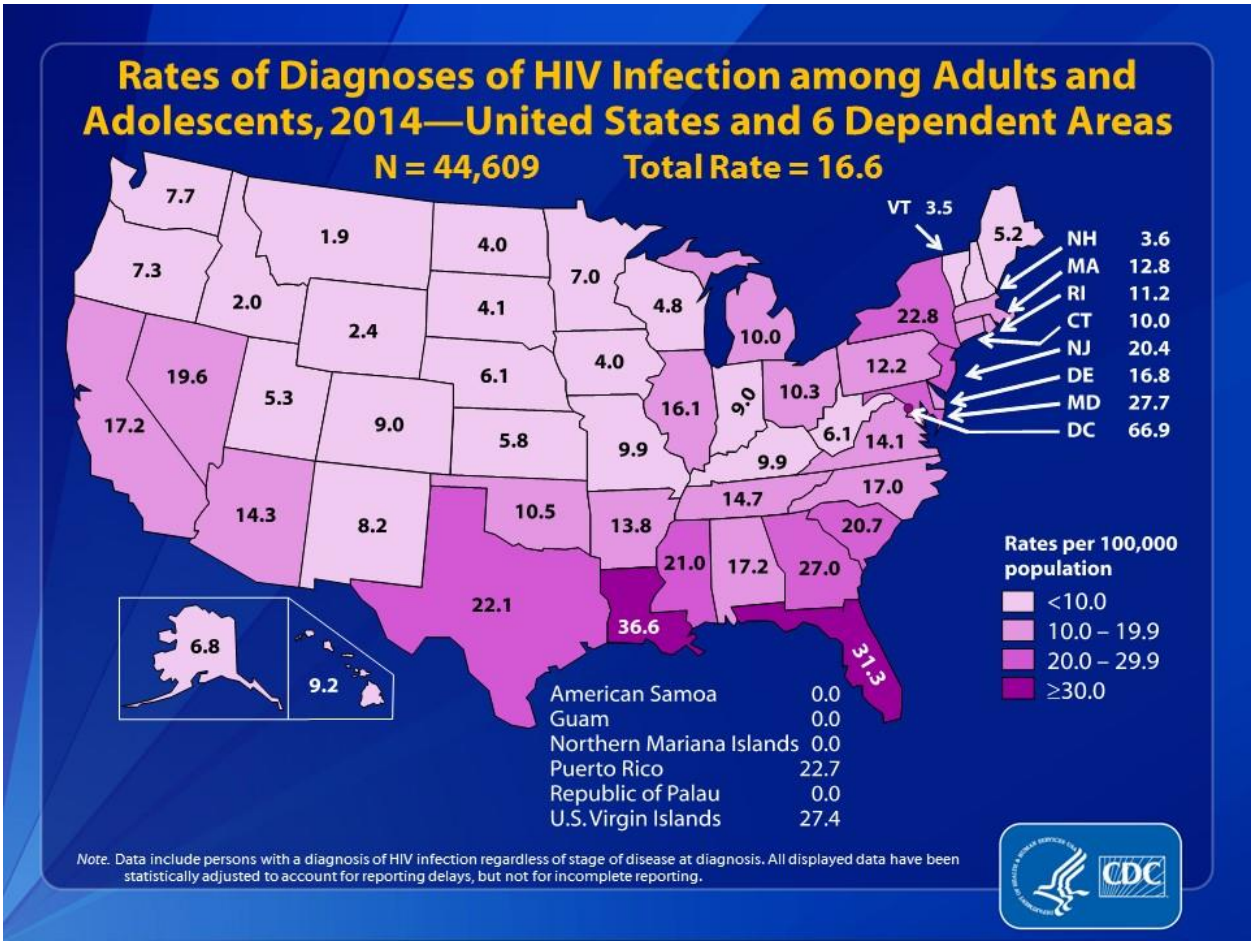

Figure 3. US HIV Incidence in 2014 [4]

As previously mentioned, HIV primarily infects CD4 T cells that are responsible for mounting an adaptive immune response to the pathogen. Illustrated in Figure 4 [5], the acute phase of HIV infection is marked by a steep increase in viral load with a corresponding precipitous drop in CD4 T cell counts. The infection can then enter a lengthy chronic phase of clinical latency when the viral load reaches a setpoint and CD4 counts stabilize. However, when HIV viral loads resurge and cause CD4 counts to plummet below 200 cells/mm^3, then the infection has reached the stage of full-blown AIDS. It is at this point that opportunisitic infections and other complications more commonly lead to death attributable to HIV infection and AIDS.

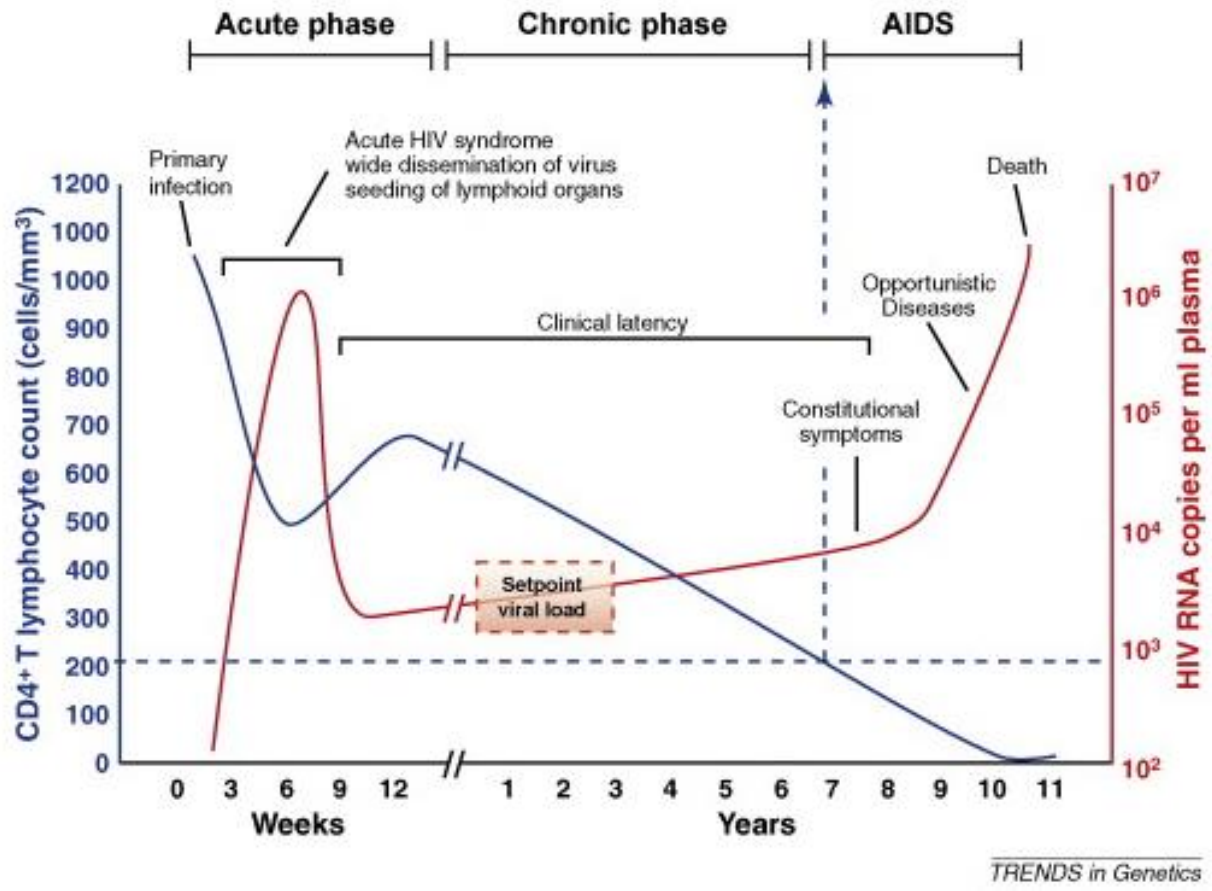

Figure 4. HIV Time Course without Treatment [5]

With the advent of combination anti-retroviral therapy (cART), viral replication can be suppressed, CD4 T cell counts may rebound, and HIV-positive individuals can live to near normal life expectancies. However, the immune system never quite returns to the same level of function as before HIV infection. Even while on effective cART, HIV patients are susceptible to secondary infections and complications, including human papillomavirus (HPV) infection and associated dysplasia/cancer [6, 7].

HUMAN PAPILLOMAVIRUS (HPV)

Human papillomavirus (HPV) is a member of the *Papillomaviridae* virus family. Its structure includes an icosahedral nucelocapsid but no envelope as illustrated in Figure 5 [8]. Its genome consists of an approximately 8,000 base-pair sequence of double-stranded deoxyribonucleic acid (dsDNA). The virus is purported to bind to heparan sulfate proteoglycans (HSPGs) to infect basal epithelial cells. Primary modes of transmission are through sex or direct skin contact although vertical transmission from mother to child can result in respiratory papillomatosis [9].

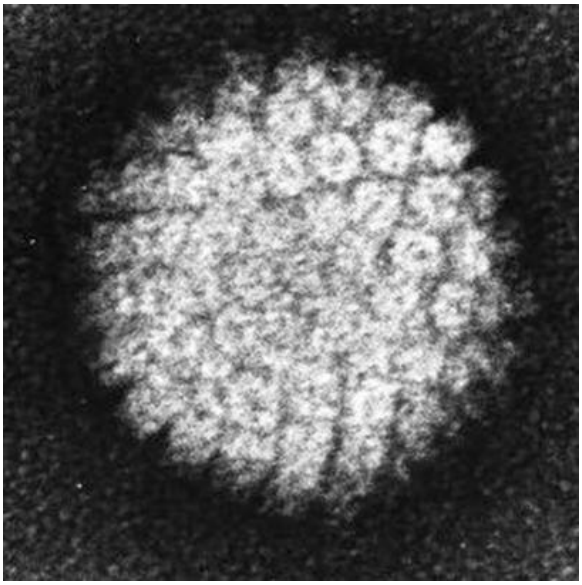

Figure 5. Micrograph of HPV showing icosahedral nucelocapsid [8]

HPV is the most prevalent sexually transmitted infection in the world. The most recent update from the National Health and Nutrition Examination Survey (NHANES) found that more than 40% of US adults aged 18 to 59 years are infected with genital HPV [10]. Chesson et al.

estimated that greater than 80% of men and women in the US would acquire HPV by the age of 45 in the absence of HPV vaccination [11].

HPV infection causes a variety of cancers -- an estimated 30,700 cases per year among men and women in the US, including 4,600 anal cancers [12]. Among HIV-positive individuals, incidence of anal HPV and anal cancer is particularly high, even while on effective cART [6, 7]. As depicted in Figure 6, even moderate reduction in CD4 count places HIV-positive men who have sex with men (MSM) at higher risk of anal HPV infection and abnormal anal cytology than HIV-negative MSM [13]. With increasing incidence of anal cancer among HIV patients, annual anal cytology screening is recommended for this high-risk population [14].

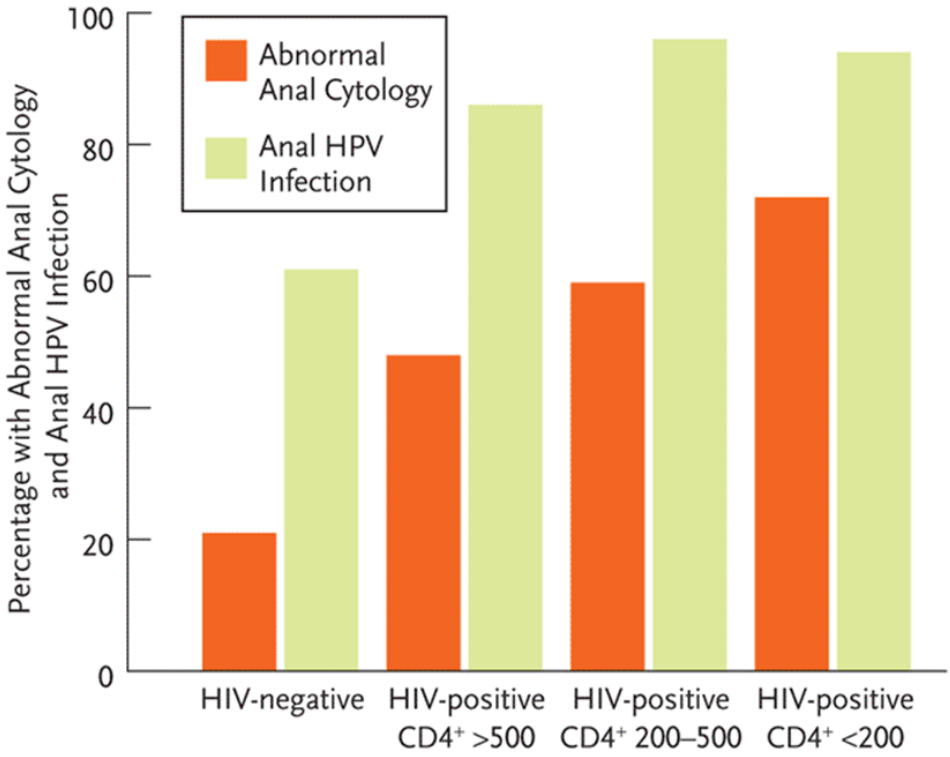

Figure 6. Percentage of HIV-positive and HIV-negative MSM with Anal HPV Infection and Abnormal Anal Cytology [13]

Although more than 200 genotypes of HPV have been discovered [15], only around 40 types are known to infect the anogenital region. Low-risk HPV genotypes 6 and 11 are associated with approximately 90% of anogenital warts. High-risk HPV genotypes 16 and 18 are responsible for about 70% of cervical cancers in the US. Several other HPV genotypes are also considered high-risk for anogenital cancers though generally detected at lower frequencies. The most important risk factor for the development of HPV-associated cancer is persistent infection with one or more oncogenic HPV genotypes.

The progression from HPV infection to dysplasia and cancer can take years or even decades. Molecular mechanisms leading to development of dysplasia and cancer involve integration of HPV's viral DNA with the host cell's own genome. Integration of viral E6 and E7 oncogenes may be accompanied by loss of the viral E2 gene, which typically represses expression of E6 and E7. When E6 oncoprotein binds to p53, it marks the cell cycle checkpoint inhibitor for ubiquitin-mediated degradation, allowing unfettered cellular proliferation. Meanwhile, when E7 oncoprotein binds the retinoblastoma protein pRb, it releases transcription factor E2F to initiate cell cycle activation and cellular proliferation. These molecular pathways are depicted in Figure 8 [16]. With the loss of E2 repression, increased expression of HPV E6 and E7 oncogenes induces the dysregulated cellular proliferation characteristic of cancer.

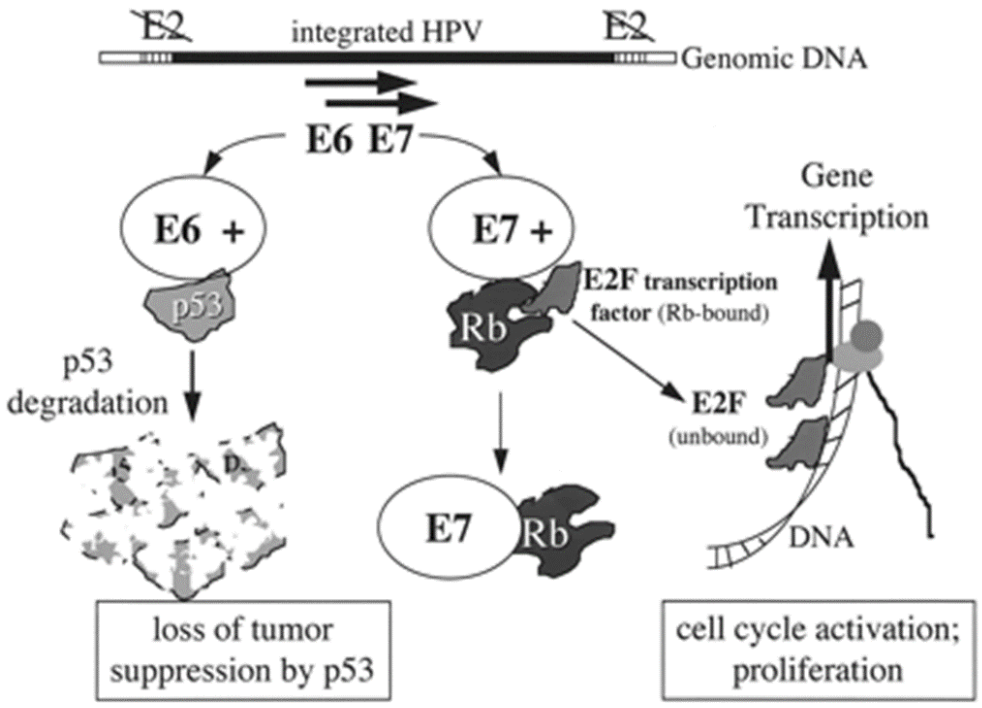

Figure 7. Increased expression of HPV E6 and E7 oncogenes induces dysregulated cellular proliferation characteristic of cancer [15]

Fortunately, effective vaccines targeting the most clinically relevant HPV types became available roughly one decade ago. Cervarix, developed by GlaxoSmithKline, targets high-risk genotypes 16 and 18 to prevent about 70% of HPV-associated cancers. Gardasil, marketed by Merck, targets high-risk genotypes 16 and 18 as well as low-risk genotypes 6 and 11 to prevent approximately 70% of HPV-associated cancers and 90% of anogenital warts. Gardasil 9 is Merck's newest entry in the HPV vaccine market. It targets five additional oncogenic HPV types to prevent approximately 90% of HPV-associated cancers and 90% of anogenital warts. HPV genotypes associated with cervical cancer and available vaccines are shown in Figure 8 [17].

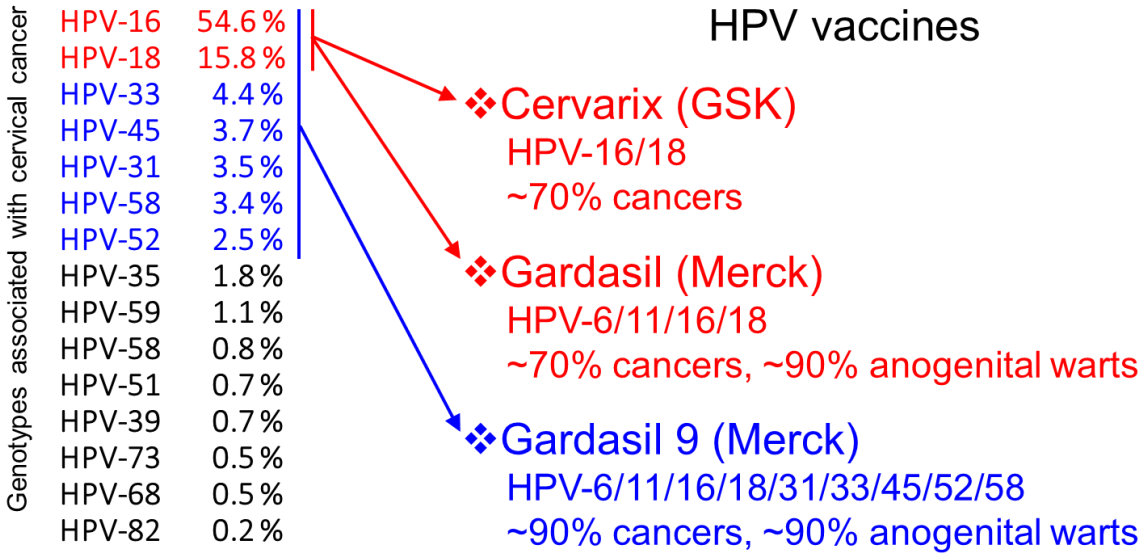

Figure 8. Frequency of HPV genotypes associated with cervical cancer, including highest risk types targeted by available vaccines [16]

Surveillance data from the past decade indicate that HPV infection has decreased in proportion with the population vaccinated against HPV. Statistics from Australia also suggest successful reduction in incidence of anogenital warts as a consequence of HPV vaccination [18]. Due the slow progression from HPV infection to associated dysplasia and cancer, data is not yet available to determine the effect of HPV vaccination on cancer incidence.

SPECIFIC AIMS

HPV is the most prevalent sexually transmitted infection in the world. The most recent update from the National Health and Nutrition Examination Survey (NHANES) found that more than 40% of US adults aged 18 to 59 years are infected with genital HPV [19]. HPV infection causes a variety of cancers -- an estimated 30,700 cases per year among men and women in the US, including 4,600 anal cancers [12]. Among HIV-positive individuals, incidence of anal HPV and anal cancer is particularly high, even while on effective cART [6, 7]. With increasing incidence of anal cancer among HIV patients, annual anal cytology screening is recommended for this high-risk population [14].

While many studies have examined HPV as a consequence of HIV infection, relatively few have scrutinized HPV as a precursor to HIV infection; however, a growing body of literature suggests that HPV does impact the acquisition of HIV [20-24]. With these bi-directional influences in mind, the overall objective of this study was to further elucidate the relationship between HIV and HPV infection in HPV-associated anal dysplasia in the context of HIV and evaluate the effect of HPV infection on subsequent HIV seroconversion. The central hypotheses stated not only that HIV-positive individuals present more frequently with HPV infection and associated anal dysplasia but also that HIV seroconversion may occur subsequent to HPV infection.

To test the central hypotheses, the following specific aims were pursued:

1. ***Determine the association between detectable HPV at various anatomical sites and atypical anal cytology among HIV-positive individuals in Hawaii.***
   The hypothesis was that anal squamous intraepithelial lesions (ASIL) are associated with detectable high-risk HPV genotypes in anal specimens as well as HPV detectable across multiple anogenital sites.

2. ***Differentiate high-grade anal intraepithelial neoplasia (HGAIN) from low-grade anal intraepithelial neoplasia (LGAIN) or negative anal biopsies using HPV type-specific DNA quantitation.***
   The hypothesis was that HGAIN is associated with higher copy numbers of HPV-16 E6 DNA than LGAIN or negative anal biopsies.

3. ***Determine whether an association exists between HIV seroconversion and acquisition or clearance of anal HPV genotypes in a Thai cohort.***
   The hypothesis was that HPV clearance is associated with HIV seroconversion in a high-risk population.

The results from this study demonstrated innovation and positive translational impact by identifying new diagnostic indicators of HPV-associated anal dysplasia and by suggesting a new approach for reducing HIV transmission.

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SPECIFIC AIM 1

Determine the association between detectable HPV at various anatomical sites and atypical anal cytology among HIV-positive individuals in Hawaii.

Hypothesis: Anal squamous intraepithelial lesions (ASIL) are associated with detectable high-risk HPV genotypes in anal specimens as well as HPV detectable across multiple anogenital sites.

Rationale: HIV-positive individuals are at higher risk for anal HPV infection and associated anal dysplasia/cancer. As the most common sexually transmitted infection in the world, HPV may be present at multiple anatomical sites, influencing the development of HPV-associated anal dysplasia/cancer.

CHAPTER 2

HUMAN PAPILLOMAVIRUS AT MULTIPLE SITES ASSOCIATED WITH ANAL SQUAMOUS INTRAEPITHELIAL LESIONS IN HIV-SEROPOSITIVE INDIVIDUALS

Chuang E, Lim E, Milne C, Zhu X, Agsalda M, Killeen J, Miller FD, Hernandez B, and Shiramizu B. Human Papillomavirus at Multiple Sites Associated with Anal Squamous Intraepithelial Lesions in HIV-Seropositive Individuals. *Ann Clin Cytol Pathol*. 2016; 2(4): 1029.

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Human Papillomavirus at Multiple Sites Associated with Anal Squamous Intraepithelial Lesions in HIV-Seropositive Individuals

Eleanore Chuang[1,2], Eunjung Lim[3], Cris Milne[1], Xuemei Zhu[4], Melissa Agsalda[1,2], Jeffrey Killeen[4,5], F. DeWolfe Miller[2], Brenda Y. Hernandez[2,4,5], and Bruce Shiramizu[1,2]*

[1]University of Hawaii, John A. Burns School of Medicine, Hawaii Center for AIDS; [2]University of Hawaii, John A. Burns School of Medicine, Department of Tropical Medicine, Medical Microbiology & Pharmacology; [3]University of Hawaii, John A. Burns School of Medicine, Office of Biostatistics and Quantitative Health Sciences; [4]University of Hawaii Cancer Center; [5]University of Hawaii, John A. Burns School of Medicine, Pathology Department

* Corresponding author: Bruce Shiramizu

University of Hawaii, John A. Burns School of Medicine

Departments of Pediatrics, Medicine, & Tropical Medicine, Hawaii Center for AIDS

651 Ilalo St. BSB 325AA, Honolulu, HI 96813

Phone: 808-692-1677

Fax: 808-692-1984;

E-mail: bshirami@hawaii.edu

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ABSTRACT

Objective. HIV-seropositive patients have higher risk of HPV infection even on anti-retroviral therapy. Infection with high-risk HPV genotypes can cause dysplasia leading to cancer. This study assessed HPV at different anatomical sites in HIV-seropositive individuals and factors associated with anal squamous intraepithelial lesions (ASIL).

Methods. Specimens were obtained from multiple anatomical sites for each participant in conjunction with routine screening for anal dysplasia. Female specimens included cervical and anal cytologies and oral wash. Male specimens included anal cytologies, oral wash, and exfoliated cells from penile head, penile shaft, scrotum, and from uncircumcised subjects, inner foreskin. Demographic and clinical characteristics were recorded. Following DNA extraction, HIV DNA copy was assessed by qPCR; HPV was genotyped. Statistical analyses included calculation of odds ratios (OR) and 95% confidence intervals (CI), t-tests or Mann-Whitney tests.

Results. Males were more likely to have ASIL: 29/50 (58%) compared to 1/11 females (9%) (OR=13.81, 95% CI: 1.64-116.32). HPV 6 or 11 in anal specimens was significantly associated with ASIL (OR= 6.29, 95% CI: 1.49-26.44). Number of HPV genotypes in anal specimens was also significant: ASIL+ (3.4±3.1) versus ASIL- (1.6±3.1) (p=0.009). Among 44 males, HPV was detected from at least one anatomical site for 33 participants (75%): 27 anus (61%), 19 oral wash (44%), 17 penile shaft (39%), 11 scrotum (26%), 10 penile head (23%), 0 foreskin. Detection of HPV in penile shaft specimens was significantly associated with ASIL (OR=6.79, 95% CI: 1.57-29.36) as was number of HPV genotypes in penile shaft specimens: ASIL+ (2.4 ± 4.0) versus ASIL- (0.6 ± 1.7) (p=0.025).

Only 1/11 females had ASIL; only 1/11 females had cervical dysplasia: OR was not estimable due to small numbers.

Conclusions. Males were more prone to ASIL than females. HPV at anal as well as non-anal sites may be indicative of ASIL.

INTRODUCTION

Individuals infected with human immunodeficiency virus type 1 (HIV) are at increased risk for human papillomavirus (HPV) infection and for anal dysplasia/cancer [1-4]. Presence of multiple HPV genotypes as well as presence of HPV at other anatomical sites potentially increase the risk for anal dysplasia/cancer [1, 2, 5, 6]. While high-risk HPV genotypes are found in more than 90% of anal cancers among HIV-infected patients, there may be additional factors that lead to anal dysplasia/cancer [6, 7]. Co-infection with HIV and HPV in the anal canal and presence of HPV at other anatomical sites may support ongoing exposure to HPV and/or HIV-related immune suppression, leading to increased risk for dysplasia/cancer. Recent data suggest that the continued persistence of HIV DNA in circulating monocytes in patients treated with combination anti-retroviral therapy (cART) leads to progression of HIV disease itself and other HIV-associated complications, which may include HPV infection and associated anal neoplasia [8-11]. The objective of this study was to evaluate specimens from various anatomical sites of the same HIV-positive individual for HPV genotypes in relation to anal squamous intraepithelial lesions (ASIL). We hypothesized that ASIL would be associated with presence of HPV at multiple anatomical sites.

MATERIALS AND METHODS

During a 12-month period, men and women, 18-65 years of age, were either self-referred or referred by community physicians for anal dysplasia/cancer screening in collaboration with the Hawaii Center for AIDS, University of Hawaii (UH), and UH Cancer Center. Subjects were included if HIV-positive regardless of previous history of HPV infection, anal dysplasia/cancer, or related treatment. Participants provided written consent in accordance with UH Institutional Review Board policy. Two anal cytology specimens were collected with a Dacron swab [12] and stored in ThinPrep collection medium (Hologic, Inc., Bedford, MA). One anal specimen was processed by a CLIA-certified clinical laboratory with cytopathology reviewed and reported by the same experienced cytopathologist (JK) according to the Bethesda system: anal cytology was evaluated, using criteria and terminology adapted from standardized cervical cytology screening [6, 13-19]. The other anal specimen was assayed for HPV and HIV DNA. Anal cytology specimens were assessed for adequacy and categorized as 1) Negative (ASIL-) if no cellular changes could be detected or if cellular changes were caused by inflammation or reparative process or 2) Positive for anal squamous intraepithelial lesions (ASIL+) if abnormal cytological changes were found, including high-grade squamous intraepithelial lesions (HSIL), low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells of undetermined significance (ASC-US), and atypical squamous cells which cannot exclude HSIL (ASC-H). Oral wash specimens were collected according to methods previously established [19]. From male participants, exfoliated cells from the penile glans/coronal sulcus, penile shaft, and scrotum were collected consecutively from each site and placed in separate collection vials as previously described [19]. Inner foreskin specimens were collected from uncircumcised subjects. Cervical cytology specimens were also obtained from female participants. Other data obtained per informed consent included plasma HIV RNA viral load and nadir CD4 cell count as well as age, gender, and ethnicity. Not all specimens and data were collected from all participants.

DNA from each specimen was extracted using QIAamp DNA Micro Kit (Qiagen Inc, Valencia, CA) and analyzed for presence or absence of HPV DNA by PCR using a modified version of the PGMY09/PGMY11 primer system [20]. β-globin-positive and HPV DNA-positive specimens were genotyped using the Linear Array HPV Genotyping Test to detect 37 different HPV types (Roche Molecular Diagnostics, Pleasanton, CA), including 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68).

Anal specimens had HIV DNA copy number quantified by real-time PCR as previously published [9]. Primers and probes included HIV gag (forward 5'-GAC ATC AAG CAG CCA TGC AA-3'; reverse 5'-CTC ATC TGG CCT GGT GCA AT-3') and β-globin (forward 5'-AGG GCC TCA CCA CCA ACT TC; reverse 5'-TCA CTA GCA ACC TCA AAC AGA CAC C-3') primers; and VIC-labeled HIV gag (5'ACC ATC AAT GAG GAA GCT GCA GAA TGG GA-3') and FAM-labeled β-globin (5'-CTC CTG AGG AGA AGT CTG CCG TTA CTG CC-3') probes. Controls included OM10.1 cells, each carrying a single, integrated HIV provirus; and water. Assays were performed in triplicate. Resulting data were analyzed using StepOne Plus software (Thermo Fisher Scientific, Waltham, MA). Copy numbers of each target gene were calculated based on the standard curve, and HIV DNA copy numbers per 1 x 10^6 cells were determined.

Statistical analyses were conducted by JABSOM Biostatistics & Quantitative Health Sciences. Mean age of participant subsets were compared using t-tests. Odds ratios (OR), 95% confidence intervals (CI), and p-values were calculated to investigate associations between anal cytology results (ASIL+ or ASIL-) and patient characteristics as well as HPV at various anatomical sites. For analyses, specimens were designated high-risk HPV-positive if one or more of 13 high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) was detected. The numbers of HPV genotypes or high-risk HPV genotypes per specimen were

compared according to anal cytology results (ASIL+ or ASIL-) using Mann-Whitney tests. Kappa coefficient and McNemar's test p-value were used to assess co-incident HPV genotypes.

RESULTS

Of 61 participants (50 males, 11 females) enrolled in the study, 30 (49%) presented with ASIL. Data was not available to determine severity of atypical cytology. Mean age with standard deviation was similar for ASIL+ (50.1 ± 8.1) compared to ASIL- (47.4 ± 8.8) participants (p=0.275) as well as males (48.8 ± 9.1) compared to females (49.4 ± 5.2) (p=0.735) (Table 1). Men had significantly higher odds of presenting with ASIL: 29/50 (58%) compared to 1/11 (9%) women (OR=13.81, 95% CI: 1.64-116.32). Race/ethnicity was not a notable factor for ASIL: 13/27 (48%) non-White versus 17/34 (50%) White participants (OR=0.93, 95% CI: 0.38-2.55).

All participants were HIV-seropositive and on combination anti-retroviral therapy (cART), with 41/48 males (85%) and 9/9 females (100%) exhibiting undetectable plasma HIV levels. Data regarding length of HIV infection and cART use was not collected for this study. Participants with detectable viral levels had higher odds of presenting with ASIL though not statistically significant (OR=2.93, 95% CI: 0.52-16.58) (Table 1). Likewise, nadir CD4 ≤200 cells/mm^3 conferred higher odds of ASIL but not statistically significant (OR=2.30, 95% CI: 0.80-6.61). Detectable HIV DNA in anal specimens was not associated with ASIL (OR=0.91, 95% CI: 0.31-2.64).

Table 1. Demographic and Clinical Characteristics of Participants and Association with ASIL

Variable	Anal Cytology, n		OR	95% CI	p-value
	ASIL+	ASIL-			
Age (yrs.), Mean ± SD	50.1 ± 8.1	47.7 ± 8.8			0.275
Gender (n=61)					
Male	29	21	13.81	1.64-116.32	0.016
Female	1	10			
Race/Ethnicity (n=61)					
White	17	17			
Asian	4	5			
Native Hawaiian	3	5			
Native American	3	2			
African-American	2	2			
Hispanic	1	0			
Nadir CD4 (n=58)*					
≤200 cells/mm^3	19	12	2.30	0.80-6.61	0.121
>200 cells/mm^3	11	16			
Plasma HIV Viral Load (n=58)*					
Detectable	5	2	2.93	0.52-16.58	0.223
Not detectable	23	27			
Anal HIV DNA (n=59)*					
Detectable	10	11	0.91	0.31-2.64	0.861
Not detectable	19	19			

ASIL = anal squamous intraepithelial lesion
OR = odds ratio
95% CI = 95% confidence interval
SD = standard deviation
* Data not available for all participants

Among 52 anal specimens collected and assayed, 29 (56%) were positive for any HPV; 20 (38%) were positive for one or more high-risk HPV genotypes (Figure 1). Genotypes detected most frequently in anal specimens included HPV 6 (17%) and 11 (15%), which are associated with genital warts; HPV 16 (19%), 52 (10%), and 68 (13%), which are high-risk types associated with cancer; as well as low-risk types like HPV 53 (12%), 55 (10%), 62 (17%), 66 (12%), 81 (13%), and 84 (12%). Other HPV genotypes were detected at frequencies below 10%.

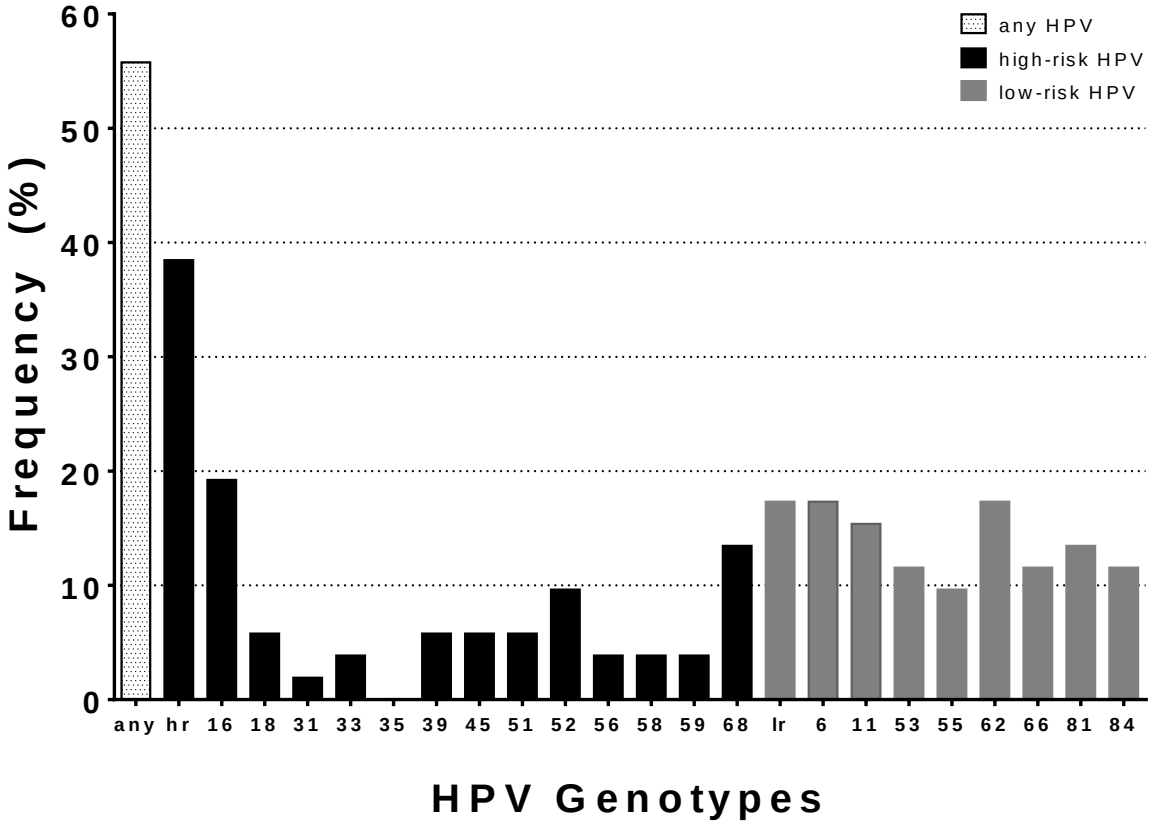

Figure 1. Percent frequency of HPV in anal specimens (n=52, male and female) containing any detectable HPV (any), one or more high-risk HPV genotypes (hr), only low-risk HPV genotypes (lr), or specific HPV genotypes, including 13 high-risk types associated with cancer as well as low-risk types 6 and 11, associated with genital warts, and others appearing at high frequency.

HPV in anal specimens conferred at least two-fold higher odds of ASIL (Table 2). Detection of HPV 6 or 11 in anal specimens was significantly associated with ASIL (OR=6.29, 95% CI: 1.49-26.44) while detection of any HPV approached statistical significance (OR=2.65, 95% CI: 0.86-8.24). The number of HPV genotypes detected in anal specimens was also significantly different for ASIL+ (3.4 ± 3.1) versus ASIL- (1.6 ± 3.1) participants (p=0.009).

Table 2. Association between HPV in Anal Specimens (n=52) and ASIL

Variable	Anal Cytology, n		OR	95% CI	p-value
	ASIL+	ASIL-			
Any HPV					
Detectable	17	12	2.65	0.86-8.24	0.091
Not detectable	8	15			
Any high-risk HPV					
Detectable	12	8	2.19	0.70-6.85	0.177
Not detectable	13	19			
HPV 6 or 11					
Detectable	11	3	6.29	1.49-26.44	0.012
Not detectable	14	24			
HPV 16 or 18					
Detectable	7	4	2.24	0.57-8.84	0.251
Not detectable	18	23			
# HPV Genotypes, Mean ± SD	3.4 ± 3.1	1.6 ± 3.1			0.009
# High-risk HPV Genotypes, Mean ± SD	1.2 ± 1.5	0.5 ± 1.2			0.105

ASIL = anal squamous intraepithelial lesion
OR = odds ratio
95% CI = 95% confidence interval
SD = standard deviation

Multiple anatomical site specimens were collected from 44 males: 25 (57%) were ASIL+ while 19 (43%) were ASIL-. HPV was detected in these specimens as follows: 27/44 (61%) anal, 19/43 (44%) oral wash, 17/44 (39%) penile shaft, 11/43 (26%) scrotum, 10/44 (23%) penile head, and 0/7 foreskin. Overall, 33/44 (75%) males had at least one HPV+ specimen. HPV was detectable at more anatomical sites among ASIL+ (1.3 ± 1.8) versus ASIL- (0.6 ± 1.1) males (p=0.045).

While 33/44 (75%) males were positive for any HPV across all anatomical sites, 25 (57%) were positive for one or more high-risk HPV genotypes (Figure 2). Across all specimens, genotypes detected most frequently among male participants included HPV 6 (30%) and 11 (18%), which are associated with genital warts; HPV 16 (25%), 18 (11%), 39 (16%), 45 (14%), 51 (11%), 52 (16%), 56 (16%), and 68 (20%), which are high-risk types associated with cancer; as well as low-risk types like HPV 53 (11%), 55 (18%), 61 (14%), 62 (30%), 66 (20%), 72 (14%), 81 (18%),

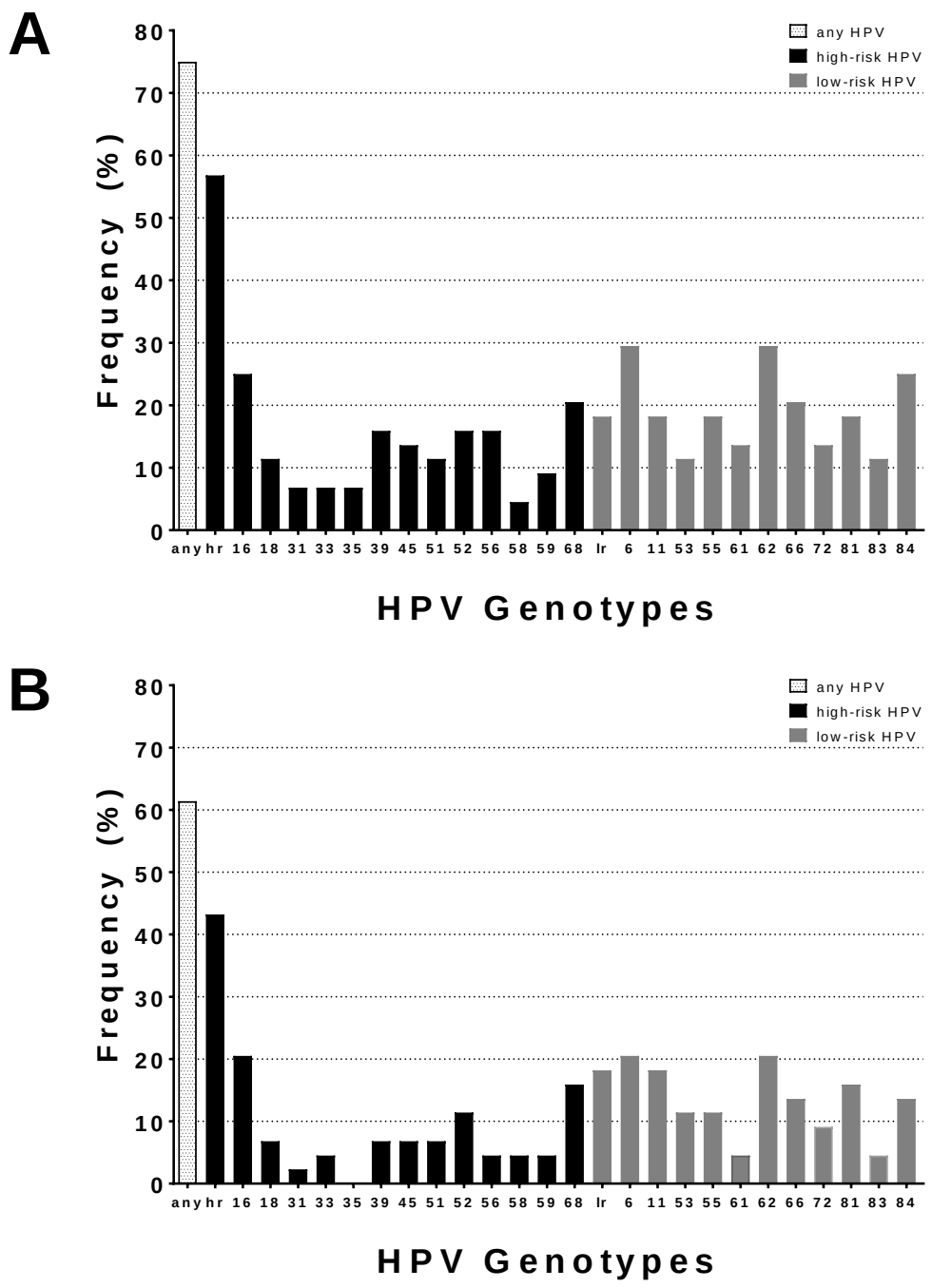

Figure 2. Percent frequency of HPV in male specimens containing any detectable HPV (any), one or more high-risk HPV genotypes (hr), only low-risk HPV genotypes (lr), or specific HPV genotypes, including 13 high-risk types associated with cancer as well as low-risk types 6 and 11, associated with genital warts, and others appearing at high frequency: A - all sites (n=44), B - anus (n=44), C - penile head (n=44), D - penile shaft (n=44), E - scrotum (n=43), F - oral wash (n=43).

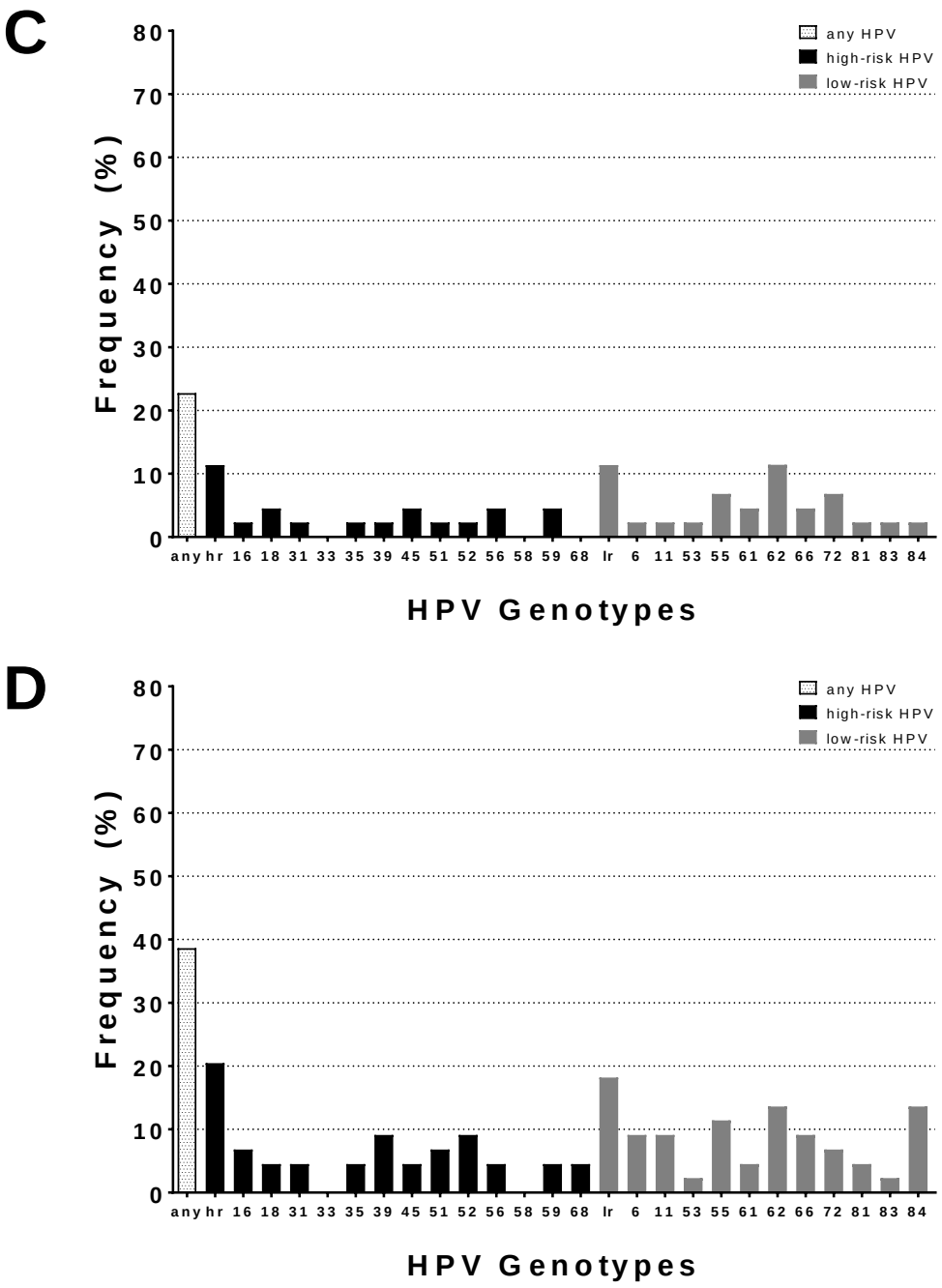

Figure 2. (continued) Percent frequency of HPV in male specimens containing any detectable HPV (any), one or more high-risk HPV genotypes (hr), only low-risk HPV genotypes (lr), or specific HPV genotypes, including 13 high-risk types associated with cancer as well as low-risk types 6 and 11, associated with genital warts, and others appearing at high frequency: A - all sites (n=44), B - anus (n=44), C - penile head (n=44), D - penile shaft (n=44), E - scrotum (n=43), F - oral wash (n=43).

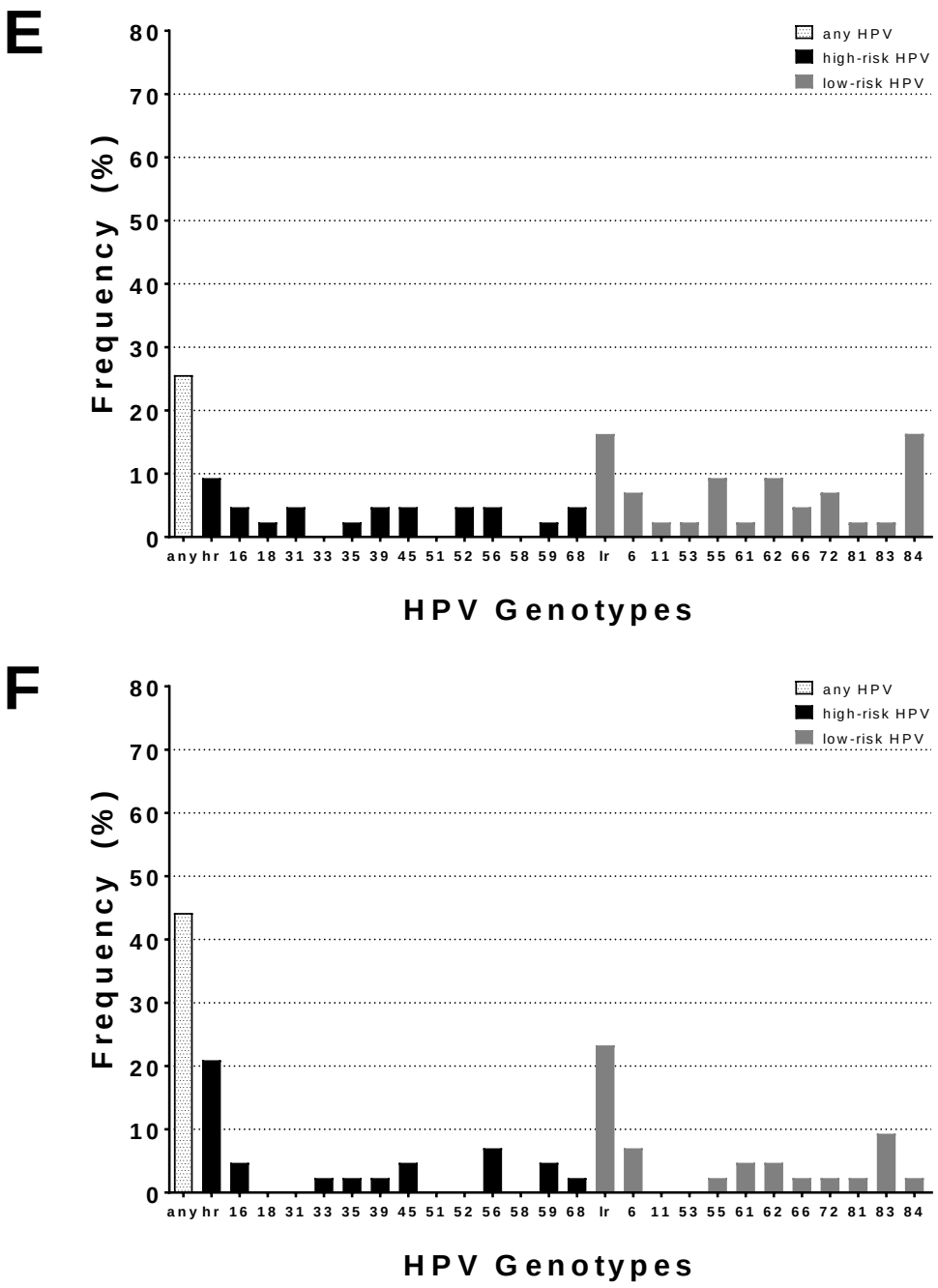

Figure 2. (continued) Percent frequency of HPV in male specimens containing any detectable HPV (any), one or more high-risk HPV genotypes (hr), only low-risk HPV genotypes (lr), or specific HPV genotypes, including 13 high-risk types associated with cancer as well as low-risk types 6 and 11, associated with genital warts, and others appearing at high frequency: A - all sites (n=44), B - anus (n=44), C - penile head (n=44), D - penile shaft (n=44), E - scrotum (n=43), F - oral wash (n=43).

83 (11%) and 84 (25%). Other HPV genotypes were detected at frequencies below 10%. In relation to ASIL, the number of unique HPV genotypes detected across all anatomical sites per participant approached statistical significance: ASIL+ (5.3 ± 5.4) compared to ASIL- (2.6 ± 4.2) males (p=0.054).

For all but one association between male specimen site and HPV variable tested, detectable HPV corresponded with increased odds of ASIL (Table 3). In particular, detection of HPV 16 or 18 proffered at least two-fold higher odds of ASIL across specimen types. Interestingly, penile shaft specimens displayed OR>3 across HPV variables. Any HPV in penile shaft specimens was significantly associated with ASIL (OR=6.79, 95% CI: 1.57-29.36) while detection of HPV 6 or 11 in anal specimens approached statistical significance (OR=4.19, 95% CI: 0.97-18.12). The number of HPV genotypes detected at some male specimen sites also differed according to ASIL status: significant for anal (p=0.0496) and penile shaft (p=0.025) specimens and approaching significance for scrotum (p=0.087) specimens (Table 4).

Table 3. Association between HPV at Male Specimen Sites and ASIL

Variable	Specimen Site, OR (95% CI)				
	Anus (n=44)	**Penile Head (n=44)**	**Penile Shaft (n=44)**	**Scrotum (n=43)**	**Oral Wash (n=43)**
Any HPV	1.91 (0.56-6.55)	4.00 (0.74-21.66)	6.79* (1.57-29.36)	2.67 (0.60-11.92)	1.71 (0.50-5.86)
Any high-risk HPV	1.58 (0.47-5.35)	3.43 (0.35-33.52)	3.31 (0.60-18.20)	2.57 (0.25-26.94)	1.78 (0.38-8.30)
HPV 6 or 11	4.19° (0.97-18.12)	0.75 (0.04-12.82)	5.68 (0.62-51.97)	1.64 (0.14-19.54)	1.64 (0.14-19.54)
HPV 16 or 18	2.07 (0.46-9.40)	4.15 (0.19-91.66)	3.43 (0.35-33.52)	6.35 (0.31-130.87)	4.33 (0.20-96.84)

ASIL = anal squamous intraepithelial lesion
OR = odds ratio
95% CI = 95% confidence interval
* Statistically significant (p=0.010)
° Approaching statistical significance (p=0.055)

Table 4. Association between Number of HPV Genotypes at Male Specimen Sites and ASIL

	# HPV Genotypes, Mean ± SD		
Specimen Site	**ASIL+**	**ASIL-**	**p-value**
Anus (n=44)	3.4 ± 3.1	1.9 ± 3.6	0.0496
Penile Head (n=44)	1.2 ± 2.9	0.5 ± 1.8	0.367
Penile Shaft (n=44)	2.4 ± 4.0	0.6 ± 1.7	0.025
Scrotum (n=43)	1.7 ± 3.6	0.5 ± 2.1	0.087
Oral Wash (n=43)	0.8 ± 1.3	0.5 ± 0.8	0.635

ASIL = anal squamous intraepithelial lesion
SD = standard deviation

Of 11 females enrolled in the study, only one presented with ASIL, and only one presented with cervical dysplasia. Odds ratios, 95% confidence intervals, and p-values were not estimable due to small numbers.

DISCUSSION

Among our participants, men presented with ASIL at a higher frequency than women. Male participants were predominantly men who have sex with men (MSM), whose behavioral factors place them at higher risk for anal dysplasia/cancer [21] though such risk factors were not assessed in this study. Consistent with literature, female participants presented with lower rates of anal and cervical dysplasia. ASIL did not differ by race/ethnicity in our diverse group of participants.

Previous studies have linked low CD4 cell counts to risk for ASIL. Bertisch et al. identified significant associations between anal cancer and low CD4 counts at nadir, at anal cancer diagnosis, and particularly 6-7 years before diagnosis [22]. While nadir CD4 and plasma HIV viral levels increased the odds of ASIL among our participants, the results were not statistically significant, perhaps owing to the small sample size. In the current cART era with effective reduction of HIV RNA to undetectable levels, presence of both HPV and residual HIV effects could establish a local, chronic inflammatory and metaplastic environment conducive to dysplastic expansion [9, 23]. Contrary to expectations, detectable HIV DNA in anal specimens was not associated with ASIL.

Analysis of HPV in anal specimens did produce some results consistent with current literature and knowledge. HIV-seropositive patients in our study exhibited a high frequency of HPV DNA present in anal specimens, with HPV 16 as the most frequently occurring genotype. Like previous studies, detectable anal HPV and the number of anal HPV genotypes was associated with ASIL [5]. Our results also showed a high degree of association between HPV 6 or 11 in anal specimens and ASIL. Further analysis demonstrated only slight agreement between anal

specimens that were positive for HPV 6 or 11 and those positive for high-risk HPV (kappa=0.20, McNemar's test p-value=0.11). Therefore, association between HPV 6 or 11 and ASIL is unlikely to be due to co-incidence of HPV 6 or 11 with high-risk HPV genotypes. Although HPV 6 and 11 are known for causing genital warts but not cancer, the pathologist's finding of abnormal cellular changes may correspond to condyloma triggered by these genotypes.

More surprising, however, were the associations between HPV in non-anal male specimens and ASIL. All but one association tested displayed higher odds of ASIL among males with HPV at non-anal sites. The association was particularly significant for detectable HPV in penile shaft specimens and less so for oral wash specimens; the variability among sites was corroborated by analysis of association between number of HPV genotypes at specimen site and ASIL. Although less well-studied, especially among HIV-positive MSM, penile HPV infection and penile intraepithelial neoplasia (PIN) have been recorded at lower rates than anal HPV infection and anal intraepithelial neoplasia (AIN) [24]. In a study of 263 HIV-positive MSM in Germany, Kreuter et al. reported 156 (59%) cases of AIN but only 11 (4%) of PIN [25]. Moreover, only 63% of penile cancers are attributable to HPV infection compared to 91% of anal cancers [26]. In light of this evidence, the high-degree of association between penile shaft HPV and ASIL in our study is unlikely to be due to co-incident penile dysplasia. Our discovery linking HPV at non-anal sites to ASIL may reflect autoinoculation -- transfer of HPV from the anus to other sites by the participant himself -- or behavioral factors among the predominantly MSM participants resulting in HPV introduction across multiple anatomical sites. Nevertheless, these results suggest that HPV at non-anal sites may also serve as indicators for ASIL among HIV-positive males.

Previous studies of women have described strong concordance of HPV and dysplasia between cervical and anal sites as well as probable autoinoculation between the two [27-29]. Only one HIV-positive female in our study presented with ASIL, and another presented with cervical dysplasia. Associations between cervical HPV and dysplasia with anal HPV and dysplasia could not be estimated due to small sample size.

Few studies have reported on the clinical significance of HPV in non-anal sites with respect to ASIL risk in HIV-seropositive individuals. Rather, studies have reported on the natural history or presence of HPV in multiple genital sites irrespective of ASIL [6, 7]. Our cross-sectional study reports for the first time that presence of HPV at multiple sites is associated with ASIL among HIV-positive males. Because HIV-seropositive individuals have an increased risk for anal dysplasia/cancer [1, 3, 30], studies that demonstrate HPV presence at non-anal sites may have important implications for diagnosis of anal dysplasia and for better understanding risk factors leading to anal cancer in the setting of HIV.

Our study was limited by the number of participants enrolled after referral for routine anal dysplasia screening and by cross-sectional design. Future investigations warrant enrollment of larger numbers of patients to assess the relationship between ASIL and HPV at non-anal sites among men and women in the context of HIV.

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SUMMARY OF SPECIFIC AIM 1 AND NEXT DIRECTION

This study aimed to determine the association between detectable HPV at various anatomical sites and atypical anal cytology among HIV-positive individuals in Hawaii. It did find that presence of HPV and the number of HPV genotypes at anatomical sites other than the anus trended toward higher odds of ASIL. Most interestingly, presence of HPV and the number of HPV genotypes at the penile shaft conferred the highest, statistically significant odds of ASIL.

These findings suggested that markers other than HPV at the anal site may be valid predictors of anal dysplasia. In search of other potential diagnostic markers, the next aim considered the utility of HPV-16 quantitation in differentiating high-grade from low-grade anal intraepithelial neoplasia.

SPECIFIC AIM 2

Differentiate high-grade anal intraepithelial neoplasia (HGAIN) from low-grade anal intraepithelial neoplasia (LGAIN) or negative anal biopsies using HPV type-specific DNA quantitation.

Hypothesis: HGAIN is associated with higher copy numbers of HPV-16 E6 DNA than LGAIN or negative anal biopsies.

Rationale: While presence of HPV at multiple anatomic sites appears to be associated with ASIL, assessment of abnormal cytology is itself dependent on the expertise and experience of the pathologist. Quantitation of HPV copy numbers may provide an objective measure to enhance current screening algorithms for diagnosis of anal dysplasia/cancer.

CHAPTER 3

HPV-16 DNA COPY NUMBERS DIFFERENTIATE HIGH-GRADE FROM LOW-GRADE ANAL INTRAEPITHELIAL NEOPLASIA

ABSTRACT

Objective. Since treatment of low-grade HPV-associated anal dysplasia is currently not recommended, high-resolution anoscopy and biopsy of these patients may be considered unnecessary invasive procedures. The study's objective was to use HPV type-specific DNA quantitation of anal cytology specimens to differentiate participants with high-grade anal intraepithelial neoplasia (HGAIN) from those with low-grade anal intraepithelial neoplasia (LGAIN) or negative anal biopsies in order to identify potentially avoidable HRAs with biopsy.

Methods. Four anal cytology specimens were collected during a single visit from patients who consented to study while undergoing routine anal cytology screening. HRA and biopsy was recommended for participants with atypical anal cytology results.

DNA extracted from anal cytology specimens was assayed for HPV-16 E6 DNA by genotype-specific quantitative real-time polymerase chain reaction (qRT-PCR).

Odds ratios (OR), 95% confidence intervals (95% CI), and p-values were calculated to investigate associations between anal cytology results and qualitative detection of HPV-16. HPV-16 copy numbers were analyzed by Mann-Whitney U test or Kruskal-Wallis non-parametric analysis of variance as appropriate.

Results. Of 75 participants enrolled, 59% presented with anal squamous intraepithelial lesions (ASIL) in cytology specimens. Gender, age, race/ethnicity, smoking status, and nadir CD4 count did not differ between ASIL+ and ASIL- groups.

Overall, 39% of participants had detectable HPV-16 DNA in anal cytology, which conferred higher odds of ASIL (OR=8.9, 95% CI: 2.7-29.7). HPV-16 DNA copy numbers per cell (mean ±

standard deviation) were significantly higher for ASIL+ (1115 ± 5110) versus ASIL- (45 ± 217) ($p<0.0001$).

When cytology results were further stratified, HPV-16 DNA copy numbers per cell still varied significantly among HSIL (4406 ± 11010), LSIL (484 ± 1397), ASC-H (89 ± 126), ASCUS (108 ± 312), and Negative (45 ± 217) grades ($p=0.0010$). When HRA biopsy results were stratified, HPV-16 DNA copy numbers per cell also differed significantly among HGAIN (7409 ± 14648), LGAIN (98 ± 192), and Negative (78 ± 136) grades ($p=0.021$)

Conclusion. In conjunction with standard anal cytology screening, HPV type-specific DNA quantitation may allow differentiation of high-grade from low-grade anal dysplasia in order to reduce the number of patients for whom treatment would not be recommended after undergoing invasive HRA with biopsy.

INTRODUCTION

Because HIV patients are at higher risk for anal HPV infection and associated dysplasia, it is recommended that they undergo annual anal cytology screening [1]. Similar to cervical cytology screening, anal cytologies are evaluated by a pathologist for abnormal anal squamous cells [2]. Atypical cytologies can be further graded as atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells but cannot exclude high-grade (ASC-H), low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL) [3]. Micrographs in Figure 1 [4] present examples of increasingly dysplastic grades of mucosal epithelia in cervical tissue.

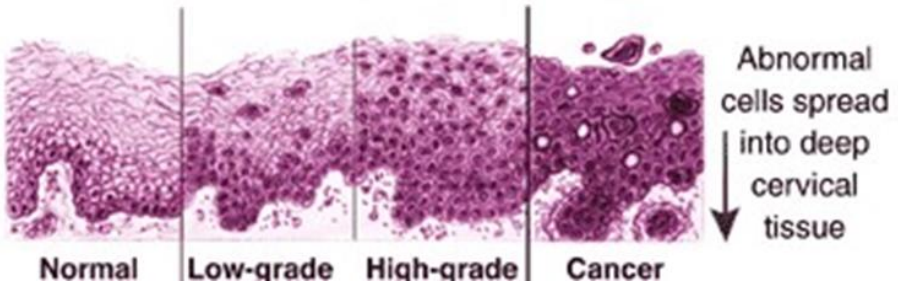

Figure 1. Histological changes from Healthy to Cancer [4]

When anal cytology results are atypical, patients are recommended to follow up with more invasive high-resolution anoscopy (HRA) and biopsy of abnormal-looking epithelia. These HRA biopsies are also assessed by a pathologist for diagnoses of low-grade anal intraepithelial neoplasia (LGAIN) or high-grade anal intraepithelial neoplasia (HGAIN). As shown in Figure 2 [5], treatment is recommended for patients with HGAIN; however patients with LGAIN are usually recommended to continue annual anal cancer screening.

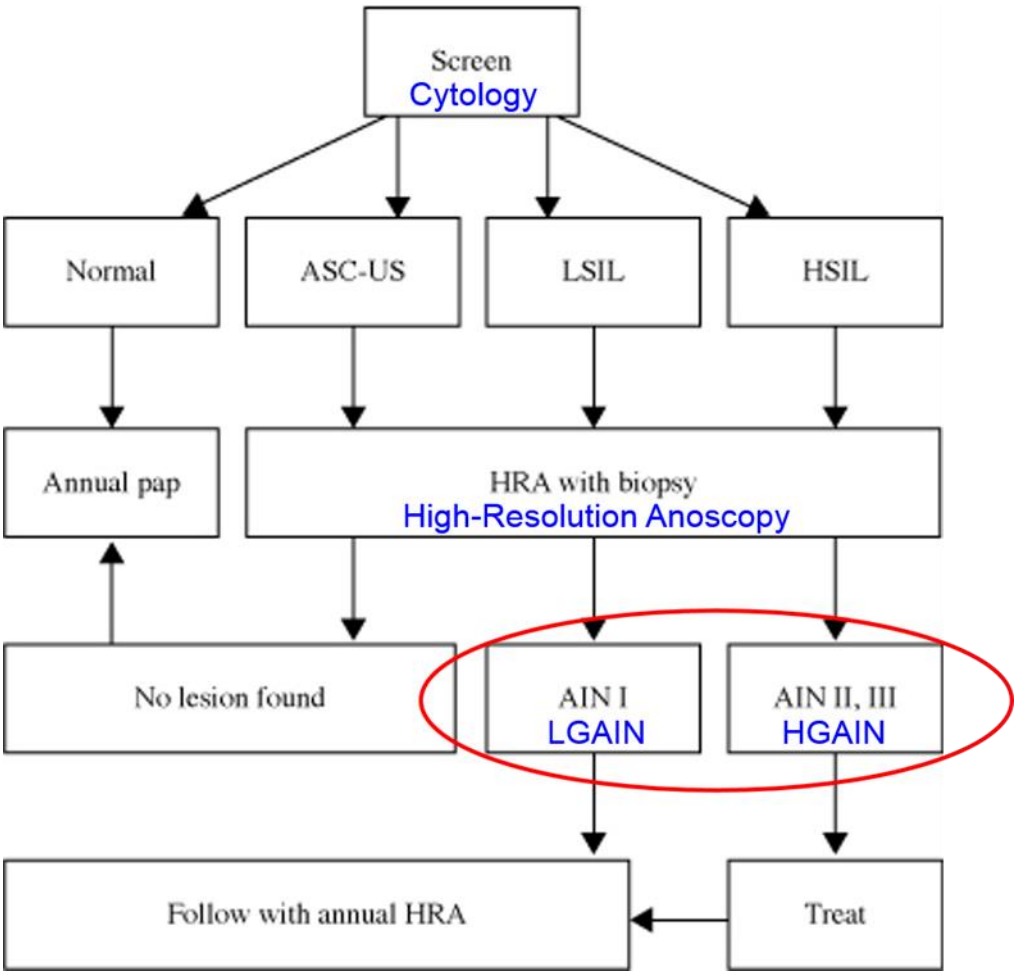

Figure 2. Anal Cancer Screening Algorithm [5]

Since treatment is not recommended, the HRA and biopsy of patients with LGAIN might be considered unnecessary in retrospect. For this reason, RMATRIX Pilot Project RM004 was initiated to distinguish low-grade from high-grade disease by detection of a triad of biomarkers related to HIV/HPV-associated anal dysplasia: HIV DNA copy number, high-risk HPV genotypes HPV-16 and HPV-18, and p16 protein. As a subcomponent of RM004, HPV-16 copy numbers were quantified to separately evaluate the utility of HPV-16 DNA viral load in distinguishing low-grade from high-grade disease.

MATERIALS AND METHODS

Specimen Collection. Study protocol (CHS #21953) was approved by the UH IRB. HIV-seropositive men and women were recruited at the Hawaii Center for AIDS Clint Spencer Clinic for RMATRIX Pilot Project RM004. Patients undergoing routine anal cytology screening were informed about the study's purpose. If they provided written consent, four anal cytology specimens were collected during a single visit: one specimen was evaluated by the pathologist, and the rest were processed for biomarker detection. Follow-up with HRA and biopsy was recommended for participants receiving atypical anal cytology results per standard of care.

Additional information was collected from participants – including gender, age, race/ethnicity, smoking status, and nadir CD4 count – per informed consent.

Methodology. DNA was extracted from specimens, using the Machery-Nagel NucleoSpin Tissue XS Kit, and quantitated on a NanoDrop 2000 instrument. HPV-16 E6 DNA was quantified by genotype-specific quantitative real-time polymerase chain reaction (qRT-PCR) and normalized as follows:

Based on literature research and preliminary experiments, genotype-specific primers targeting the HPV-16 E6 gene were selected [6]. A HPV-16 E6 gene-specific probe was designed by analysis of the E6 region in a published HPV-16 whole genome sequence [7], using online software Primer3 [8]. Primers and probe targeting β-globin have previously been published [9]. Primer and probe sequences are listed in Table 1. Standard curves were derived from ten-fold serial dilutions of β-globin plasmid and HPV-16 plasmid – p1203 PML2d HPV-16 was a gift from Peter Howley (AddGene #10869) (unpublished data) – at calculated quantities from 10 copies to 1 million copies. Controls included DNA from SiHa cells (American Type Culture Collection

ATCC® HTB-35™), positive for HPV-16; and water. All qRT-PCR assays were performed in triplicate, using TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA). Representative amplification plots of HPV-16 plasmid (standard), SiHa cell DNA (positive control), and water (negative control) are show in Figure 3. Resulting data were analyzed using StepOne™ version 2.0 software (Thermo Fisher Scientific, Waltham, MA). Copy numbers of each target gene were calculated based on the standard curve, and HPV-16 copy numbers per cell were determined.

Table 1. Primer/Probe Sequences for qRT-PCR

Target Gene	**Primer/Probe**	**Sequence (5'→ 3')**
HPV-16 E6	Forward Primer Reverse Primer Probe	TCAAAAGCCACTGTGTCCTGA CGTGTTCTTGATGATCTGCAA VIC-ATATAAGGGGTCGGTGGACC-TAMRA
β-globin	Forward Primer Reverse Primer Probe	AGGGCCTCACCACCAACTTC TCACTAGCAACCTCAAACAGACACC 6FAM-CTCCTGAGGAGAAGTCTGCCGTTACTGCC-TAMRA

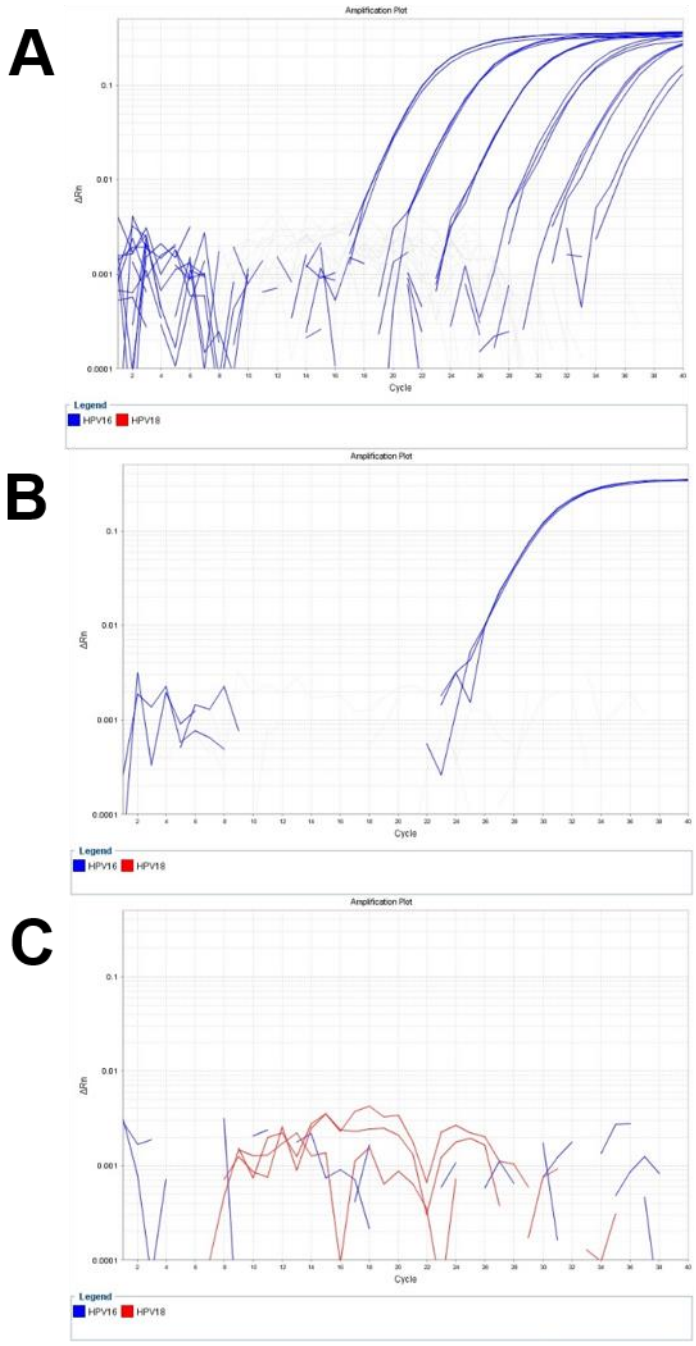

Figure 3. Quantitative real-time polymerase chain reaction (qRT-PCR) targeting HPV-16 E6 gene. Each amplification plot displays PCR cycle number on the *x*-axis and ΔRn on the *y*-axis: A - ten-fold serially diluted HPV-16 Plasmid (standard), B - DNA from SiHa cells (positive control), C - water (negative control).

Statistics. Statistical analysis was conducted in consultation with the Office of Biostatistics and Quantitative Health Sciences at UH JABSOM. Based on power calculation, a sample size of 75 was deemed sufficient to determine whether biomarker detection could differentiate high-grade from low-grade disease. Odds ratios (OR), 95% confidence intervals (95% CI), and p-values were calculated to investigate associations between anal cytology results (ASIL+ or ASIL-) and detectable HPV-16 in anal cytology specimens. HPV-16 copy numbers were compared between ASIL+ and ASIL- groups by Mann-Whitney U test. Furthermore, HPV-16 copy numbers were compared among patients stratified by anal cytology grade and by HRA biopsy grade, using Kruskal-Wallis non-parametric analysis of variance.

RESULTS

Out of 75 participants enrolled in the study, 67 (89%) were male. Mean age was 51 years with a standard deviation of 10.6 years. Among this racially/ethnically diverse group, 38 (51%) were White, 10 (13%) Asian, 11 (15%) Hawaiian/Pacific Islander, 4 (5%) African-American, 2 (5%) Native/Alaskan American, and 10 (13%) identified as more than one race/ethnicity. Participants' smoking status was as follows: 17 (23%) current smokers, 32 (43%) past smokers, 20 (28%) non-smokers. Median CD4 count at nadir was 209 cells/mm^3. A summary of participant characteristics and association with ASIL is shown in Table 2.

Table 2. Participant Characteristics and Association with ASIL

Variable	All Patients (n=75)	ASIL+ (n=44)	ASIL- (n=31)	p-value
Age, mean (SD)	51 (10.6)	50 (9.7)	51.0 (11.9)	0.73
Gender, n (%) Male	67 (89)	39 (89)	28 (90)	0.99
Race/ethnicity, n (%)				0.79
White	38 (51)	22 (50)	16 (52)	
Asian	11 (15)	7 (16)	4 (13)	
Hawaiian/Pacific Islander	10 (13)	4 (9)	6 (19)	
African American	4 (5)	3 (7)	1 (3)	
Native/Alaskan American	2 (3)	1 (2)	1 (3)	
More than One	10 (13)	7 (16)	3 (10)	
Smoking, n (%)				0.53
Current Smoker	19 (25)	13 (30)	6 (19)	
Past Smoker	36 (48)	19 (43)	17 (55)	
Never Smoked	20 (27)	12 (27)	8 (26)	
CD4 nadir count, median	209	193	233	0.35

ASIL = anal squamous intraepithelial lesion
SD = standard deviation

Of the 75 participants, 44 (59%) presented with anal squamous intraepithelial lesions (ASIL) in cytology specimens. None of the characteristics listed above – gender, age, race/ethnicity, smoking status, and nadir CD4 count – differed statistically between those with normal versus atypical anal cytology.

Overall, 29 (39%) participants had detectable HPV-16 DNA in anal cytology specimens. Of the 44 participants with ASIL, 25 (57%) had detectable HPV-16 DNA in anal cytology specimens. Those with detectable HPV-16 DNA had significantly higher odds of presenting with ASIL (OR=8.88, 95% CI: 2.66-29.72) (p=0.0001). HPV-16 DNA copy numbers (mean ± standard deviation) also differed significantly between the ASIL+ (1115 ± 5110) and ASIL- (45 ± 217) groups (p<0.0001). These statistics are summarized in Table 3.

Table 3. Association between HPV-16 in Anal Specimens (n=75) and ASIL

	Anal Cytology, n				
Variable	**ASIL+**	**ASIL-**	**OR**	**95% CI**	**p-value**
HPV-16					
Detectable	25	4	8.88	2.66-29.72	0.0001
Not detectable	19	27			
HPV-16 copies per cell, Mean ± SD	1115 ± 5110	45 ± 217			<0.0001

ASIL = anal squamous intraepithelial lesion
OR = odds ratio
95% CI = 95% confidence interval
SD = standard deviation

When atypical cytology results were further stratified, 9 (12%) were graded HSIL, 15 (20%) LSIL, 2 (3%) ASC-H, 18 (24%) ASCUS, and 31 (41%) Negative. As shown in Figure 4, the proportion of participants with detectable HPV-16 DNA varied according to anal cytology grade: 7 (78%) of

HSIL, 9 (60%) of LSIL, 1 (50%) of ASC-H, 8 (44%) of ASCUS, and 4 (13%) of Negative. However, these differences were not statistically significant.

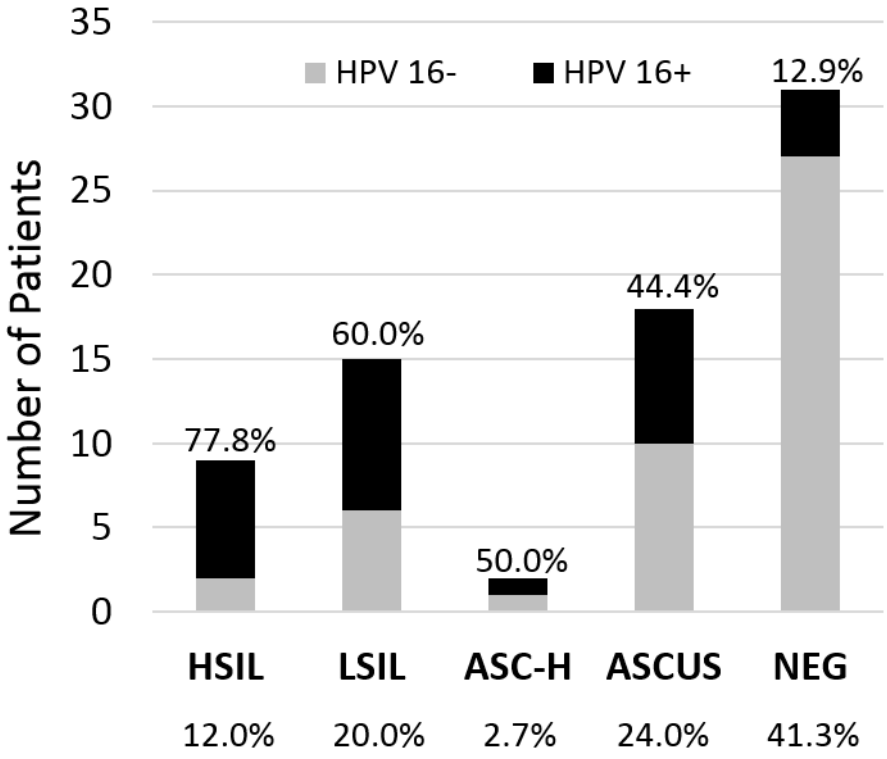

Figure 4. HPV16+ Proportion and Percent of Cytology Specimens by Anal Cytology Grade. Detectable HPV-16 DNA varied according to anal cytology grade, but differences were not statistically significant.

As pictured in Figure 5, HPV-16 DNA copy numbers per cell (mean ± standard deviation) also varied according to anal cytology grade: HSIL (4406 ± 11010), LSIL (484 ± 1397), ASC-H (89 ± 126), ASCUS (108 ± 312), and Negative (45 ± 217) grades differed significantly (p=0.0010) by Kruskal-Wallis non-parametric analysis of variance.

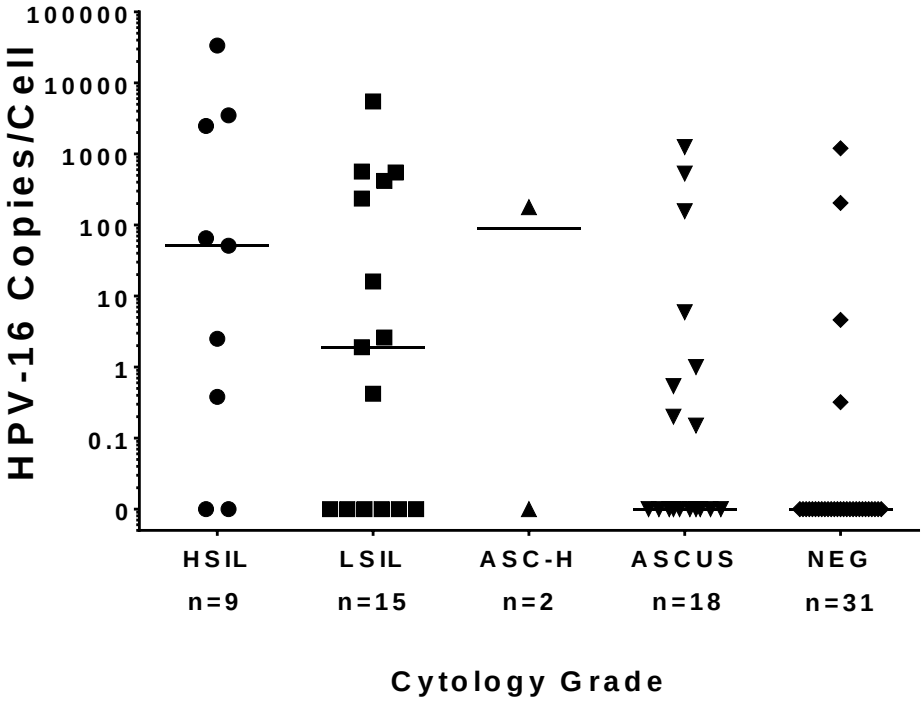

Figure 5. HPV16 Copy Number by Anal Cytology Grade. HPV-16 copies per cell differed significantly among anal cytology grades (p=0.0010). Bar represents median value in group.

Follow-up HRA and biopsy was recommended for participants with atypical anal cytology results. For the 16 participants who did undergo HRA, biopsy grades were as follows: 5 (31%) HGAIN, 8 (50%) LGAIN, and 3 (19%) Negative. As depicted in Figure 6, the proportion of participants with detectable HPV-16 DNA varied according to anal cytology grade: 5 (100%) of HGAIN, 6 (75%) of LGAIN, and 1 (33%) of Negative. However, these differences were not statistically significant.

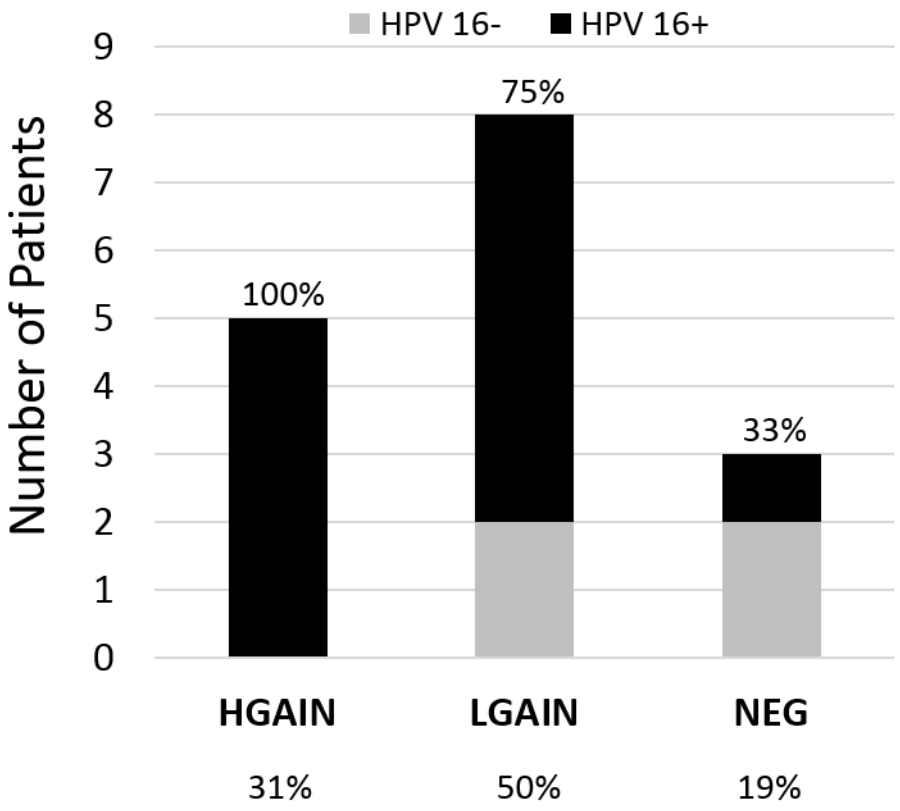

Figure 6. HPV16+ Proportion and Percent of HRA Biopsy Specimens by Grade. Qualitative differences in HPV-16 detection among HRA biopsy grades were not statistically significant.

As illustrated in Figure 7, HPV-16 DNA copy numbers per cell (mean ± standard deviation) also varied according to HRA biopsy grade: HGAIN (7409 ± 14648), LGAIN (98 ± 192), and Negative (78 ± 136) grades differed significantly (p=0.021) by Kruskal-Wallis non-parametric analysis of variance. A threshold line drawn at 10 copies per cell neatly demarcates all participants with HGAIN while three others with LGAIN and another with Negative biopsy results also had HPV-16 E6 copy numbers above 10 copies per cell.

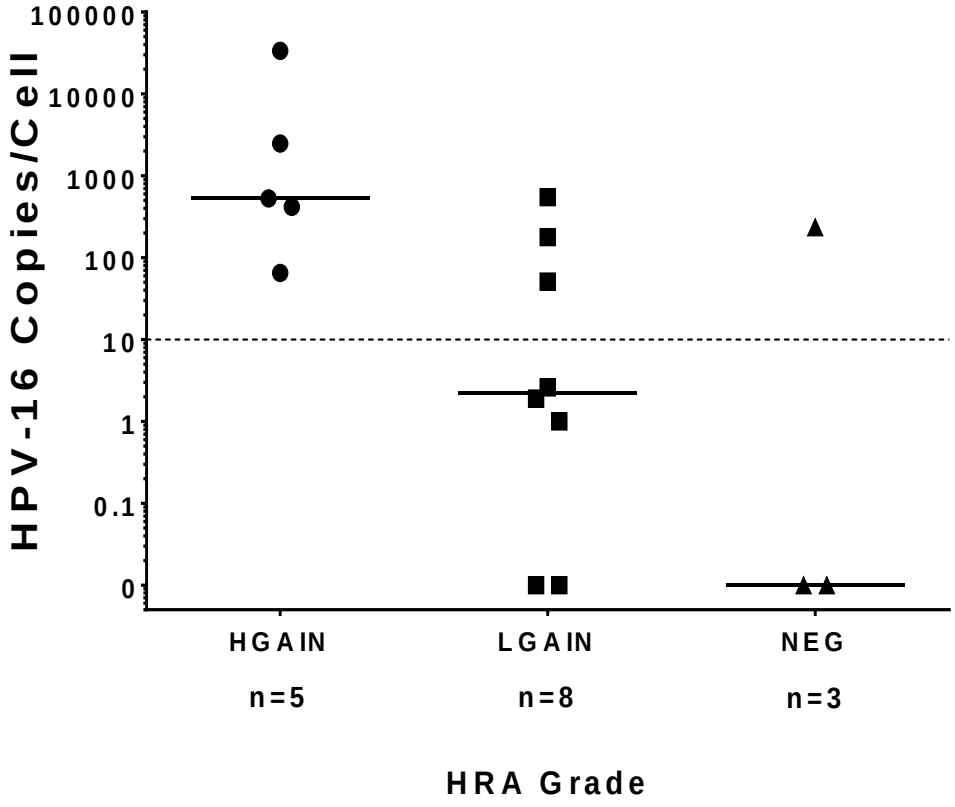

Figure 7. HPV16 Copy Number by HRA Biopsy Grade. HPV-16 copies per cell differed significantly among HRA biopsy grades (p=0.021). Bar represents median value in group.

DISCUSSION

Consistent with a previous study of anal dysplasia among HIV+ patients in Hawaii [9], participants in this study were predominantly male and racially/ethnically diverse, and there was no significant difference in age or race/ethnicity between participants with ASIL and those without. Unlike the previous study, females in this study presented with ASIL at approximately the same frequency as males. This discrepancy may be attributable to the low number of females enrolled in both studies.

Other researchers have noted an association between cigarette smoking and HPV infection or HPV-associated dysplasia. In a study of HIV+ MSM, Wieland et al. found higher frequency of anal dysplasia and higher HPV-16 viral loads among smokers compared to non-smokers [10]. This study revealed no difference in ASIL occurrence among HIV+ participants who were current smokers, past smokers, or never smokers. Further analysis is necessary to investigate whether HPV-16 viral loads differ among these groups. Other researchers have also linked nadir CD4 cell counts of HIV+ patients to risk for ASIL [11]. No association between ASIL and nadir CD4 counts appeared in this study.

Overall, the proportion of participants who presented with ASIL (59%) in anal cytology specimens was also similar to the previous study of anal dysplasia among HIV+ patients in Hawaii [9]. As expected, ASIL+ participants were also more likely to have detectable HPV-16 E6 DNA as well as greater HPV-16 E6 copies per cell than ASIL- participants. When integrated into infected host cells, the HPV E6 oncogene can be transcribed and translated into oncoprotein that disrupts p53-regulated cell cycle checkpoints to induce unregulated cellular proliferation indicative of cancer [12]. Presence of HPV-16 E6 DNA, particularly at higher copy numbers,

suggests replication of viral oncogenes with potential to transform infected host cells. Such cellular transformation may be recognized as dysplasia by pathologists assessing cytology and biopsy specimens.

Upon further stratification of anal cytology results, participants with HSIL were most likely to have detectable HPV-16 E6 DNA, trailed by those with LSIL, then ASC-H, ASC-US, and Negative cytology results. Although higher grades of anal dysplasia corresponded to greater frequency of HPV-16 detection, differences among grades were not statistically significant. HPV-16 E6 copy numbers followed a similar trend with highest mean copies per cell in participants with HSIL then LSIL, ASC-US, ASC-H, and Negative cytology results. These differences in copy number were statistically significant.

Likewise, stratification of HRA biopsy results revealed greater frequency of HPV-16 detection in specimens with higher grades anal dysplasia: 100% of those with HGAIN tested positive for HPV-16 E6 DNA in anal cytology. Here again, mean HPV-16 E6 copies per cell were significantly higher in those with HGAIN relative to LGAIN and Negative biopsy results.

Although only the five participants with HGAIN (31%) out of sixteen who underwent HRA with biopsy would have been referred for treatment, three others with LGAIN and another with Negative biopsy results had HPV-16 E6 copy numbers within range of those with HGAIN as illustrated in Figure 7. While the screening algorithm recommends annual HRA follow-up for those with LGAIN, it may be reasonable to recommend the same for the individual with Negative biopsy results but high HPV-16 copy numbers. Furthermore, it is worth considering whether invasive HRA with biopsy was necessary for the remaining seven (44%) with LGAIN or Negative biopsy results and low HPV-16 E6 copy numbers.

Assessment of cytology and biopsy specimens is known to be somewhat subjective, dependent on the experience and expertise of the pathologist. As part of the ASCUS-LSIL Triage Study, Stoler et al. found only moderate agreement among even well-trained pathologists examining cervical cytology (kappa = 0.46, 95% CI: 0.44-0.48) and biopsy (kappa = 0.46; 95% CI, 0.43-0.49) specimens [13]. Baena et al. recently published an article describing similar inter-observer reproducibility (median kappa = 0.51) for cervical cytology evaluation, with overall false-positive rate of 31% and false-negative rate of 11% [14]. Incorporating HPV quantitation in anal dysplasia screening may improve diagnostic accuracy with the benefit of reducing patient discomfort from potentially unnecessary invasive procedures and anxiety induced by false-positive results.

ACKNOWLEDGEMENTS

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SUMMARY OF SPECIFIC AIM 2 AND NEXT DIRECTION

This study aimed to differentiate high-grade anal intraepithelial neoplasia (HGAIN) from low-grade anal intraepithelial neoplasia (LGAIN) or negative anal biopsies using HPV type-specific DNA quantitation. Based on the HPV-16 qRT-PCR data and available biopsy reports, all participants with HGAIN also had detectable HPV-16 E6 DNA above a threshold of 10 copies per cell. However, some participants with LGAIN or Negative biopsies also had detectable HPV-16 E6 DNA above a threshold of 10 copies per cell. Since cytology and biopsy results are dependent on the pathologist's expertise and experience, HPV quantitation may prove useful as an objective supplemental diagnostic marker.

Thus far the relationship between HPV and anal dysplasia has been examined in the context of prior HIV infection. The next aim probed the converse relationship on this axis – the acquisition of HIV after HPV infection.

SPECIFIC AIM 3

Determine whether an association exists between HIV seroconversion and acquisition or clearance of anal HPV genotypes in a Thai cohort.

Hypothesis: HPV clearance is associated with HIV seroconversion in a high-risk population.

Rationale: As previously demonstrated, HIV-positive individuals frequently present with HPV at multiple anatomical sites in association with anal dysplasia/cancer, and HPV quantitation may enhance current screening methods for diagnosis of HPV-associated anal dysplasia/cancer in the context of HIV infection. Better understanding of HPV's role in subsequent HIV seroconversion could suggest a novel strategy for reducing HIV transmission.

CHAPTER 4

ANAL HPV ACQUISITION INCREASED HIV SEROCONVERSION RISK IN THAILAND TEST & TREAT COHORT

ABSTRACT

Objective. Previous studies suggest an association between HPV acquisition or clearance and HIV seroconversion. This study sought to determine whether anal HPV acquisition or clearance was associated with HIV seroconversion among the Thai Test & Treat cohort.

Methods. From Thai MSM and TG women, ages 18 and older, with no previous HIV-positive test, who were enrolled in the parent Test & Treat study, 24 HIV serconverter cases were selected and matched to 48 HIV-negative controls. Cases and controls had anal cytology specimens collected twice within a twelve-month period. DNA previously extracted from anal cytology specimens were assayed, using Roche's LINEAR ARRAY HPV Genotyping Test

Conditional logistic regression models were applied; and odds ratios, 95% confidence intervals, and p-values were calculated to determine whether an association existed between anal HPV acquistion or clearance and HIV seroconversion.

Results. Acquisition of any HPV genotype (OR=4.75, 95% CI: 1.01-22.32, p=0.049) or any high-risk HPV genotype (OR=4.93, 95% CI: 1.36-17.96, p=0.015) conferred higher odds of HIV seroconversion. Clearance of any HPV genotype (OR=1.64, 95% CI: 0.56-4.85, p=0.37) or any high-risk HPV genotype (OR=1.16, 95% CI: 0.46-2.91, p=0.75) was not significantly associated with HIV seroconversion.

Conclusion. HPV acquisition not clearance was associated with HIV seroconversion in the Thai Test & Treat cohort. Clarification of HPV's role in HIV acquisition may suggest a new approach for reducing HIV transmission.

INTRODUCTION

Thailand is one of five Southeast Asian countries that together account for 99% of the region's HIV burden [1]. Despite declining incidence, Thailand witnessed an estimated 8800 new HIV infections in 2012; adult HIV prevalence remains about 1% [2]. Populations at higher risk of acquiring HIV infection include men who have sex with men (MSM) and transgender (TG) people. HIV prevalence among MSM is 7.1% in Thailand and 24.7% in Bangkok [3]. HIV prevalence among TG people is 10.4% in Thailand and 7.7% in Bangkok [4].

In order to further suppress transmission of HIV, the Thai Red Cross AIDS Research Centre (TRCARC) with support from the Thai Ministry of Public Health initiated a "Test & Treat" protocol to encourage regular visits for voluntary counseling and testing (VCT) among MSM and TG women and to make available anti-retroviral therapy (ART) to those who test HIV-positive, regardless of CD4 count. Although participants entered the study with no previous HIV-positive test, some acquired HIV during the two-year follow-up period. The primary outcome measure for Test & Treat was ART uptake at HIV seroconversion.

HIV-positive patients are at higher risk of acquiring HPV [5] even while on ART [6]. Infection with high-risk HPV genotypes in the anogenital region can lead to dysplasia, which can progress to cancer. HIV and HPV can both be contracted via sexual transmission; deposition or infection by both viruses can occur at the same tissue sites. While many studies have examined HPV as a consequence of HIV infection, few have scrutinized HPV as a precursor to HIV infection. Two meta-analyses suggest an association between HPV infection and HIV acquisition [7, 8]. Two other studies demonstrate an association between HPV clearance and HIV seroconversion [9, 10]. This substudy of the Test & Treat protocol sought to determine whether anal HPV

acquisition or clearance was associated with HIV seroconversion among Thai MSM and TG women. Clarification of HPV's role in HIV acquisition may suggest a new approach for reducing HIV transmission.

MATERIALS AND METHODS

Specimen Collection. The parent Test & Treat protocol was approved by the Chulalongkorn University IRB, and enrollment began at TRCARC's Anonymous Clinic. Inclusion criteria were Thai MSM and TG women, ages 18 and older, no previous HIV-positive test. An amendment to the Test & Treat protocol to allow specimen access for this substudy was also approved by the Chulalongkorn University IRB. The proposal submitted to the UH IRB was granted exempt status. This substudy utilized DNA previously extracted from anal cytology specimens for the parent Test & Treat protocol. Participants included in the substudy had at least two anal cytology specimens collected within a twelve-month period.

Methodology. HIV serconverter cases from whom anal cytology specimens were collected at HIV seroconversion and within twelve months prior were selected and matched to HIV-negative controls from whom anal cytology specimens were collected twice within a twelve-month period. Matching criteria included the following: age, number of partners during the previous month, and inconsistent condom use during the previous month. DNA previously extracted from anal cytology specimens were assayed, using Roche's LINEAR ARRAY HPV Genotyping Test to detect up to 37 HPV genotypes, including 13 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) associated with cancer.

Statistics. A power calculation found that 24 HIV seroconverter cases matched to 48 HIV-negative controls would be sufficient to determine whether an association between HPV acquistion or clearance and HIV seroconversion existed. Change in HPV genotypes detected at timepoint1 (T1) versus timepoint2 (T2) for each HIV seroconverter case compared to matched HIV-negative controls was analyzed. Conditional logistic regression models were applied; and

odds ratios, 95% confidence intervals, and p-values were calculated to determine whether an association existed between anal HPV acquistion or clearance and HIV seroconversion.

RESULTS

Out of 498 MSM and TG women enrolled in the parent Test & Treat study in Bangkok, 112 (22%) tested HIV-positive at baseline and were excluded from the retrospective case-control substudy. Of the remaining participants, 37 seroconverted, testing HIV-positive during follow-up. As shown in Figure 1, a total of 24 HIV serconverter cases from whom anal cytology specimens were collected at HIV seroconversion and within twelve months prior were selected and matched to 48 HIV-negative controls from whom anal cytology specimens were collected twice within a twelve-month period.

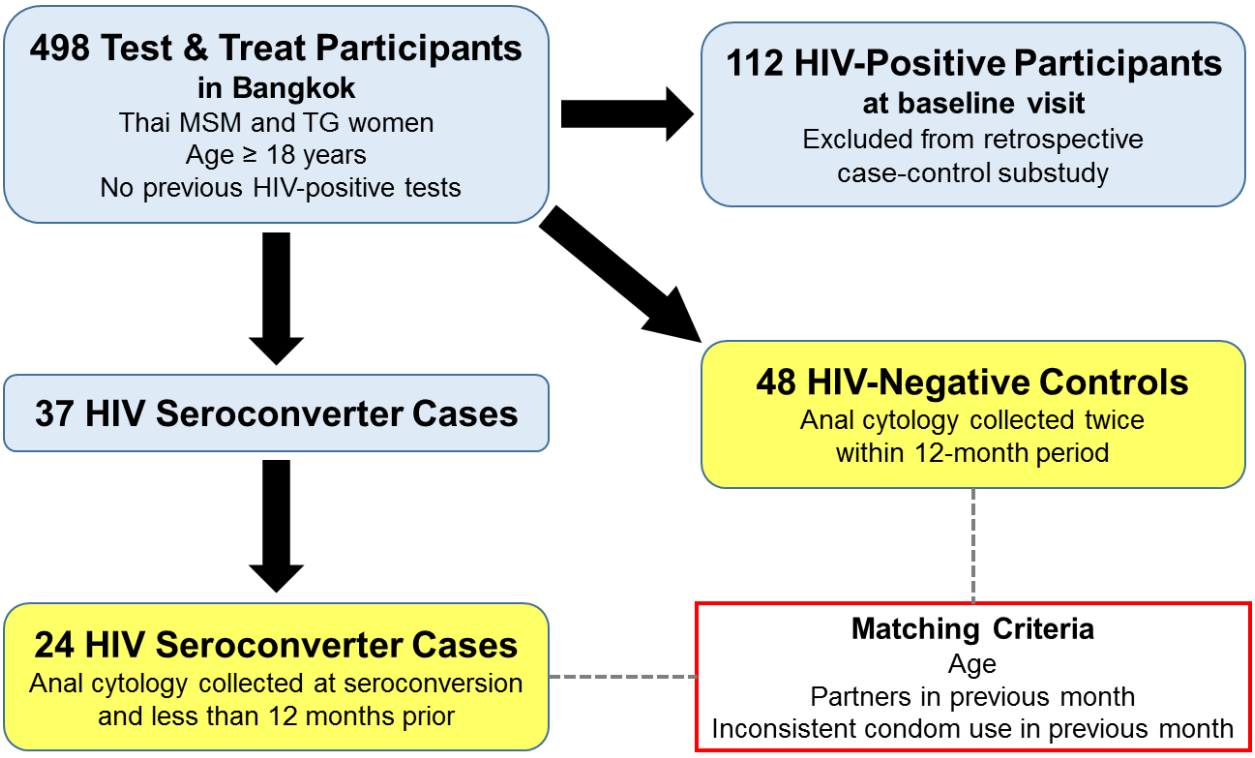

Figure 1. Retrospective Case-Control Substudy Design

DNA from anal cytology specimens at both timepoint1 (T1) and timepoint2 (T2) for all 24 selected HIV seroconverter cases produced valid HPV genotyping results. However, among 48 matched HIV-negative controls, two produced invalid HPV genotyping results at T1. A third matched control was found not to have met the criterion for two anal cytology specimens collected within a twelve-month period; therefore DNA from that participant's anal cytology specimens was not tested. All valid HPV genotyping tests were included for the purpose of calculating genotype frequency. Three controls were excluded from analysis of HPV association with HIV seroconversion due to the aforementioned invalid HPV genotyping tests and unmet inclusion criterion.

Among cases, the frequency of high-risk HPV genotypes 18, 45, 51, 52, 59, and 68 in anal cytology specimens increased during the period between T1 to T2 while frequency of high-risk HPV genotypes 31, 33, 35, and 56 decreased. In particular, types 18 and 52 displayed the overall highest frequency (33.3%) among cases at T2. A few high-risk HPV genotypes were detected at consistent frequencies from T1 to T2: types 16 (25.0%), 39 (12.5%), 58 (20.8%). During the same timeframe, low-risk HPV genotype 6 increased while 11 decreased in frequency among cases. Frequency in anal cytology specimens of 37 HPV genotypes detected by the Roche LINEAR ARRAY HPV Genotyping Test is shown in Table 1. Frequency of 13 high-risk HPV genotypes and select low-risk genotypes are graphed in Figure 2.

Table 1. Frequency in anal cytology specimens of 37 HPV genotypes detected by the Roche LINEAR ARRAY HPV Genotyping Test

	Frequency (%)			
HPV Genotype	Cases T1 n=24	Cases T2 n=24	Controls T1 n=45	Controls T2 n=47
6	4.2	16.7	4.4	4.3
11	25	12.5	6.7	6.4
16	25	25	20	21.3
18	12.5	33.3	11.1	8.5
26	4.2	8.3	4.4	0
31	16.7	8.3	6.7	2.1
33	8.3	4.2	0	2.1
35	4.2	0	2.2	2.1
39	12.5	12.5	11.1	14.9
40	4.2	4.2	2.2	6.4
42	8.3	0	8.9	6.4
45	4.2	16.7	4.4	2.1
51	4.2	16.7	11.1	10.6
52	4.2	33.3	4.4	8.5
53	0	4.2	4.4	4.3
54	0	4.2	2.2	6.4
55	4.2	0	2.2	6.4
56	8.3	0	4.4	4.3
58	20.8	20.8	8.9	12.8
59	4.2	20.8	6.7	10.6
61	12.5	16.7	4.4	12.8
62	4.2	0	2.2	10.6
64	0	0	0	0
66	4.2	8.3	4.4	2.1
67	4.2	0	0	0
68	8.3	20.8	4.4	4.3
69	0	0	0	2.1
70	4.2	4.2	0	4.3
71	0	0	0	0
72	4.2	0	0	6.4
73	4.2	4.2	4.4	4.3
81	4.2	4.2	0	4.3
82	4.2	12.5	0	0
83	0	0	6.7	4.3
84	12.5	4.2	6.7	12.8
CP6108	12.5	12.5	4.4	4.3
IS39	0	0	2.2	2.1

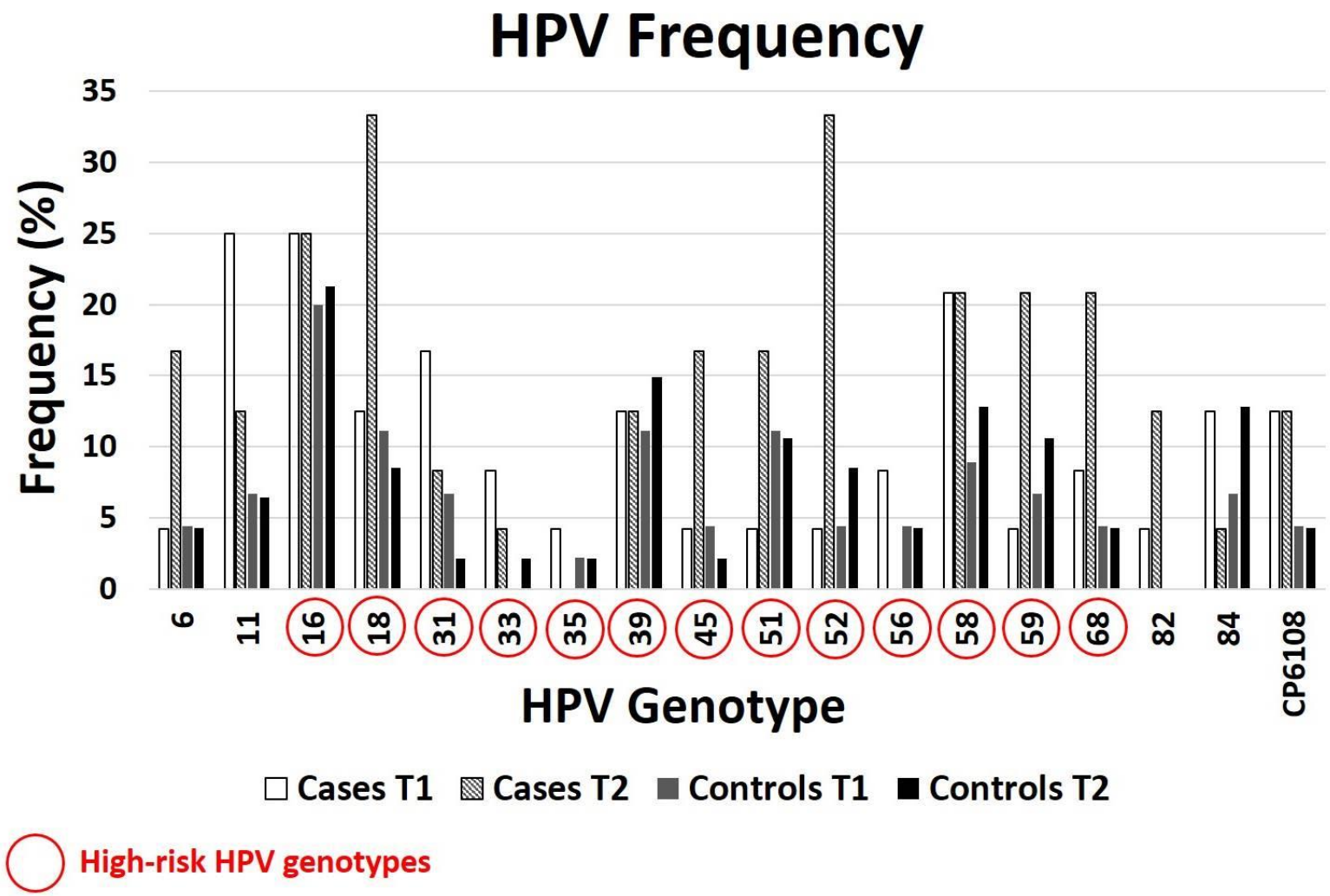

Figure 2. Frequency of 13 high-risk HPV genotypes and select low-risk genotypes in anal cytology specimens

In contrast, frequency of HPV genotypes in anal cytology specimens from matched controls exhibited rather less dramatic change from T1 to T2. High-risk HPV genotypes 16, 33, 39, 52, 58, and 59 increased modestly while high-risk HPV genotypes 18, 31, and 45 decreased modestly. High-risk types 35, 51, 56, and 68 changed negligibly as did low-risk HPV genotypes 6 and 11. Nonetheless, HPV 16 was the most frequently detected HPV genotype in anal cytology specimens from matched controls at both T1 and T2.

Overall, detection of any HPV genotype in anal cytology specimens from cases increased from 79.2% to 91.7% while any high-risk (HR) HPV genotype increased from 66.7% to 83.3% during a mean interval of 34.5 weeks between T1 and T2. For matched controls, any HPV genotype rose moderately from 71.1% to 76.6% while any HR HPV genotype rose slightly from 62.2% to 63.8% during a mean interval of 48.2 weeks between T1 and T2. These statistics are summarized in Table 2 and graphed in Figure 3.

Table 2. Frequency in anal cytology specimens of any detectable HPV genotype or any detectable high-risk HPV genotype during interval T1 to T2

	Frequency (%)			
	Cases T1	**Cases T2**	**Controls T1**	**Controls T2**
Any HPV	79.2	91.7	71.1	76.6
Any HR HPV	66.7	83.3	62.2	63.8
Mean time from T1 to T2	34.5 weeks		48.2 weeks	

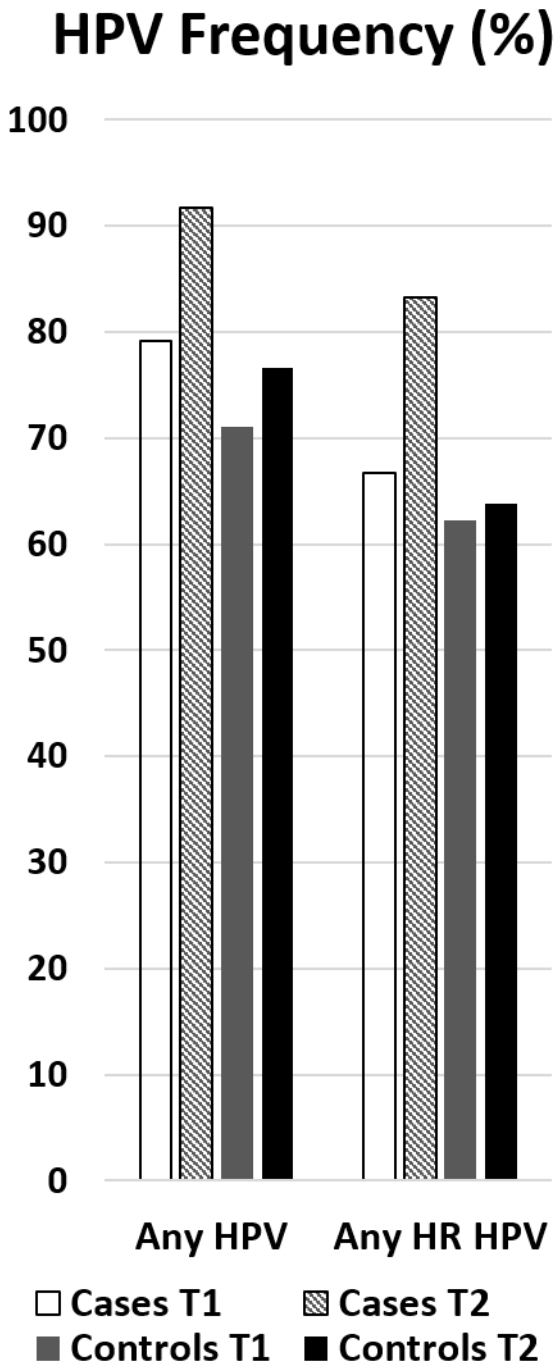

Figure 3. Frequency in anal cytology specimens of any HPV genotype or any high-risk HPV genotype for cases and matched controls at T1 and T2

When conditional logistic regression models were applied, HPV acquisition not clearance was associated with HIV seroconversion. Acquisition of any HPV genotype and acquisition of any HR HPV genotype conferred higher odds for HIV seroconversion: OR=4.75, 95% CI: 1.01-22.32 (p=0.049) and OR=4.93, 95% CI: 1.36-17.96 (p=0.015), respectively. In comparison, clearance of any HPV genotype or clearance of any HR HPV genotype was not significantly associated with HIV seroconversion: OR=1.64, 95% CI: 0.56-4.85 (p=0.37) and OR=1.16, 95% CI: 0.46-2.91 (p=0.75), respectively. These statistics are summarized in Table 3.

Table 3. Conditional logistic regression models for odds of HIV seroconversion

Covariate	OR	LCI	UCI	p-value
Cleared any HPV genotype	1.64	0.56	4.85	0.37
Cleared any HR HPV genotype	1.16	0.46	2.91	0.75
Acquired any HPV genotype	4.75	1.01	22.32	0.049
Acquired any HR HPV genotype	4.93	1.36	17.96	0.015

OR = odds ratio
LCI = lower 95% confidence interval
UCI = upper 95% confidence interval

DISCUSSION

Among cases in this study, more oncogenic HPV genotypes increased than decreased in frequency in anal cytology specimens by HIV seroconversion visit, with types 18 and 52 in particular rising dramatically by T2 to 33.3% each. Meanwhile, frequency of low-risk genotypes associated with genital warts also changed significantly: HPV-6 surged (4.2% to 16.7%) while HPV-11 declined sharply (25% to 12.5%) among cases during the same time period. In contrast, the shift in frequency of detectable HPV genotypes in anal cytology specimens from matched controls generally occurred in only modest increments. Interestingly, oncogenic HPV-16, which is typically the most prevalent type in HPV-associated dysplasia/cancer, maintained relatively stable frequencies among both HIV seroconverter cases (25%) and matched HIV-negative controls (20% to 21.3%).

The overarching trends are reflected in the changing frequencies of any detectable HPV genotype and any high-risk (HR) HPV genotype, with more evident increases among HIV seroconverter cases (>12%) compared to matched HIV-negative controls (<6%). Analysis using conditional logistic regression models did find an association between acquisition of any HPV genotype or any HR HPV genotype and HIV seroconversion. Clearance of any HPV genotype or any HR HPV genotype was not associated with HIV seroconversion.

Due to its retrospective case-control design, this study was limited to specimens previously collected for the parent Test & Treat study. T2 anal cytology specimens for cases were collected at HIV seroconversion visit. More frequently collected anal cytology specimens and corresponding HIV serology were not available to ascertain timepoint of HIV acquisition relative to HPV detection. While this study did find an association between HPV acquisition and HIV

seroconversion, it is possible that increased HPV acquisition occurred not as a precursor but as a consequence of acute HIV infection. In addition, analyzing the acquisition or clearance of any detectable HPV genotype or any HR HPV genotype as a group may dampen the apparent effect of acquisition or clearance of any individual HPV genotype. Unfortunately, the study was not designed to perform this type of analysis, and its sample size and statistical power were inadequate for such.

Nonetheless, a growing body of literature now exists, including two meta-analyses [7, 8] supporting the interpretation that HPV infection increases the risk of subsequent HIV acquisition. While the majority of original research citations examine HPV infection and associated HIV acquisition among women [10-15], others reach similar conclusions about HPV infection and HIV acquisition among men. Chin-Hong et al. found an association between anal HPV infection and HIV acquisition among MSM, independent of sexual activity, substance use, and other sexually transmitted infections [16]. Meanwhile, Tobian et al. established an association between clearance of penile HPV infection and HIV acquisition among Ugandan men [9].

Although the exact nature of HPV infection's influence on HIV acquisition is not yet understood, some researchers believe there is sufficient evidence to investigate the effect of HPV vaccination on HIV incidence. In journal commentaries, van der Loeff et al. have advocated for rigorous randomized control trials [17] while Rositch et al. have promoted mass vaccination and surveillance in countries with high HIV incidence as a more ethical and cost-effective approach [18]. In the absence of an effective HIV vaccine, HPV vaccination could prove a novel strategy for reducing the global incidence of HIV.

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SUMMARY OF SPECIFIC AIM 3

This study aimed to determine whether an association exists between HIV seroconversion and acquisition or clearance of anal HPV genotypes. Based on data from the Thai Test & Treat cohort, HPV acquisition not clearance was associated with HIV seroconversion. While some researchers have published similar results, others concluded that HPV clearance was associated with HIV acquisition. If further research confirms HPV's role in subsequent HIV acquisition, HPV vaccination could prove a novel strategy for reducing the global incidence of HIV.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The central objective of this study was to further elucidate the relationship between HIV and HPV infection in anal dysplasia/cancer by assessing HPV infection in the context of HIV and vice versa. A substantial catalog of scientific literature has consistently found increased incidence of HPV in the wake of HIV infection. Even while on effective cART, HIV patients are more susceptible to HPV infection and associated dysplasia/cancer. Although less well-studied, a growing body of literature suggests that the reverse is also true: HPV infection increases the risk of subsequent HIV acquisition. This study was designed with these bi-directional interactions in mind.

First, the study demonstrated that HPV at non-anal sites may be associated with anal dysplasia/cancer among HIV-positive males. The presence of HPV and the number of HPV genotypes at anatomical sites other than the anus trended toward higher odds of ASIL. Surprisingly, presence of HPV and the number of HPV genotypes at the penile shaft conferred the highest, statistically significant odds of ASIL – greater odds than conferred by presence of HPV and the number of HPV genotypes at the anus itself. HPV at non-anal sites may be linked to ASIL due to autoinoculation – transfer of HPV between sites by the participant himself – or to behavioral factors among the predominantly MSM participants resulting in HPV introduction across multiple anatomical sites. Regardless, these data suggest that HPV detection at non-anal sites may be useful indicators of ASIL risk among HIV-positive males.

Second, the study showed the potential of HPV quantitation for enhanced screening and diagnosis of anal dysplasia/cancer in the context of HIV infection. Here, all participants with HGAIN had HPV-16 E6 DNA levels above 10 copies per cell. Three participants with LGAIN and

one with a Negative biopsy also had detectable HPV-16 E6 DNA above a threshold of 10 copies per cell. While the anal cancer screening algorithm recommends annual HRA follow-up for those with LGAIN, perhaps the same recommendation should apply for those with Negative biopsy results but high HPV-16 copy numbers. For the remaining seven participants with LGAIN or Negative biopsy results, their low HPV-16 E6 copy numbers beg the question whether invasive HRA with biopsy could have been avoided. Since cytology and biopsy results are known to be dependent on the pathologist's expertise and experience, HPV quantitation may prove useful as an objective supplemental diagnostic marker.

Third, the study explored the effect of prior HPV infection on acquisition of HIV. Among MSM and TG women in the Thai Test & Treat cohort, HPV acquisition appeared to increase the risk of subsequent HIV seroconversion. While there are previous publications supporting this conclusion, others instead link HPV clearance to HIV acquisition. Despite remaining ambiguity over HPV's role in HIV acquisition, some researchers are advocating for study of HPV vaccination as a means for reducing HIV incidence.

In its entirety, this study has contributed to the body of knowledge in the field by identifying new diagnostic indicators of HPV-associated anal dysplasia and by providing additional evidence for HPV's influence on HIV acquisition. Further investigation will permit validation of these novel diagnostic markers and innovative strategy for reducing the global incidence of HIV.

FUTURE DIRECTIONS

The Hawaii HIV Laboratory (HHIVL) continues to explore the relationship between HIV and HPV infection. An upcoming project will investigate differences or similarities in biomarkers and risk factors influencing development of anal neoplasia between HIV-serodiscordant partners. An additional component will evaluate Raman spectroscopy of anal biopsies as a potential diagnostic tool in screening for anal cancer.

The second year of RMATRIX Pilot Project RM004 is in progress with anal cytology specimens collected outside the clinic by participants themselves, using a mail-in kit designed by HHIVL. As in year one, the same triad of biomarkers (HIV DNA copy number, high-risk HPV copy numbers, and p16 protein) are being assessed for association with anal dysplasia. In addition to HPV-16 E6 DNA copy numbers, HPV-18 E7 DNA copy numbers are also being assayed. If comparable to data from year one when specimens were collected in the clinic, results from year two will validate the feasibility of self-collection mail-in kits as well as the utility of biomarkers including HPV-16 E6 DNA copy numbers for anal cancer screening. It is also hoped that a kit for self-collection of specimens will reduce stigma and other barriers to access for anal cancer screening.

A currently unfunded research project proposes to exploit recent advances in flow cytometry to distinguish cells from anal cytology specimens that express high versus low levels of HPV type-specific mRNA and protein. High-expression cells would be sorted separately from low-expression cells. Extracted total nucleic acid could be assayed not only for HPV type-specific DNA copy number but also for differential expression of other genes not typically linked to HPV

infection. Network analysis could pinpoint signaling pathways that influence development of HPV-associated anal dysplasia and cancer.

Meanwhile, as more evidence supporting the association between prior HPV infection and subsequent HIV acquisition has accumulated, some researchers have begun to advocate for studies examining the use of HPV vaccination to reduce HIV incidence. The debate about best approach – randomized control trial versus mass vaccination and surveillance – has already begun. In the absence of an effective HIV vaccine, HPV vaccination could prove a novel strategy for reducing the global incidence of HIV.