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# Host genetic factors associated with cervical human papillomavirus clearance

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# HOST GENETIC FACTORS ASSOCIATED WITH CERVICAL HUMAN PAPILLOMAVIRUS CLEARANCE

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A DISSERTATION

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ABSTRACT

This dissertation research focuses on how variants in various host genes are associated with clearance of HPV in three longitudinal cohorts from the United States. I first examined the definition of the intermediate phenotype to cervical cancer, HPV persistence and then I incorporated this phenotype in all my three aims by examining variations in xenobiotic metabolism genes in HIV-1 negative and immune-related genes in HIV-1 positive females and how these contribute to HPV infection outcomes. Several significant variants were associated with HPV clearance for the three aims.

For the first aim, in ALTS assessing functional variants within xenobiotic metabolism genes and clearance of HR-HPV, I observed a functional variant allele in *CYP1A1* was significantly associated with lower clearance rates of HR-HPV and the *GSTM1* null variant was significantly associated with higher clearance rates of HR-HPV (Chapter 3). In the second aim, using the REACH cohort assessing SNPs within Interleukin family of cytokines and Toll-like Receptors (TLRs) and their influence on HR-HPV clearance, I observed HR-HPV clearance rates were significantly associated with five SNPs that mapped to coding and regulatory regions in three genes (*IL2RB*, *IL1RN*, and *IL7R*) (Chapter 4). Finally in the third aim, using the HERS cohort assessing SNPs within several immune related genes and their influence on HR-HPV, I observed

several significant associations between SNPs, specifically those located on chromosome 6 in the HLA-G region was associated with higher clearance rates (Chapter 5).

Host genetic factors associated with higher clearance rates of HR-HPV could serve as potential biomarkers for future HPV related disease. Also, identification of susceptibility loci may allow earlier diagnosis based on an individual's genetic constitution and may facilitate identification of disease subtypes amenable to therapeutic approaches.

Keywords: Host genetics, HPV, HIV

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# DEDICATION

For my husband Tyler, my parents Michele and Joe, and Doug and Denise, my siblings Eric and Matt, and the rest of my family.

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IMMUNOGENETICS OF HPV CLEARANCE AMONG HIV-1 POSITIVE WOMEN IN THE HIV EPIDEMIOLOGY RESEARCH STUDY (HERS)

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AIDS | acquired immunodeficiency syndrome |
| ALTS | atypical squamous cells of undetermined significance-low-grade squamous intraepithelial lesion triage study |
| ASC-US | atypical squamous cells of undetermined significance |
| CD4+ | CD4+ T lymphocyte cells |
| CIN | cervical intraepitheilial neoplasia |
| CNV | copy number variation |
| CYP1A1 | cytochrome P450, family 1, subfamily A, polypeptide 1 |
| DHFR | dihydrofolate reductase |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immune sorbent assay |
| FDR | false discovery rate |
| GSTM1 | glutathione S- transferase mu 1 |
| GSTT1 | glutathione S- transferase theta 1 |
| GWAS | genome-wide association study |
| HAART | highly active antiretroviral therapy |
| HERS | HIV epidemiology research study |
| HIV-1 | human immunodeficiency virus type 1 |
| HLA | human leukocyte antigens |
| HPV | human papillomavirus |
| HR-HPV | high risk HPV |
| HWE | Hardy Weinberg equilibrium |

| | |
|---|---|
| IL | interleukin |
| LD | linkage disequilibrium |
| LR-HPV | low risk HPV |
| LSIL | low grade squamous intraepithelial lesions |
| MAF | minor allele frequency |
| MHC | major histocompatibility complex |
| OC | oral contraceptive |
| Pap | papanicolaou test |
| PCA | principal component analysis |
| PCR | polymerase chain reaction |
| QC | quality control |
| Q-Q | quantile-quantile |
| REACH | reaching for excellence in adolescent care and health |
| SNP | single nucleotide polymorphism |
| STI | sexually transmitted infection |
| TLR | toll-like receptors |
| UTR | untranslated region |
| VNTR | variable number tandem repeats |
| WLW | Wei-Lin-Weisfeld |

# CHAPTER 1

# INTRODUCTION

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted infections (STIs) in both men and women. Annually, 160 million incident infections of HPV are estimated worldwide although the rates of dysplasia and cervical cancer (529,409 cases) performed by clinical diagnosis are significantly lower.[1] Approximately 80% of the female population is exposed to HPV sometime in their lifetime, but the infection is usually transient, with 70-90% of infected individuals "clearing" the virus (HPV DNA undetectable by assays) within 12-24 months and only a small proportion will progress to cervical cancer.[2, 3] The main consequence of persistent infection with HPV is the development of precancerous cervical lesions that may progress to malignancy in the next 5-15 years after infection, and this subsequently could result in invasive cervical cancer.[4]

HPV persistence has been consistently and strongly associated with precancerous lesions and predicting the risk for cervical cancer.[5-7] HPV infections occurring at older ages could have little impact on cancer cases compared to persistent infections occurring at earlier age, due to the years required for cancer development. The role of persistent infection in predicting future risk of cancer further emphasizes the need for earlier detection of disease progression by monitoring the persistent infection with HPV, before the development of lesions. Therefore the research for my dissertation focused on HPV persistence, which I define as the intermediate phenotype to cervical cancer.

The importance of this intermediate phenotype, HPV persistence is described in detail in Chapter 2 of my thesis. This chapter describes why HPV persistence is the intermediate phenotype to cervical cancer and the challenges that come along with using this intermediate phenotype in research studies.[8] Chapter 2 lays the foundation for the background for my thesis as well as the methods that were incorporated into the research manuscripts for Chapter 3, 4 and 5 (Table 1). A recurring theme of assessing host genetics factors associated with HPV clearance will be seen in those three chapters and similar methods will be used that were provided in detail in Chapter 2.

Functional variants in xenobiotic metabolism genes have been associated with cervical cancer risk. This association made me question whether this association with cervical cancer could be a surrogate for a true association with HPV persistence because this inflammation could result in DNA damage, which would allow HPV to integrate. My chapter 3 specific aim was: t*o assess whether functional variants in xenobiotic metabolism genes are associated with cervical HPV infection in the ALTS study.* The functional variants are in three genes *CYP1A1*, *GSTT1*, and *GSTM1*, which were chosen based on their previous association with cervical cancer risk. The cohort is comprised 450 women from the Atypical squamous cells of undetermined significance-low-grade squamous intraepithelial Lesion Triage Study (ALTS), which was designed to determine the optimal clinical management for low-grade cervical cytological abnormalities.[9, 10]

Most HPV infections are eventually cleared due primarily to a strong localized cell-mediated immune response; however, the virus persists in a subset of the population.[11] Host genetics factors of immune related genes may explain differences in the ability to clear the HPV infection.

Table 1. Study design characteristics for the three cohorts used for the three aims

| | **ALTS** | **REACH** | **HERS** |
|---|---|---|---|
| Study purpose | Determine the optimal clinical management for low-grade cervical cytological abnormalities | Study of HIV disease progression in adolescents infected through sex or drug behaviors | Natural history of HIV in women |
| Recruiting location | 4 US cities | 13 US cities | 4 US cities |
| years | 1996-1998 | 1995-1999 | 1993-1995 |
| Ethnicity | Self-report | Aims markers | Principal component analysis |
| Follow up | every 6 months | every 3 months HIV-related data; every 6 months for HPV testing | every 6 months |
| Total Sample size | 1549 women | 435 females and 143 males; HIV+/- | 830 HIV+/- women |
| HPV Detection | L1 consensus primer PGMY09/11 | consensus primers MY09/11 and HMB01 | consensus primers MY09/11 and HMB01 |
| HPV types | 31 | 32 | 26 |
| Subset used for this analysis | 450 ancillary study from Birmingham | Subset comprised of 134 HIV+ African American females | Subset 258 African-American HIV+ |
| Study Aim | Aim 1 | Aim 2 | Aim 3 |

My Chapter 4 aim was: *to assess host genetic variants related to clearance/persistence of HR-HPV in HIV-1 positive African-American adolescent females, selected based on two groups of candidate genes encoding Interleukin family of cytokines and Toll-like Receptors (TLRs)* that have been reported to have functional consequences or associations with cervical cancer, HPV infection or other infections. There were 267 SNPs within the exons, 5' UTR and 3' UTR sequences of 35 immune-related candidate

genes encoding interleukin family of cytokines and toll-like receptors included in the analysis. There were 134 HIV-1 African-American adolescent participants from the Reaching for Excellence in Adolescent Care and Health (REACH) cohort.[12, 13]

Lastly, I wanted to assess genetic variants in immune related genes and their association with HPV clearance on a broader scale by using the Illumina ImmunoChip that is comprised of densely spaced SNP variants, developed by a consortium of specialists in the fields of immunology and inflammation. [14, 15] The chip is comprised of assays for 196,070 SNPs (of which 5,001 are non-synonymous coding, 1,926 are synonymous coding, and 4,065 are in the UTR). My chapter 5 aim was*: to assess host genetic variants related to clearance/persistence of HR-HPV in HIV-1 positive women using the Human ImmunoChip.* African-American HIV-1 positive women from the HIV Epidemiology Research Study (HERS) cohort were included in this study. [16]

In summary, the aims of my dissertation involve a comprehensive examination of variations in xenobiotic metabolism genes in HIV-negative (chapter 3), and immune-related in HIV co-infected individuals (chapters 4 and 5) and how these contribute to HPV infection outcomes. The results from these aims hold important public health significance because they focus on prevention of persistent HPV infection which could potentially reduce incident cases of precancerous lesions as well as cancer.

# KEY CONSIDERATIONS AND CURRENT PERSPECTIVES OF EPIDEMIOLOGICAL STUDIES ON HUMAN PAPILLOMAVIRUS PERSISTENCE, THE INTERMEDIATE PHENOTYPE TO CERVICAL CANCER

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**Abstract**

Persistent infection with human papillomavirus (HPV) causes essentially all precancerous cervical lesions and cervical cancer in females and thus is an important intermediate phenotype to cervical cancer. A majority of infected individuals naturally clear HPV viral infection, but the virus persists in a subset of infected hosts and the mechanism for this differential outcome is not well described. Most of the epidemiological studies have been cross-sectional in nature, and even with longitudinal studies, the definition of HPV persistence or clearance has not been well defined. There is no consensus on the correct time interval between HPV DNA testing or how to utilize HPV persistence information in clinical management because there is no treatment for HPV. While most studies are performed with the endpoints of cancer, the intermediate phenotype has been overlooked. Epidemiological studies of HPV persistence suffer with several challenges in definitions, study designs and analyses that undermine its importance in identifying and understanding the interactions between the viral and host genomes in the process of HPV infection pathogenesis. We have evaluated the current status of HPV persistence and provided perspectives on how the field would benefit with a research focus on intermediate phenotype in epidemiological studies.

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted infections (STIs) in both men and women. The virus is highly contagious and studies have estimated HPV transmission probability to be as high as 40-60% following unprotected sexual intercourse.[1] Annually, 160 million incident infections of HPV are estimated worldwide; however, as you progress to dysplasia and then to cervical cancer (529,409) the rates significantly decrease (Figure 1). Incident HPV infections seem to be age dependent where HPV infections peak soon after the age when most young women become sexually active (average age of 20 years) and is usually followed by a gradual decline. Approximately 80% of the female population is exposed to HPV sometime in their lifetime, but the infection is usually transient, with 70-90% of infected individuals "clearing" the virus (HPV DNA undetectable by assays) within 12-24 months and only a small proportion will progress to cervical cancer (Figure 1).[2, 3] The main consequence of persistent infection with HPV is the development of precancerous cervical lesions that may progress to malignancy in the next 5-15 years after infection, and this subsequently could result in invasive cervical cancer.[4]

HPV persistence has been consistently and strongly associated with precancerous lesions.[5, 6] A recent 16-year longitudinal study also confirmed the critical role of persistent carcinogenic HPV infections in predicting risk of cervical cancer in women.[7] Based on this study, a 16-year risk of cervical cancer was 6.2% among women infected with any carcinogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) HPVs and 13.5%, 10.3%, or 4.0% for women infected with HPV16, HPV58, or other carcinogenic HPVs (without HPV 16 and 58), respectively. However, the rates were lower with other HPV type infections; 2.1%, 1.1% and 0.26% among women infected with

possibly/probably carcinogenic HPV types, other non-carcinogenic HPV infections, and HPV-negative women, respectively. HPV infections occurring at older ages could have little impact on cancer cases compared to persistent infections occurring at earlier age, due to the years required for cancer development. These observations further emphasize the need for earlier detection of disease progression by monitoring the persistent infection with HPV, before the development of lesions. Research focus should include persistent HPV infection since this is the known precursor of cervical cancer. In this paper, we will focus on HPV persistence, which we define as "intermediate phenotype", and provide perspectives in epidemiological study designs and analyses.

**Why HPV persistence is the intermediate phenotype?**

Epidemiologic and virologic data demonstrate that 13-15 high risk, or oncogenic HPVs are the primary and necessary causal agents of cervical cancer;[8, 9, 10] HPV infection is attributable to 99.9% of cervical cancers, and oncogenic HPV types 16/18 are responsible for 70% of all cervical cancers.[11] Individual HPV infections are not independent from each other in either sex, meaning that acquisition of multiple HPV types occurs more often than expected.[12] However, no two HPV types are more likely to be acquired together than any other HPV types in several populations.[12-14] While these cross-sectional studies show strong associations, HPV is required to persist to cause necessary cellular changes in the host to progress to cancer. Since most HPV infections do not clear, persistent HPV infection is a prerequisite and thus we consider it as an "intermediate phenotype". An infection persisting for more than four years has only a small chance of remission.[15] Women who have persistent infection can develop CIN lesions; however, a

proportion of high-grade cervical lesions may never progress to cervical cancer and can even regress without treatment (Figure 2). However, the status of HPV is not known during the cervical lesion regression process, i.e. whether regression is linked to clearance of HPV.

Most epidemiological studies have described CIN or cervical cancer as the outcome in the host and only few have incorporated HPV persistence as the main outcome. HPV pathogenesis with respect to the cervical carcinogenesis in the host should be viewed in two separate phases; first, the biology related to virological and host immunological process of HPV persistent infection; and second, the functionally important stages in cervical cancer progression. It should be noted that the first phase is a prerequisite to the second phase. The limited studies on HPV persistence suggest that multiple HPV infections,[16] smoking,[17] and multiple lifetime sexual partners[18, 19] are the main factors associated with persistent HPV infection. However, some of the epidemiological and biological factors associated with cervical cancer could be a surrogate of HPV persistence, where it is confounded by the selection bias of HPV persistent individuals among cancer patients. For instance, human leukocyte antigens (HLA) have shown to be associated with cervical cancer in several studies,[20] but of note, HLA and other immune related genes may be more involved with persistence or clearance of HPV.

The importance of HPV persistence as the intermediate phenotype has been acknowledged in the clinical settings, resulting in performing HPV tests into several screening programs. In a large longitudinal study, Castle, et al, recently described that while both baseline Pap and HPV tests predicted development of CIN3 within the first 2

years of follow-up, only HPV testing predicted CIN3 in 10 to 18 years.[21] Precancerous lesions and cervical cancer have often been the public health focus and recently HPV testing has been recommended in clinical screening.[4] Similarly, persistent HPV should also be carefully considered in research settings to understand the dynamics of how some are susceptible to persistent infection while most are able to overcome.[22] Phenotypically, it is extremely important for epidemiological studies to accurately define the intermediate phenotype, determine the correct HPV types, and systematically be able to analyze the complex data.

## Challenges in research studies of HPV persistence, the intermediate phenotype

### *Epidemiological Study Designs*

There has been considerable heterogeneity in study design and methodological approaches in various cohort studies examining the natural history and persistence of HPV. The most common epidemiological study design for HPV is a cross-sectional design that estimates the prevalence at any time point, but does not provide information regarding HPV persistence and clearance. Prevalent cases may have had the infection for few days to years making these women significantly different than those with an incident infection during follow up. In that sense, longitudinal data are more powerful and are better predictors for the outcome of interest. Ideally, a study assessing persistence of HPV in the population needs to follow women before their first HPV infection and for an extended period in order to be certain that the HPV infections are truly incident infections and not latent. Including only those with incident HPV infections allows the researchers a clearer understanding of when the individual was infected. While everyday sampling is

theoretically possible with self-sampling approaches, it is logistically not quite feasible; thus, data from shorter visit periods will be more informative. There have been very few long-term longitudinal studies that have actually followed persistent HPV and assessed the risk of cervical cancer among women.[7, 23]

*Definitions of HPV persistence*

Correctly defining the intermediate phenotype is critical for accurate study design and analysis. "HPV viral persistence" is often defined as detection of the same HPV type at two or more intervals.[5, 6, 24, 25] In a recent meta-analysis by Rositch, et al, reported that the definition of HPV persistence was mediated by study region, detection method, and HPV type.[25] They estimated that approximately half of the HPV infections persist past 6-12 months. These findings coincide with the current ASC/ASCCP/ASCP guidelines that recommend a one year repeat screening interval for women over 30 years who are HPV-positive with normal cytology.[25] If a woman is going to clear an HPV infection, it will likely occur within a year and future follow up is needed if HPV persists longer than one year. This meta-analysis emphasizes the need for a concise definition of HPV persistence and time interval between HPV DNA tests in order to more effectively determine clinical and treatment outcomes.

After detection of the HPV virus, "HPV viral clearance" is often defined as not detecting the same HPV type at two subsequent visits following a HPV positive visit, thus requiring "two consecutive visits".[24] The main issue with these definitions is that there is no standard biologically relevant duration. While most studies use "two consecutive visits" for clearance to rule out false negatives, this process may also inflate

the time to clearance since the exact date of clearance is unknown. It is also possible that negative HPV diagnostic results could indicate shedding of HPV virus at quantities below the limit of detection or the latent phase of HPV. Latency will change the definition of clearance, persistence and reinfection and this concept of HPV is still poorly understood and further research is warranted.

*HPV testing methods*

There are several HPV genotyping methods currently being used. Molecular methods have been developed for HPV detection, including those based on signal amplified hybridization, polymerase chain reaction (PCR), DNA sequencing, type-specific probes, reverse line-blot hybridization, *in situ* hybridization, southern blot hybridization and immunological techniques, including ELISA and western-blot. The gold standard is based on PCR, but still the results can vary across methods. PCR-based methods are quite sensitive to minimal amounts of HPV, and even if the virus is not totally cleared from the system, it is still capable of detecting trace amounts of virus. Yet, it is not quite possible to characterize and differentiate a new infection from reinfection with the same HPV type as some virus could remain latent in basal cells at undetectable levels.[24] Other parameters of the virus, such as viral variants, viral load, expression and genomic integration capacity,[24] specifically in relation to co-infections, will need to be assessed as possible markers in future studies.

*Analytical methods*

A major analytical challenge has been the prevalent cases and censoring of events; since, as in any prospective study, it is not clear how long HPV persists after the

end of the study. To account for some of these, Cox model has been the standard approach; however, this model cannot simultaneously analyze time to clearance of several types of HPV because it does not address possible correlations between incident HPV infections. Cox model with the Wei-Lin-Weisfeld (WLW) extension accounts for the correlation between HPV subtypes within a person and has population-level interpretations.[26] While the Cox model with the frailty term also accounts for the correlation between HPV subtypes within a person, it has individual-level interpretations, which is difficult to comprehend in epidemiological studies.[26, 27] Other methods, such as the model based on transitional probability,[28] the framework model based on the clustered longitudinal binary data structure,[29] and the discrete-time semi-Markov models[30] have also been used to account for both prevalent and incident infections.

Additionally, missing HPV data in longitudinal studies can have a significant impact in correctly identifying persistence and clearance. Certain assumptions are made based on the data, but in several instances, a premature censoring or exclusion of the individual episode are often required to outweigh the benefit of larger samples size. For example, if the lower threshold of assay detection is in question, one would want HPV negative results in two consecutive testing intervals and the ideal time to clearance will be the midpoint between the last positive visit and the first negative visit (Table 1. Scenario 1). In another example (Table 1 Scenario 7), the individual could be positive for HPV at visit 2, negative at visit 3, data missing at visit 4 and negative at visit 5. One would assume that the HPV type remains the same as the previous visit, which indicates that the HPV virus cleared in this individual and this assumptions hold at visit 5. However, missing data scenarios could be quite complex and thus, the interpretation

could be very subjective. For consistent use of the intermediate phenotype, a clear consensus should be made on the biological definition of HPV persistence.

## Conclusion

Throughout this paper, we have described how the focus on cervical cancer and precancerous lesions are too little too late because the virus has already evaded the immune system and initiated the integration process. In clinical settings, HPV screening would be the first step and confirmation of persistent infection from follow-up HPV testing would complement cytology testing. While prophylactic HPV vaccine to 4 types is available, challenges remain for researchers to understand the pathogenesis of HPV, specifically among those already infected, those who get infected with other oncogenic types and those who do not get vaccinated. There is consensus that persistence of HPV is the known, main factor for progression to precancer and cancer. Thus, research based on the well-defined intermediate phenotype could provide valuable information for prevention and alternatives to progression of cancer. The technology to detect the virus is feasible in most settings and with careful follow-up plans would greatly assist scientists with their research and clinicians with their screening programs to help recognize, monitor and manage the burden of the disease.

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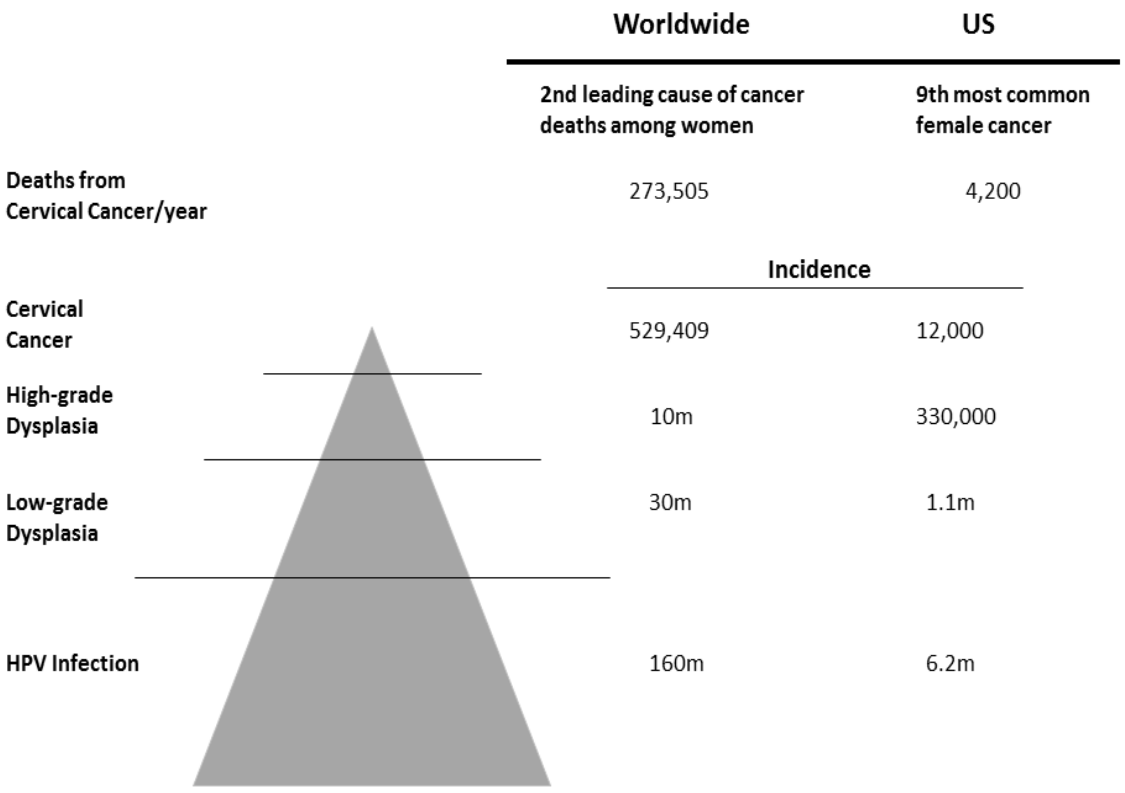
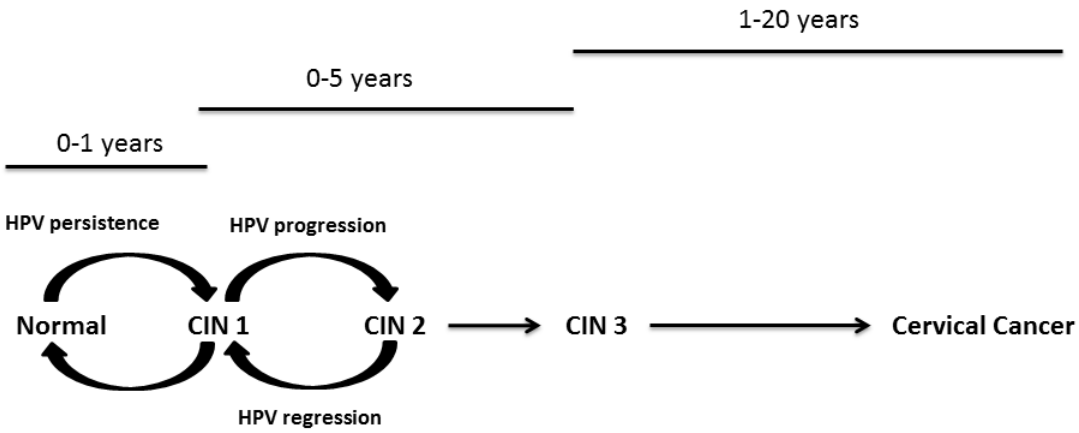



**Figure 1.** HPV infection and cervical abnormalities epidemiology in the United States (US) and Worldwide. [31-33]




| Cervical Histological Lesion | Regression to normal | Lesion persists but unchanged | Progress to CIN3 | Progress to CC |
|---|---|---|---|---|
| CIN1 | 60% | 20-30% | 5-10% | 1% |
| CIN2 | 40% | 40% | 15-20% | 5% |
| CIN3 | 33% | | | 10-12% |

**Figure 2.** HPV persistence and progression to cervical abnormalities timeline and estimated likelihoods of regression and progression of cervical histological lesions (data based on various resources.[33-35]

**Table 1. HPV clearance and definitions of prevalence, incidence, clearance, left censoring, and right censoring**

| | | | V1 | | V2 | | V3 | | V4 | V5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | → | | | | | | | | | |
| **Scenario 1** | | **I** | **-** | | **+** | | **+** | * | **-** | **-** | | **C** |
| **Scenario 2** | | **P-LC** | + | | + | * | - | | - | - | | **C** |
| **Scenario 3** | | **P-LC** | + | | + | | + | * | - | - | | **C** |
| **Scenario 4** | **baseline** | **P-LC** | + | | + | | + | | + | + | **end of follow-up** | **RC** |
| **Scenario 5** | | **I** | - | | - | ¥ | + | | + | - | | **RC** |
| **Scenario 6** | | **I** | - | ¥ | + | | - | | + | - | | **RC** |
| **Scenario 7** | | **I** | - | | + | | - | * | M | - | | **C** |

**P-LC = prevalence, left censored; I = Incidence (¥); RC = right censored; C = cleared (*); V1-V5 = visits 1 to 5 as examples; M=Missing**

# FUNCTIONAL VARIANTS IN XENOBIOTIC METABOLISM GENES ARE ASSOCIATED WITH CLEARANCE OF HPV INFECITON

by

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**Abstract**

Chronic inflammation is a well-established factor in the pathogenesis of cancer. We evaluated time to clearance of any human papillomavirus (HPV) infection in relation to functional variants in three genes (*CYP1A1*, *GSTT1*, and *GSTM1*) that are involved in xenobiotic metabolism, as well as a subset analysis of time to clearance of high risk (HR-HPV) HPV types. The study group consisted of 450 HPV infected women from the Atypical squamous cells of undetermined significance-low-grade squamous intraepithelial Lesion Triage Study (ALTS) cohort followed up at the clinical center at Birmingham, Alabama. The Cox proportional hazard model with the Wei-Lin-Weisfeld (WLW) approach was used, controlling for relevant covariates. The majority of HPV infected women were African American (65%), reported having a high school degree or less (59%), currently smoked (84%), and had children (80%). Women who were polymorphic for CYP1A1 experienced a HR-HPV clearance rate that was 20% (HR=0.80, p=0.04) lower than women without the polymorphism for *CYP1A1*, adjusting for all other cofactors. The *GSTM1* null genotype was associated with higher HR-HPV clearance rate (HR= 1.39, p=0.006). The polymorphism in *GSTT1* was not significantly associated with time to clearance of HR-HPV. Many carcinogens require metabolic activation enzymes like *CYP1A1*, which are then detoxified by enzymes like *GSTT1* and *GSTM1*. Reactive metabolites that are not detoxified may result in DNA damage, which may facilitate HPV integration resulting in lower likelihood of its clearance.

**Introduction**

Chronic inflammation is a well-established factor in the pathogenesis of cancer (1-4). Inflammation can promote cancer by: inducing cell proliferation, recruiting inflammatory cells, increasing reactive oxygen leading to oxidative DNA damage, and reducing DNA repair (Figure 1) (1). Functional variants of three xenobiotic metabolism genes: cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*), glutathione S-transferase mu 1 (*GSTM1*), and glutathione S-transferase theta 1 (*GSTT1*) have been associated with several cancers and other diseases. The *CYP1A1* gene functions in metabolic activation of polycyclic hydrocarbons (5). An association between *CYP1A1* polymorphism and increased risk for development of cervical cancer was reported in two of three studies (5-7). The enzyme-encoding genes of the glutathione S-transferase (GST) family have a critical function in the detoxification of a variety of both endogenous products of oxidative stress and exogenous carcinogens. Both *GSTM1* and *GSTT1* genes exhibit an inherited homozygous deletion polymorphism (null genotype) that is associated with absence of enzyme activity (8). The association between cervical cancer risk and polymorphism in *GSTM1* and *GSTT1* has not been consistently documented, as two meta-analyses (8, 9) show differences in risk among similar ethnic populations for *GSTM1* and hazardous versus null findings for *GSTT1*. Previous research has also found increased risk of cervical disease associated with combinations of these three polymorphisms, suggesting that interaction between these polymorphisms may be important (5). Inconsistent findings on the association of these polymorphisms with cervical cancer also suggest that studying this association at an early endpoint, HPV infection, may help elucidate the carcinogenic process (10).

HPV is the most common sexually transmitted infection in the United States (U.S.) (11). HPV infection is a necessary but not sufficient cause of cervical cancer. Whereas the vast majority of infected individuals naturally clear their infection(s), further research is warranted to distinguish risk factors that lead a subset of individuals infected with oncogenic HPV types to have persistent infection and later develop cervical lesions (12). It is unknown how polymorphisms in the three genes involved in the biotransformation pathway interact with HPV infection. HPV infection may modulate expression of cellular xenobiotic metabolizing enzymes, affecting the ability of cells to handle environmental carcinogens (13). Based on this background, we hypothesized that the three genes coding for xenobiotic metabolizing enzymes influence HPV clearance. In this study we examined the association of the three host gene polymorphisms with HPV infection outcomes taking into account other risk factors, multiple co-infections, and repeated HPV infections.

## Methods

### *Study Population*

The parent study, the Atypical squamous cells of undetermined significance-low-grade squamous intraepithelial Lesion Triage Study (ALTS) was designed to determine the optimal clinical management for low-grade cervical cytological abnormalities (14, 15). Between October 1996 and December 1998, 1549 women were recruited from clinical centers located in Birmingham, Alabama; Oklahoma City, Oklahoma; Pittsburgh, Pennsylvania; and Seattle, Washington. The women in this study were enrolled based on having cytology diagnoses of atypical squamous cells of undetermined significance (ASC-US) and low grade squamous intraepithelial lesion (LSIL). Participants with

cytology diagnoses of ASC-US and LSIL from referring community laboratories were followed every 6 months for 2 years (16). This study includes a subset of the ALTS cohort, from the clinical center at Birmingham, Alabama, who also agreed to participate in an ancillary study of nutrient interactions and risk of developing cervical intraepithelial neoplasia (CIN). At the enrollment visit, blood samples were obtained from 709 women who consented to participate in the ancillary study, and a brief questionnaire was administered to assess the use of vitamin supplements. The administration of a risk-factor questionnaire, pelvic examinations, collection of specimens for cytology and HPV testing, and cervicography were carried out by the main ALTS personnel, and the results were made available for the ancillary study.

*HPV detection and classification*

Cervical brush samples were collected every 6 months for two years. The viral DNA from cervical lavage were identified through L1 consensus primer PGMY09/11 polymerase chain reaction amplification and reverse line blot hybridization to detect 38 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 81, 85, 91, 73 [PAP238A], 82 [W13B], 83 [PAP291], and 84 [PAP155]) and a β-globin internal control (15, 17). The results were classified as negative or positive for each of the 38 HPV types at each visit and β-globin negatives were excluded from the analyses. Two consecutive HPV-negative tests were required to confirm clearance to minimize the influence of false-negative test results. HPV-infected women whose test became negative at the last study visit were censored at that time. Type-specific HPV status was assumed to remain unchanged across single

missing visits. Infections spanning more than two consecutive missing visits were excluded because of the increased uncertainty regarding HPV status.

Of the 709 women sampled, 259 were excluded from the longitudinal analysis in this study for the following reasons: HPV infection status known only at baseline (N=96), pregnancy during follow–up (N=1), HPV-negative for all 34 types throughout the study period (N=61), and inadequate follow up (2 consecutive missing visits, N=101) (Figure 2). Among the 450 HPV-positive (positive for any of the 38 HPV types) women included in this study, 118 of them were able to clear their HPV infection(s) during follow up, 67 had persistent HPV infection(s), and 265 had mixed outcomes, i.e., cleared an infection with a specific HPV type while continuing with a different HPV type through follow up.

*Laboratory methods*

*Genotyping CYP1A1*

The DNA sample was amplified in a 25-μl volume containing genomic DNA, 2 μM primers [5'-CAGTGAAGAGGTGTAGCCGC-3' (forward) and 5'-TAGGAGTCTTGTCTCATGCC-3' (reverse)], 0.2 mM of each deoxynucleotide triphosphate, 1 X PCR buffer G (Epicentre, Madison, WI), and 2.5 units of Taq polymerase (Promega, Madison, WI) (18). Amplification was performed by initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 61°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

An *MspI* restriction fragment length polymorphism in the 3' noncoding region of the *CYP1A1* gene was detected by PCR amplification as described by Hayashi, *et al* (18). An aliquot of the amplified mixture was digested with *Msp*I (New England Biolabs, Beverly, MA). The 340-bp PCR product from individuals with the wild-type allele is not

digested by *Msp*I. The 340-bp PCR product is digested by *Msp*I when the variant allele is present, to yield two fragments of 140- and 200-bp. Heterozygous individuals have all three bands (340-, 140- and 200-bp) after PCR and *Msp*I digestion. Individuals were categorized as either homozygous for the wild-type allele or heterozygous/homozygous for the variant allele.

*Genotyping GSTM1 and GSTT1*

A multiplex PCR assay described by Xiong *et al*, was used to genotype both the *GSTT1* and *GSTM1* polymorphism (19). The *DHFR* gene was amplified as an internal control to ensure that DNA degradation was not responsible for lack of amplification of *GSTM1* and *GSTT1*. *GSTM1, GSTT1*, and *DHFR* were coamplified in a 25-µl reaction mixture containing 100 ng of genomic DNA as the template, 3.5 pmol of each *GSTM1* primer, 2.9 pmol of each *GSTT1* primer, 6.2 pmol of each *DHFR* primer, 0.2 mM of each deoxynucleotide triphosphate, 1 X PCR buffer G (Epicentre, Madison, WI), and 2.5 units of Taq polymerase (Promega, Madison, WI). The conditions for PCR amplification were as follows: an initial melting step at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s, with a final step of 72°C for 10 min for elongation. The PCR products were separated on 1.2% agarose gels, and the gels were stained with ethidium bromide and photographed with a photodocumentation camera (Fisher Scientific, Pittsburgh, PA).

The *GSTT1, GSTM1* and *DHFR* primers generate 480-base pair (bp), 215-bp and 280-bp fragments, respectively. In this assay, the absence of a 480-bp band or a 215-bp

band indicates the *GSTT1* null or *GSTM1* null genotypes, respectively, provided that the 280-bp fragment of the *DHFR* internal control is amplified.

*Statistical Analysis*

*CYP1A1* did not deviate from Hardy-Weinberg Equilibrium (HWE) in all participants and also in a sub-population of African-Americans. HWE was not evaluated for GSTM1 and GSTT1 polymorphism because the PCR technique used in this study only recognizes the presence (wild-type or heterozygous genotype) or the absence (null genotype) of the genes, but does not distinguish between heterozygous and homozygous wild-type.

The Cox proportional hazard model was used to assess all type specific HPV clearance with functional variants in *CYP1A1, GSTM1,* and *GSTT1*. Due to the possible correlation between co-infected HPV types, the Wei-Lin-Weisfeld (WLW) extension was used with the Cox models. The WLW approach can simultaneously analyze time to HPV clearance of several types of HPV either at the same or at different visits (20). This method produces a population-averaged interpretation and allows different baseline hazards functions for each HPV type. The multivariable model was adjusted for cofactors that have been associated with cervical cancer development: age, education, smoking, parity, Bethesda classification diagnosis, age at first sexual intercourse, number of lifetime sexual partners and use of oral contraceptives (21). All analyses were adjusted for self-reported race to account for confounding by population stratification. Stratified analysis by each race was also performed to examine if there were variations.

Regression coefficients of the interaction between any two of the three genotypes were tested to examine the joint association of the polymorphisms with the HPV outcomes.

Clearance of high-risk (HR-HPV) HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was also assessed using the Cox proportional hazard model with the WLW extension adjusting for low-risk (LR-HPV) HPV subtypes that an individual may have been infected with while they were infected with a HR-HPV type. This subset analysis consisted of 392 individuals (Figure 2). Stratified analyses by smoking were also done since previous studies have shown associations with smoking and the genetic variants.

**Results**

Among the 450 HPV infected women, the majority were black/non-Hispanic (65%), reported a high school degree or less (59%), did not smoke (68%), had children (80%) and had used oral contraceptives in the past two years (55%). There were 1654 different HPV infections/co-infections (768 prevalent infections and 886 incident infections) among the 450 women and 939 (601 prevalent infections and 338 incident infections) of these cleared during follow-up. The top five most common infections by type were: HPV-16 (138 women), HPV-52 (111 women), HPV-62 (80 women), HPV-53 (77 women), and HPV-35 (73 women). Of the 939 infections that cleared during follow up, the average time to clearance was 108 days (range 197 days, min 46 days, max 243 days).

For the analysis assessing clearance of any HPV type (LR and HR) the Cox proportional hazard with the WLW model incorporated HPV type-specific baseline

hazard functions while assuming a common exposure effect (Table 1 and 2). In the multivariable analysis of non-genetic factors, the hazard ratio estimates indicate that the rate of HPV clearance increases by 6% (HR=1.06, p=0.02) for every one-year increase in age at first intercourse. Women with some college had a HPV clearance rate that is 26% (HR=0.74, p=0.003) lower than women with a high school diploma or less. Likewise, in the multivariable analysis of the genetic variants, women who were homozygous/heterozygous for the *CYP1A1* variant allele had a HPV clearance rate that was 20% (HR=0.80, p=0.02) lower than women that were homozygous for the wild-type allele, adjusting for known HPV cofactors (Table 2). The *GSTT1* null and *GSTM1* null genotypes were not significantly associated with time to HPV clearance. The interaction between the three polymorphisms was not statistically associated with time to HPV clearance.

For the subset analysis assessing clearance of HR-HPV infection while controlling for other LR infections using the Cox proportional hazard with the WLW model, we found similar significant results for education level (HR= 0.65, p=0.0007) and age at first intercourse (HR=1.09, p=0.001) compared to the analysis for any HPV clearance. Similar to the results for clearance of any HPV type, women who were homozygous/heterozygous for the *CYP1A1* variant allele had a HR-HPV clearance rate that was 21% (HR=0.79, p=0.04) lower than women that were homozygous for the wild-type allele, adjusting for known HPV cofactors and LR HPV types (Table 2). The *GSTM1* null genotype was associated with higher HR-HPV clearance rate (HR= 1.39, p=0.006) adjusting for known HPV cofactors and LR HPV types (Table 2). The *GSTT1* null genotype was not significantly associated with time to HR-HPV clearance. The

interaction between the three polymorphisms was not statistically associated with time to HR-HPV clearance.

There were significant differences between African American and white women in the frequencies of the *CYP1A1* variant allele (45% vs. 19%, p-value <0.001) and of the *GSTM1* null genotype (26% vs. 55%, p-value <0.001). Cox proportional hazard models stratified by race yielded similar hazard ratios for the three polymorphisms in both the analyses for any HPV type and for HR-HPV (data not shown). Although race was not a significant factor in the univariate analysis, all analyses were adjusted for race to avoid any confounding by population stratification. In a stratified analysis based on smoking status was assessed, the association between the *CYP1A1* variant and HPV clearance was not significant among smokers but remained significant among the nonsmokers in the any HPV type, this difference was not seen in the HR-HPV analysis.

**Discussion**

Many carcinogens require metabolic activation by enzymes like *CYP1A1*, and are detoxified by other enzymes like *GSTT1* and *GSTM1*. Individuals with genetically determined high metabolic activity and low detoxification enzyme activity would theoretically produce higher levels of metabolites and consequently potentially cause more DNA damage. This DNA damage could then facilitate HR-HPV integration and high levels of oncoprotein expression (21). We assessed the effect of functional variants in the three xenobiotic metabolism genes on HPV type specific clearance rates among women with a baseline cytology diagnosis of ASC-US or LSIL.

We found that the variant allele homozygous/heterozygous for *CYP1A1* was associated with a decreased clearance rate of any HPV type and HR-HPV, controlling for known HPV risk factors in women with ASC-US or LSIL. While previous research has focused on this variant and its association with cervical cancer risk, in which two studies found an increased risk for development of cervical cancer (5, 6) and a third found a null association (7), we found it to be significantly associated with lower clearance rates of HPV, which result in a persistent HPV infection, the intermediate phenotype on the causal pathway of cervical cancer. The effect of the polymorphism in *CYP1A1* results in increased enzyme activity, although the results have not been consistent (22-24). The increased *CYP1A1* enzyme activity could result in oxidative stress, which could lead to DNA damage, which may potentially facilitate HR-HPV integration and result in lower likelihood of its clearance.

We found a significant association between the *GSTM1* null genotype and HR-HPV clearance but did not observe the same significant association when assessing clearance of any HPV type. The differential effects of *GSTM1* on clearance of HR-HPV vs. low-risk HPV genotypes are largely unknown, but it is plausible that HR-HPVs may be more susceptible to oxidative stress than the LR-HPV types. Further research is needed to validate this finding and understand the mechanisms involved in explaining the observed results. The *GSTM1* null genotype results in the absence of enzyme activity, which would decrease the detoxification of oxidative stress. Before HR-HPV integrates, oxidative stress could facilitate the clearance of HR-HPV infections. There seems to be a delicate balance between production and removal of oxidative stress, since there was an association with the *CYP1A1* variant, which has been shown to have increased enzyme

activity and resulted in lower clearance of HR-HPV infection. The interaction between these variants was not significant in our models, but this may be due to small sample size. Further research is warranted to evaluate the interaction between these polymorphisms and HR-HPV clearance.

While we assessed the association between HPV clearance rates and the null genotypes of *GSTT1* and *GSTM1*, a recent meta-analysis found no increased risk of cervical neoplasia between the interaction of *GSTT1* and *GSTM1* polymorphisms and HPV infection status (8). The association between cervical cancer risk and polymorphism in *GSTM1* and *GSTT1* has not been consistent. Two meta-analyses have recently attempted to address this issue (8, 9), and both found that the null genotype of *GSTM1* polymorphism was associated with a significantly increased risk of cervical neoplasia; however the results differed when stratifying by population (Gao, et al found a significant increased for Chinese populations, while Economopoulos, et al did not found a significant increased risk for Asian populations)(8, 9). The association between *GSTT1* polymorphism and cervical neoplasia was inconsistent between the two meta-analyses (Gao, *et al* found a significant increased risk, while Economopoulos, *et al* found a null association) (8, 9). The *GSTM1* null genotype is associated with faster clearance of HR-HPV in our population, but is associated with an increased risk of cervical cancer in several other populations.

One limitation of our genotyping methods for *GSTT1* and *GSTM1* is that our methods can only distinguish between individuals with the null genotype versus those with a functional copy (25, 26). While new methods using gene copy number assays (0, 1 or 2 copies of the gene), have been recently developed, our genotyping method is still

commonly used including meta-analyses that have used similar analytical approach (8, 9). We found that having no enzyme activity versus functional activity for *GSTT1* and *GSTM1* did not significantly affect the time to clearance of any HPV, but did find a significant association between the *GSTM1* null genotype and HR-HPV clearance.

In conclusion, we found a functional variant in *CYP1A1* and *GSTM1* null genotypes were associated with clearance rates of HR-HPV infection, while the variant in *GSTT1* was not significantly associated with HPV clearance. Further research is warranted to confirm the role of these xenobiotic metabolism genes and the intermediate phenotype to cervical cancer, HPV persistence.

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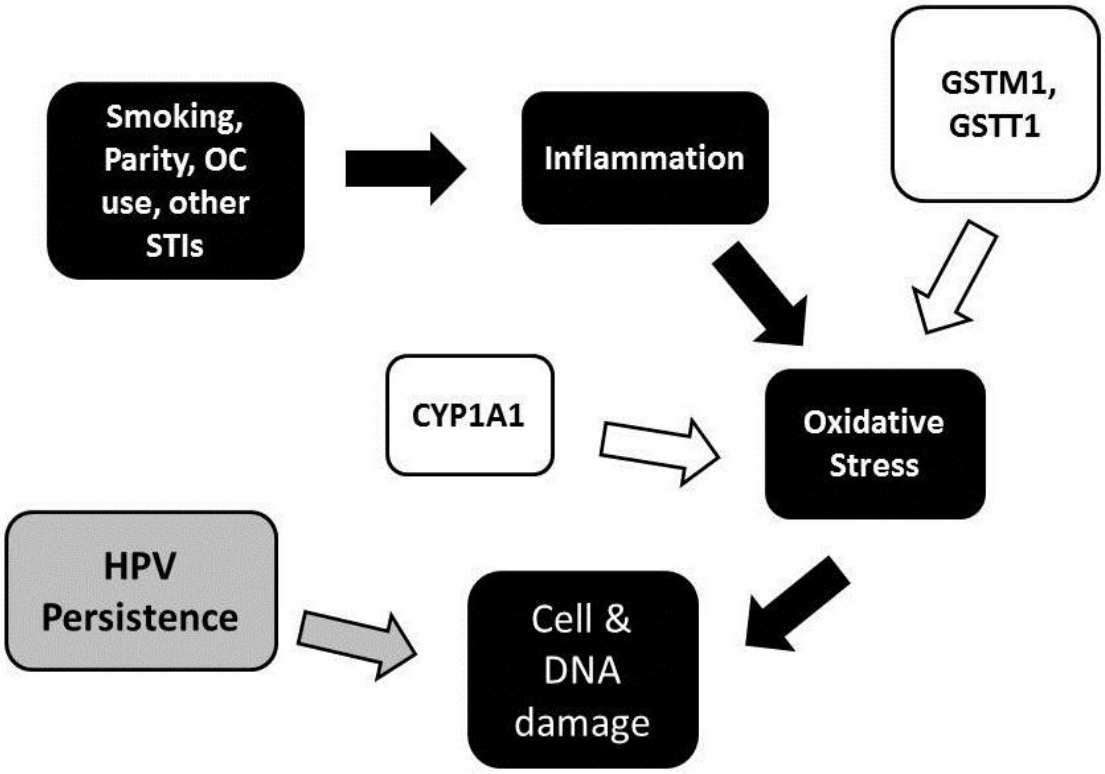


**Figure 1.** Environmental factors like smoking induce inflammation and therefore require the generation of reactive oxygen species (ROS) to reduce the inflammation. The main source of ROS in cells is *CYP1A1*. In order to maintain the beneficial effects of ROS, the cell must balance the production of ROS with its removal. *GSTM1* and *GSTT1* are activated in the antioxidant system to reduce the levels of ROS. If the oxidative stress is not dealt with then this will have deleterious effects in the cell, which can cause DNA damage and creates DNA adducts. This DNA damage in turn could facilitate HPV integration and high levels of oncoprotein expression.

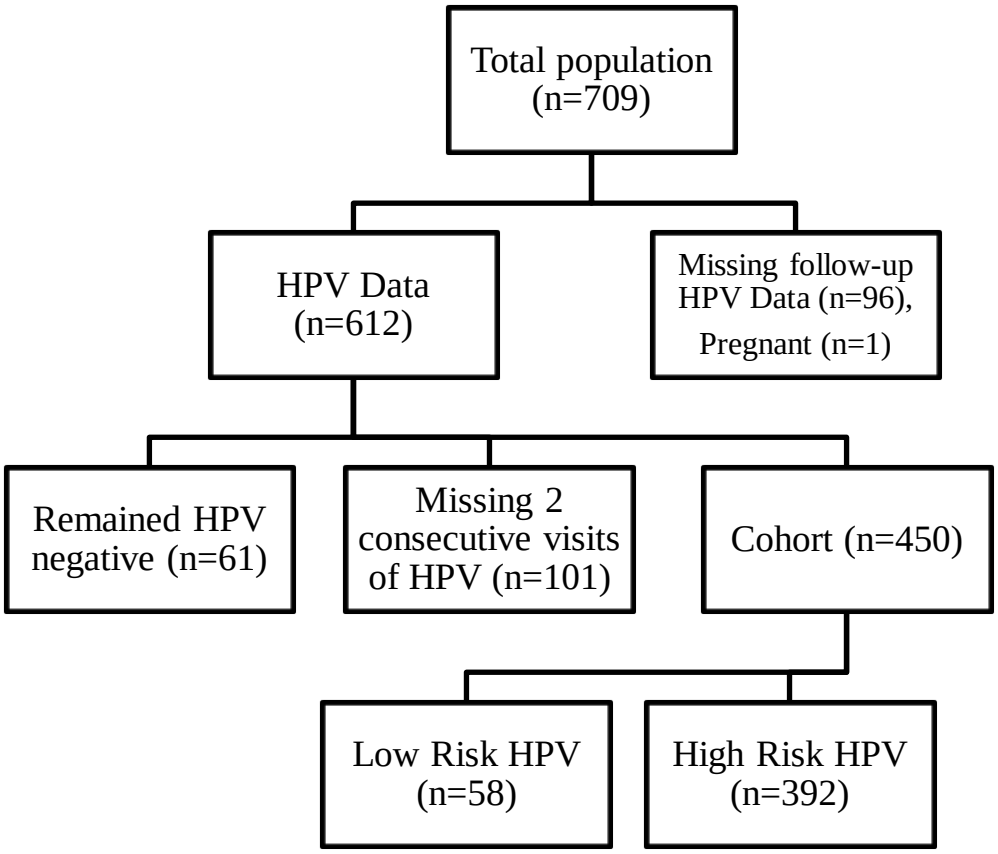


**Figure 2.** Distribution of study subjects based on exclusion criteria

**Table 1. Cox proportional hazard ratios for time to clearance of HPV infection**

| | Any HPV | | | | | High Risk HPV | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Unadjusted | | Adjusted* | | | Unadjusted | | Adjusted* | |
| | %(n=450) | HR | P | HR | P | %(n=392) | HR | P | HR | P |
| Age at Enrollment | 25.65 ± 7.15 | 1.01 | 0.41 | 1.01 | 0.42 | 25.3 ± 6.7 | 1.01 | 0.38 | 1.00 | 0.78 |
| Race | | | | | | | | | | |
| *White* | 35.33 (159) | ref | | | | 35.46 (139) | ref | | ref | |
| *African American* | 64.67 (291) | 0.9 | 0.24 | 1.07 | 0.59 | 64.54 (253) | 0.93 | 0.51 | 1.19 | 0.25 |
| Highest Education | | | | | | | | | | |
| *HS Graduate or Less* | 58.80 (264) | ref | | | | 59.95 (235) | ref | | Ref | |
| *College* | 41.20 (185) | 0.82 | 0.03 | 0.74 | 0.003 | 40.05 (157) | 0.77 | 0.02 | 0.65 | 0.0007 |
| Age at First Intercourse | 16.06 ± 2.25 | 1.03 | 0.17 | 1.06 | 0.02 | 16.0 ± 2.2 | 1.05 | 0.09 | 1.09 | 0.001 |
| Total Number of Male Sex Partners | 6.40 ± 6.30 | 1.01 | 0.11 | 1.01 | 0.07 | 6.6 ± 6.5 | 1.05 | 0.69 | 1.01 | 0.16 |
| Smoking Status | | | | | | | | | | |
| *Never/Former* | 68.22 (307) | ref | | | | 66.84 (262) | ref | | ref | |
| *Current* | 31.78 (143) | 1.18 | 0.08 | 1.12 | 0.35 | 33.16 (130) | 1.22 | 0.08 | 1.16 | 0.30 |
| Parity | | | | | | | | | | |
| *0* | 19.56 (88) | ref | | | | 20.15 (79) | ref | | ref | |
| *1+* | 80.44 (362) | 0.92 | 0.41 | 0.88 | 0.2 | 79.85 (313) | 0.94 | 0.64 | 0.97 | 0.84 |
| Bethesda Classification (combo of histology and cytology) | | | | | | | | | | |
| *Normal/ASC-US* | 37.78 (170) | Ref | | | | 38.78 (152) | ref | | ref | |
| *LSIL/HSIL* | 62.22 (280) | 0.92 | 0.34 | 0.93 | 0.43 | 61.22 (240) | 0.94 | 0.55 | 1.01 | 0.92 |
| Used Birth Control in Past 2 Years | 54.73 (243) | 1.15 | 0.15 | 1.12 | 0.24 | 54.08 (212) | 1.2 | 0.11 | 1.27 | 0.06 |
| Infected with any STD | 58.89 (265) | 0.99 | 0.95 | | | 59.69 (234) | 1.03 | 0.8 | | |
| Randomized Study Arm | | | | | | | | | | |
| *Immediate colposcopy* | 34.00 (153) | 0.94 | 0.57 | 0.93 | 0.51 | 33.93 (133) | 0.91 | 0.48 | 0.87 | 0.25 |
| *HPV triage* | 27.11 (122) | 0.94 | 0.56 | 0.97 | 0.79 | 27.30 (107) | 0.91 | 0.49 | 0.91 | 0.49 |
| *Conservative management* | 38.89 (175) | ref | | ref | | 38.78 (152) | ref | | ref | |

*Adjusted for study arm, age at enrollment, education, smoking, parity, Bethesda classification diagnosis, age at first sexual intercourse, number of lifetime sexual partners and use of oral contraceptives

**Table 2. Cox proportional hazard ratios for time to clearance of HPV infection**

| | Any HPV type | | | | | High Risk HPV | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Unadjusted | | Adjusted* | | | Unadjusted | | Adjusted* | |
| | %(n=450) | HR | P | HR | P | %(n=392) | HR | P | HR | P |
| *CYP1A1* | | | | | | | | | | |
| *Heterozygous (+/-)/ Homozygous (-/-)* | 36.02 (161) | 0.78 | 0.01 | 0.8 | 0.02 | 34.36 (134) | 0.79 | 0.02 | 0.79 | 0.04 |
| *Wild Type (+/+)* | 63.98 (286) | ref | | | | 65.64 (256) | ref | | | |
| *GSTM1* | | | | | | | | | | |
| *Null (-/-)* | 36.24 (158) | 1.04 | 0.69 | 1.04 | 0.7 | 65.45 (250) | 1.19 | 0.14 | 1.39 | 0.006 |
| *Wild Type (+/+ or +/-)* | 63.76 (278) | ref | | | | 34.55 (132) | ref | | ref | |
| *GSTT1* | | | | | | | | | | |
| *Null (-/-)* | 18.58 (81) | 0.89 | 0.33 | 0.93 | 0.56 | 81.94 (313) | 0.88 | 0.4 | 0.88 | 0.41 |
| *Wild Type (+/+ or +/-)* | 81.42 (355) | ref | | | | 18.06 (69) | ref | | ref | |

*Adjusted for study arm, age at enrollment, education, smoking, parity, Bethesda classification diagnosis, age at first sexual intercourse, number of lifetime sexual partners and use of oral contraceptives

# VARIANTS IN INTERLEUKIN FAMILY OF CYTOKINES AND TOLL-LIKE RECEPTOR GENES INFLUENCE CLEARANCE OF HIGH RISK HPV IN HIV CO-INFECTED AFRICAN-AMERICAN ADOLESCENTS

by

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**Abstract**

**Background:** The majority of women infected with human papillomavirus (HPV) never progress to the disease and naturally clears the infection. Our work aimed to examine the potential influence of host genetic factors on high-risk (HR-HPV) HPV clearance.

**Methods:** HPV viral DNA fragments from the samples were amplified by use of consensus primers MY09/11 and HMB01 and hybridized with a consensus probe and 30 different HPV strain-specific probes by use of a chemiluminescent dot-blot format. Clearance of genital HR-HPV infection was evaluated for 134 HIV-1 seropositive African-American youth from the Reaching for Excellence in Adolescent Care and Health (REACH) cohort. Genotyping targeted 267 SNPs within the exons, 5' UTR and 3' UTR sequences of 35 immune-related candidate genes encoding interleukin family of cytokines and toll-like receptors. Cox proportional hazard models were used to determine the association of type-specific HPV clearance adjusting for time-varying CD4+ T-cell count and low-risk (LR-HPV) HPV co-infections using Wei-Lin-Weisfeld approach.

**Results:** Among the 134 eligible participants, there were 400 different HR-HPV infections and 255 of these cleared during follow-up. HR-HPV clearance rates were significantly ($p< 0.001$ and FDR $<0.05$) associated with five SNPs (rs228942, rs419598, rs315950, rs7737000, rs9292618) mapped to coding and regulatory regions in three genes (*IL2RB*, *IL1RN*, and *IL7R*).

**Conclusion:** These data suggest that the analyzed genetic variants in interleukin family of cytokines and toll-like receptors have important associations with HR-HPV clearance in HIV-positive African-Americans that warrants replication.

**Impact:** These genotype-specific differences in immune-related genes may help explain the biology related to variation in HR-HPV clearance.

**Introduction**

Host genetic factors for cervical cancer have been relatively well assessed in several populations, specifically with genes in the HLA region (1-13). While these factors are important in understanding cervical cancer, prevention of the disease should focus on the precursor or the intermediate phenotype of cervical cancer, persistent human papillomavirus (HPV) infection (14). This intermediate phenotype has more practical implications because the persistence of high-risk (HR-HPV) HPV is required for the development of cervical cancer. Approximately 80% of the female population is exposed to HPV sometime in their lifetime, but infection is usually transient, with 70–90% of infected individuals "clearing" the virus within 24 months (15, 16). Risk factors associated with HR-HPV persistent infection and progression to cervical lesions and cancer are not well understood and further research is warranted to assess these factors (17). Infection with HR-HPV is therefore the necessary but insufficient cause of cervical cancer.

Data derived from immunodeficient hosts including HIV-1 infected individuals and those with iatrogenic immunosuppression such as renal transplant recipients, indicate that cellular immune defects are associated with persistence of HPV (18, 19). HIV-1 infected women have a higher prevalence and lower clearance of HPV than HIV-1 uninfected women (20). The rate of HPV clearance is lowest among severely immunocompromised individuals (CD4+ T lymphocyte cells (CD4+) <200/mm), which clearly shows the influence of host immune response (21-23). However, even after immune reconstitution of CD4+ cells for HIV-1 positive individuals with the use of antiretroviral therapies, specifically highly active antiretroviral therapy (HAART), the

incidence of HPV-related diseases has not declined (24-26). Therefore, it is likely that other factors including host genes related to immune response are influencing clearance of HPV among HIV-1 positive individuals. To account for the effect of CD4+ cells, we controlled for CD4+ count measured at every visit in our population in order to effectively decipher the independent association between variations in specific immune-related genes and HPV clearance.

Most HPV infections are eventually cleared due primarily to a strong localized cell-mediated immune response; however, the virus persists in a subset of the population (23). Host genetics factors of immune related genes may explain differences in the ability to clear the HPV infection. Our objective was to assess host genetic variants related to clearance/persistence of HR-HPV in HIV-1 positive African-American adolescent females, selected based on two groups of candidate genes encoding Interleukin family of cytokines and Toll-like Receptors (TLRs) that have been reported to have functional consequences or associations with cervical cancer, HPV infection or other infections.

## Material and Methods

### *Study Population*

Participants from the Reaching for Excellence in Adolescent Care and Health (REACH) cohort were included in this study (27, 28). Between 1996 and 2000, adolescents who acquired HIV-1 through risk behaviors, mainly sexual activities (perinatal transmission or blood product contamination were excluded), and comparable HIV-1 seronegative adolescents (aged 12–19 years) were recruited into a longitudinal

study at 15 clinical sites in the U.S. to investigate the natural history of HIV-1 (27). A subset of the REACH cohort was used for this analysis in order to perform the genetic analysis in a very homogenous population. Of the 535 adolescents enrolled in the REACH study, the analysis was restricted to 226 African-American HIV-1 positive females. The study design and methods for quarterly follow up, HIV-1 testing and viral-load measurement, and immunophenotyping of CD4+ counts, along with demographics, risk behavior, and other clinical data, have been previously described in detail (27, 28). Additionally, HPV was also tested at baseline and each semi-annual follow up visit (29).

***HPV DNA Detection and Classification***

At enrollment and every six months thereafter, cervical lavage samples were tested for HPV infection. HPV viral DNA fragments from the samples were amplified by use of consensus primers MY09/11 and HMB01 and hybridized with a consensus probe and 30 different HPV strain-specific probes (some probes were, however, specific for more than one HPV type) by use of a chemiluminescent dot-blot format (24, 30, 31). The 30 different HPV types were: HPV2/57, HPV 6/11/42/44, HPV13/32, HPV16, HPV18, HPV26/29, HPV31/33/35, HPV39, HPV45, HPV51, HPV52, HPV53/66, HPV54/40, HPV55, HPV56, HPV58, HPV59/68/70, HPV62/72, and HPV67. There were 10 types that were classified as high risk (HR-HPV) HPV types: HPV16, HPV18, HPV31/33/35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59/68/70.

Prevalent and incident HR-HPV infections were used in this analysis. Two consecutive HPV-negative tests after a positive test were required to confirm clearance because of the possibility of false-negative test results. Since the exact point of infection

with HPV cannot be determined in this dataset, we took the midpoint in time between the prior negative visit and the positive visit. Prevalent infections had an additional 90 days added on to the initial positive visit time. Similarly, the exact date of clearance of the HPV infection cannot be determined so we again took the midpoint between the last positive visit and the first negative visit if there were two consecutive negative tests. HPV infections that became negative at a woman's last study visit were censored at the last visit. Type-specific HPV status was assumed to remain unchanged across single missing visits. Two consecutive missing visits were censored at the visit prior to the first missing visit. Of the 226 African-American HIV-1 positive females, 55 remained HPV-negative throughout follow-up, and 8 only had a low-risk (LR-HPV) HPV infection and both of these groups were excluded from the analysis since the outcome of interest is time to clearance of HR-HPV, which left 163 individuals eligible for the study.

***Genotyping***

High-molecular-weight genomic DNA was extracted from whole blood and was used for the genotyping of 349 SNPs in the coding, 5' and 3' UTR within 35 immune-related genes that broadly can be categorized into two major groups – Interleukin family of cytokines and Toll-like Receptors (Table 1). Genes within these groups were chosen based on their previous association with HIV and other infectious diseases, as well as their known involvement in several different innate and adaptive immune systems. Genotyping of the single nucleotide polymorphisms (SNPs) were performed using the custom GoldenGate assay (Illumina, San Diego, CA) using the standard commercial platforms (Illumina, San Diego, CA) (32).

***Statistical Analysis***

All SNPs were checked for completeness (by SNP and by subject), rare variants (frequency), and deviation from Hardy-Weinberg Equilibrium (HWE) for standard quality control (QC) thresholds. SNP genotype completeness by subject was set at 95% coverage and 29 individuals with high overall missing genotype were removed from the analysis, leaving 134 individuals in the analysis. Three of the 349 SNPs were removed for not being in HWE (p-value <0.001). Additionally, 12 SNPs were removed since they were missing in more than 10% of the individuals. The minor allele frequency (MAF) for each SNP was calculated and 67 rare SNPs with a frequency < 0.05 were removed from the analysis, which left 267 SNPs. Initially, multiple testing of SNPs in linkage disequilibrium (LD) with each other within a gene was performed using the matrix spectral decomposition method, as previously described (33). Out of the 267 SNPs in 35 genes, 79 SNPs were in LD with other SNPs within individual genes, thus a bonferroni corrected p-value was set at 0.05/188, p= 0.0003.

Cox proportional hazard model was used to assess influence of variants in immune-related genes on type specific HR-HPV clearance using an additive model. All of the models were adjusted for CD4+ count as a time-varying covariate during the HPV infected periods because all of the women were HIV+ and this served as a marker for disease status as well as a surrogate for HIV treatment. LR-HPV infections were adjusted for in the model when an individual was co-infected with any HR-HPV type, which also allowed LR-HPV to serve as a time-varying covariate when infection time over lapped. The Wei-Lin-Weisfeld (WLW) extension was used with the Cox

proportional hazard model because this approach can simultaneously analyze time to HPV clearance of several types of HPV either at the same or different visits, taking into account possible correlation between the types (34). The WLW model was implemented in SAS using PHREG procedure, selecting the STRATA option to allow different baseline hazards function for each HPV type and robust variance through the option [COVS(AGGREGATE)]. These options produce a population-averaged interpretation. Hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated.

**Results**

Among the 134 African-American HIV-1 positive females, there were 400 different HPV infections and 255 of these cleared during follow-up. The most common HR-HPV types were HPV59 (n=65), HPV31 (n=62), HPV58 (n=55), and HPV16 (n=54). Of the 255 infections that cleared during follow up, the average time to clearance was 319 days (median 210 days). Among the HR-HPV infections that cleared during follow-up the average CD4+ count was 574.2 cells/$mm^3$ (median 540.0 cells/$mm^3$) and among the HR-HPV infections that persisted during follow-up the average CD4+ count was 485.1 cells/$mm^3$ (median 464.4 cells/$mm^3$).

Cox proportional hazard models were assessed for each SNP using an additive model for an association with time to clearance of HR-HPV, adjusting for CD4+ count and other LR-HPV infections over time. The βeta-values of 267 SNPs were plotted by –log p-values of associations observed in the analytical model (Figure 1). To account for multiple testing using the bonferroni correction, a negative-log p-value of greater than 3.52 would be needed for the association test to reach statistical significance in our

population; however, none of the SNPs in our analysis reached this stringent threshold. A false discovery rate (FDR, q=0.04) for multiple testing was calculated using the Benjamini-Hochberg procedure and the Storey Qvalue procedure and five of the SNPs in our analysis reached this statistical threshold (Figure 1) (35-37).

The five SNPs that met the FDR threshold, along with SNPs that were marginally associated with time to clearance of HPV, p-value <0.05 and q-value > 0.05 are listed in Table 2. HIV-1 positive African American women infected with HPV that have minor allele A for SNP rs228942 in the *IL2RB* gene had a HPV clearance rate that is 1.69 times (HR=1.69, 95% CI 1.40-1.97, p= 0.0003) higher than those with the wild type allele controlling for CD4+ count and other LR-HPV infections. Similarly, individuals with C allele for SNP (rs419598) in *ILRN*, a T allele for SNP (rs7737000) in *IL7R*, or an A allele for SNP (rs9292618) in *IL7R* had a HPV clearance rate that is higher than those with a wild type allele (Table 2). HIV-1 positive African American women infected with HR-HPV that have minor allele C for SNP rs315950 in the *IL1RN* gene had a HPV clearance rate that is 1.49 times (HR=0.67, 95% CI 0.44-0.90, p= 0.0006) lower than those with the wild type allele.

**Discussion**

We report several variants within the two candidate gene groups Interleukin family of cytokines and Toll-like receptors that are associated with clearance of HR-HPV infection in HIV-1 African American adolescent females adjusting for CD4+ count and other LR-HPV infections. The most significant SNP (rs228942) in our analysis is located in the coding region of the *IL2RB* gene was associated with a higher clearance rate of

HR-HPV infection. Additionally, two other SNPs (rs3218273 and rs3218329) located in the coding and 3'UTR of *IL2RB* were marginally ($p<0.05$) associated with higher clearance rate of HR-HPV infection. IL2 is important for stimulating T-cell proliferation through the T-cell producing and secreting IL2, which stimulates the interleukin-2 receptor (IL2R). The SNP (rs228942) has been previously associated with susceptibility to kidney allograft rejection and the authors hypothesized that this polymorphism may influence T-cell proliferation or cause $T_{reg}$ proliferation to be reduced (38). Their hypothesis coincides with our findings in that those with the minor allele for those SNPs in *IL2RB* have faster clearance rates of HR-HPV, which may result in higher T-cell proliferation. The importance of IL2 and cervical HPV is further elucidated in another finding where recombinant human IL2 (rhIL2) was used as a treatment on HPV-associated tumor cells and shown to have inhibited cell growth in a dose-dependent manner (39). However, Rangel-Corona et. al. found that IL2 in cervical cancer cells act as a growth factor for these cells (40). Therapeutic approaches with IL2 on cervical carcinoma need further exploration as well as the potential for IL2 therapy and HR-HPV clearance.

We found that a SNP (rs315950) located in the 3' UTR of *IL1RN* gene was associated with a lower clearance rate of HR-HPV infection while SNP (rs419598) located in the coding region of *IL1RN* was associated with higher clearance rate of HR-HPV infection. *IL1RN* gene encodes for a protein interleukin-1 receptor antagonist (IL1RA), a naturally occurring anti-inflammatory cytokine produced by monocytes, macrophages, and epithelial cells that regulates the biological activities of IL1A and IL1B, which are the most potent pro-inflammatory cytokines (41-46). The IL1RA and

IL1 levels at an inflammatory site determines whether a pro-inflammatory response site will be initiated and persist or will be terminated (46). If IL1RA is under expressed, then this could result in damage to the host cells and create DNA adducts, which could potentially favor HPV to persist and integrate into the host genome (47, 48).

The SNP (rs419598) and VNTR have previously been shown to be in high LD (49). Variable number tandem repeats (VNTRs) within the intron 2 of *IL1RA* has been associated with increased risk for cervical cancer (41, 50, 51). This association between cervical cancer and variation with *IL1RN* could be a surrogate for a true association between HPV and *IL1RN*. This same VNTR in *IL1RA* is also associated with HPV infection in women with cervical cancer (52). To date, the function of the SNP in *IL1RN* (rs315950), which we found to be associated with clearance of HR-HPV is unknown. However, based on the present results in the literature, *IL1RN* is associated with HPV outcomes, including cervical cancer. If a woman is unable to clear the HPV infection due to variation in host gene immunity then it likely that this association could potentially be seen in the likelihood for progression of lesions to cervical cancer due to the inability to regulate the immune response.

The two SNPs (rs315950 and rs419598) in *IL1RN* had opposite associations with HR-HPV clearance in our population. We assessed the interaction between the two SNPs and time to clearance of HR-HPV infection controlling for CD4+ and LR-HPV. The minor allele C for SNP rs419598 was associated with faster HR-HPV clearance rate, while the minor allele C for SNP rs315950 was associated with lower HR-HPV clearance rate. Therefore, we assumed that an individual that was CC for SNP rs419598 and TT for SNP rs315950 would have faster HR-HPV clearance rates (refer to as High group). The

lower HR-HPV clearance rates would be TT for SNP rs419598 and CC for SNP rs315950 (refer to as Low group). Any other combination of the genotypes for the two SNPs was considered to be the intermediate of the two other groups (refer to as Intermediate group). Comparing the intermediate group to the low group, a faster HR-HPV clearance rate (HR=3.01, $p=8.5x10^{-13}$, 95% CI 2.71, 3.30) was observed. Comparing the high group to the low group, we found a faster HR-HPV clearance rate (HR=5.12, $p=3.16x10^{-20}$, 95% CI 4.77, 5.47). However, the interpretation of this interaction with smaller sample size needs caution and require replication. Of note, out of the 134 individuals, 3.03% (n=4) were in the low group, 87.88 (n=116) were in the intermediate group, and 9.09% (n=12) were in the high group.

Two SNPs (rs7737000 and rs9292618, but also in LD) located in the coding and the flanking 3' UTR of *IL7R* were associated with a higher clearance rate of HR-HPV. The SNP rs7737000 has been associated with an increased risk for non-small cell lung cancer among African-American and Caucasian women (53). However, this association was not seen in a Chinese population (54). To date, the function of the coding SNP rs7737000 is not known so the biological relation of IL7R cannot be deciphered with HR-HPV clearance. There were two SNPS in TLRs (*TLR3* and *TLR7*) that were marginally associated with time to clearance of HR-HPV. However, the majority of significant polymorphisms were found in the interleukin family of cytokines.

There are several limitations to this study. First, our sample was comprised of 134 women, which reduces our power to detect the significance level needed for multiple testing with genetic data using a stringent bonferroni correction. However, we were able to observe association with significant significance using the FDR. Second, our

population was extremely homogenous in that all study participants were African-American HIV-1 positive adolescent females. Thus, our study findings may not be generalizable to the general population since our cohort was composed of only African-American HIV-1 positive adolescents. Third, we included prevalent HPV infections in the analysis. However, these adolescent women were likely to be infected recently with HIV and HPV due to their age and sexual risk factors that predisposed them to these infections.

Inflammation has been shown to be associated with cancer and HPV infection. Interleukin family of cytokines and TLRs associated with the ability to clear HPV infection need to be investigated further. Understanding the pathogenesis of HPV infection will have a major public health impact and be cost effective if these genetic variants could help target woman for treatment. Since HPV persistence is the major risk factor for cervical cancer, identification of susceptibility loci may allow earlier diagnosis based on an individual's genetic constitution and may facilitate identification of disease subtypes amenable to therapeutic approaches.

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**Table 1. The thirty five genes listed by group with total number of SNPs per gene**

| Group Name | Gene | Chromosome | Target SNPs |
|---|---|---|---|
| **Interleukin family of cytokines** | | | |
| | *IL10* | 1q31-q32 | 8 |
| | *IL10RA* | 11q23 | 12 |
| | *IL10RB* | 21q22.11 | 12 |
| | *IL12A* | 3q25.33 | 6 |
| | *IL12B* | 5q31.1-q33.1 | 5 |
| | *IL13* | 5q31 | 9 |
| | *IL15* | 4q31 | 4 |
| | *IL17A* | 6p12 | 16 |
| | *IL17RB* | 3p21.1 | 5 |
| | *IL19* | 1q32.2 | 6 |
| | *IL1A* | 2q14 | 11 |
| | *IL1B* | 2q14 | 9 |
| | *IL1RN* | 2q14.2 | 10 |
| | *IL2* | 4q26-q27 | 4 |
| | *IL20* | 1q32 | 8 |
| | *IL24* | 1q32 | 9 |
| | *IL2RA* | 10p15-p14 | 4 |
| | *IL2RB* | 22q13.1 | 10 |
| | *IL4* | 5q31.1 | 7 |
| | *IL4R* | 16p12.1-p11.2 | 14 |
| | *IL5* | 5q31.1 | 7 |
| | *IL6* | 7p21 | 7 |
| | *IL7* | 8q12-q13 | 19 |
| | *IL7R* | 5p13 | 11 |
| | *IL8* | 4q13-q21 | 8 |
| | *IL8RA* | 2q35 | 2 |
| | *IL8RB* | 2q35 | 2 |
| **Toll-like receptor** | | | |
| | *TLR1* | 4p14 | 4 |
| | *TLR2* | 4q32 | 2 |
| | *TLR3* | 4q35 | 5 |
| | *TLR4* | 9q33.1 | 7 |
| | *TLR6* | 4p14 | 3 |
| | *TLR7* | Xp22.3 | 3 |
| | *TLR8* | Xp22 | 13 |
| | *TLR9* | 3p21.3 | 5 |

**Table 2. Cox proportional Hazard Ratios (HR) for the SNPS associated with time to clearance of high risk HPV infection controlling for CD4+ count and low risk HPV.**

| Gene | SNP | MAF | location | B-value | HR | 95%CI | p-value | FDR |
|---|---|---|---|---|---|---|---|---|
| **p-value <0.001, q-value <0.05** | | | | | | | | |
| *IL2RB* | rs228942 | 0.07 | coding | 0.52 | 1.69 | (1.40, 1.97) | 0.0003 | 0.04 |
| *IL1RN* | rs419598 | 0.06 | coding | 0.50 | 1.65 | (1.36, 1.93) | 0.0005 | 0.04 |
| *IL1RN* | rs315950 | 0.17 | flanking 3UTR | -0.40 | 0.67 | (0.44, 0.90) | 0.0006 | 0.04 |
| *IL7R* | rs7737000 | 0.12 | coding | 0.44 | 1.55 | (1.29, 1.80) | 0.0007 | 0.04 |
| *IL7R* | rs9292618 | 0.10 | flanking 3UTR | 0.43 | 1.54 | (1.29, 1.79) | 0.0008 | 0.04 |
| **p-value <0.05, q-value >0.05** | | | | | | | | |
| *IL1RN* | rs315948 | 0.16 | flanking 3UTR | 0.35 | 1.42 | (1.18, 1.66) | 0.004 | 0.18 |
| *IL17A* | rs3915558 | 0.08 | flanking 5UTR | 0.34 | 1.40 | (1.15, 1.66) | 0.009 | 0.31 |
| *IL17A* | rs3927607 | 0.08 | flanking 5UTR | 0.34 | 1.40 | (1.15, 1.66) | 0.009 | 0.31 |
| *IL2RB* | rs3218273 | 0.08 | coding | 0.33 | 1.39 | (1.12, 1.66) | 0.016 | 0.39 |
| *TLR3* | rs3775291 | 0.07 | coding | 0.41 | 1.51 | (1.17, 1.85) | 0.017 | 0.39 |
| *IL7R* | rs1494558 | 0.27 | coding | -0.28 | 0.75 | (0.52, 0.99) | 0.020 | 0.39 |
| *IL1B* | rs4849126 | 0.31 | flanking 5UTR | -0.24 | 0.79 | (0.59, 0.99) | 0.020 | 0.39 |
| *IL2RB* | rs3218329 | 0.20 | 3UTR | 0.29 | 1.34 | (1.09, 1.58) | 0.020 | 0.39 |
| *IL1RN* | rs315949 | 0.33 | flanking 3UTR | -0.23 | 0.80 | (0.60, 0.99) | 0.024 | 0.39 |
| *IL8* | rs13142454 | 0.16 | flanking 3UTR | -0.32 | 0.73 | (0.45, 1.00) | 0.024 | 0.39 |
| *IL6* | rs10242595 | 0.48 | flanking 3UTR | 0.22 | 1.25 | (1.06, 1.45) | 0.025 | 0.39 |
| *IL7* | rs2717536 | 0.32 | flanking 3UTR | 0.27 | 1.30 | (1.07, 1.54) | 0.025 | 0.39 |
| *IL17A* | rs6922427 | 0.05 | flanking 5UTR | 0.40 | 1.49 | (1.14, 1.85) | 0.026 | 0.39 |
| *IL10RA* | rs4252243 | 0.31 | flanking 5UTR | 0.20 | 1.23 | (1.03, 1.42) | 0.040 | 0.52 |
| *IL1B* | rs13032029 | 0.29 | flanking 5UTR | 0.20 | 1.22 | (1.03, 1.42) | 0.043 | 0.52 |
| *TLR7* | rs179007 | 0.11 | flanking 3UTR | -0.33 | 0.72 | (0.39, 1.04) | 0.046 | 0.52 |
| *IL4R* | rs9302448 | 0.08 | flanking 3UTR | -0.39 | 0.68 | (0.30, 1.06) | 0.047 | 0.52 |
| *IL1B* | rs4848306 | 0.31 | flanking 5UTR | 0.19 | 1.21 | (1.02, 1.39) | 0.048 | 0.52 |
| *IL8* | rs16849958 | 0.21 | flanking 3UTR | 0.24 | 1.27 | (1.03, 1.51) | 0.049 | 0.52 |
| *IL6* | rs2069849 | 0.13 | coding | 0.27 | 1.32 | (1.04, 1.59) | 0.050 | 0.52 |

The results for each SNP using an additive model, the with major allele frequency used as the reference

Abbreviations: SNP=single nucleotide polymorphism, MAF=minor allele frequency, HR= hazard ratio, 95% CI= 95% confidence interval. FDR=False Discovery Rate q-value

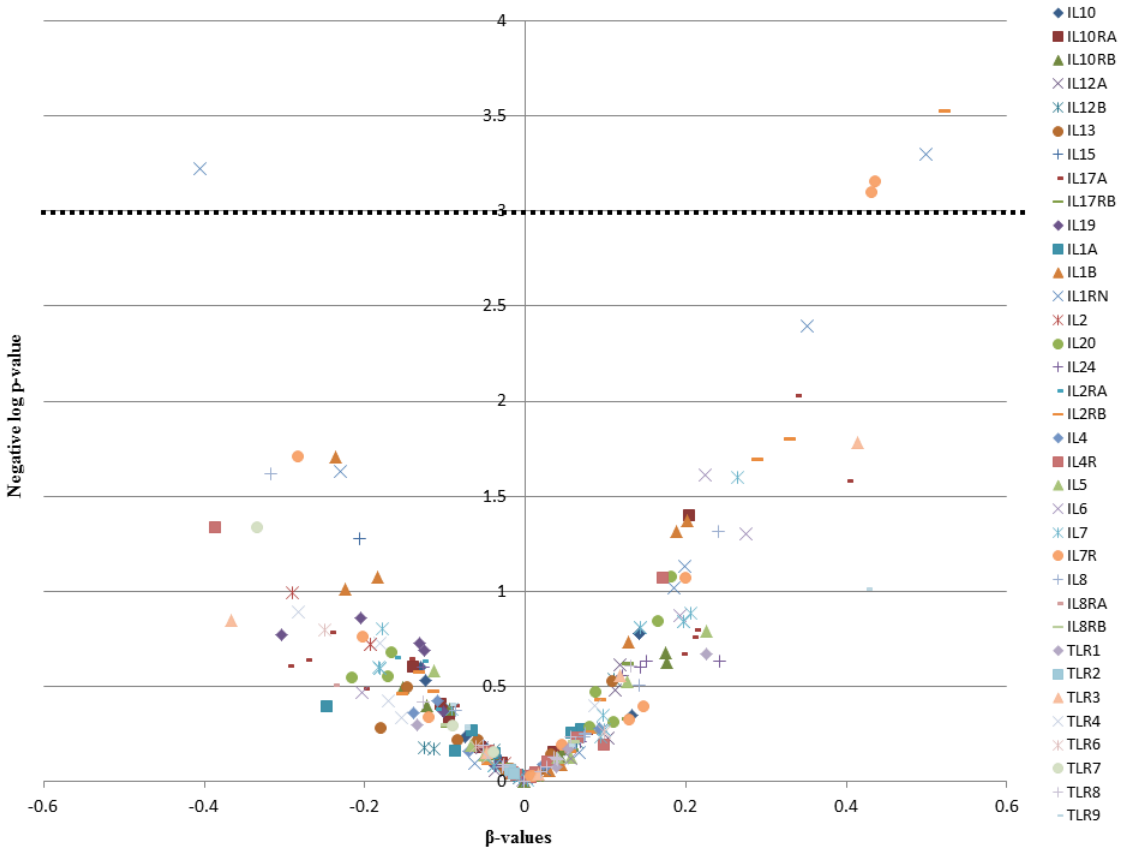


**Figure 1.** Negative Log p-values by beta-values of SNP association of time to HR-HPV clearance, using Cox proportional hazard WLW model for all 267 SNPs. The symbol represents each of the 35 genes examined. Dotted line represents the false discovery rate (FDR) q <0.05 cut-off for statistical significance.

# IMMUNOGENETICS OF HPV CLEARANCE AMONG HIV-1 POSITIVE WOMEN IN THE HIV EPIDEMIOLOGY RESEARCH STUDY (HERS)

by

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**Abstract**

**Background:** To assess the influence of variants in immune-related genes associated with clearance of high risk (HR-HPV) infections in African-American HIV-1 positive women.

**Methods:** DNA was extracted from stored PMBC from 258 African-American HIV-positive women in the HIV Epidemiology Research Study (HERS) with HR-HPV infection(s) identified by hybridization of PCR amplified virus from cervicovaginal lavage to type-specific HPV probes. DNA was genotyped for variants in immune related genes using the Illumina ImmunoChip. Longitudinal data on HIV and HPV was collected at HERS follow-up visits every 6 months. To examine the influence of single nucleotide polymorphisms in immune related genes with time to clearance of HR-HPV, we used the Cox proportional hazards model with the Wei-Lin-Weissfeld (WLW) approach and adjusted models for CD4 count, low risk (LR-HPV) HPV, principal components analysis for ancestry, and genotyping facility.

**Results:** The most common HR-HPV types were HPV18 (n=71), HPV51 (n=61), HPV58 (n=58), and HPV16 (n=57). There were three SNPs (rs1633038, rs6571225, and rs2524035) located on chromosome 6 that were also associated with higher clearance rates. Additionally, there were three other SNPs (rs1256215, rs17781894, and rs7802321) located on chromosome 1, 19, and 7, respectively that were also associated with higher HR-HPV clearance rates.

**Conclusion:** This hypothesis generating analysis assessing SNPs in immune related genes and their association with HR-HPV clearance brought forth several significant SNPs.

**Introduction**

Cervical cancer is third most common cancer in women worldwide and more than 85% of the global burden of this cancer occurs in developing countries [1]. Human papillomavirus (HPV) DNA is present in 99.7% of all cervical carcinomas with HPV types 16, 18, 45 and 31 being the most predominant [2-5]. HPV is a common sexually transmitted infection with 80% of women in the United States being infected sometime in their lifetime. However, the majority of those infected are able to clear the infection and only a small proportion will progress to cervical cancer [6, 7]. A persistent HPV infection is therefore a necessary but an insufficient cause of cervical cancer. While cervical cancer is a definitive end to the stages of progression associated with HPV infection, it is important to understand the intermediate process. Persistent HPV infection, the intermediate phenotype, is considered the most important factor for high-grade lesion development and progression to cervical cancer in HPV infected women [8].

The factors that lead to the development of a persistent HPV infection in some women, but not others, remain unclear. Investigation into the role of host genetics and HPV persistence is important, given the different responses to infection among women. To our knowledge, most publications on host genetics have focused on cervical cancer as the outcome, and few have incorporated the intermediate phenotype to cervical cancer. Even with a few studies including the genome-wide association study with HPV as the outcome, the study design predominantly used in the past has been cross-sectional, with genetic variants compared between women with cervical HPV+ cancer and randomly selected controls [9-11]. The results from publications thus far generally show the

association with HLA variants (i.e., *DRB1*13* and *DRB1*1501-DQB1*06*), although associations have not been consistent across studies [12].

Although the mechanism for HPV persistence is unknown, several studies implicate immune evasion [13, 14] involving genetically mediated determinants of the host immune response [15]. However, most HPV infections are eventually cleared due primarily to a strong localized cell-mediated immune response [16]. Host genetics factors of immune related genes may explain differences in the ability to clear the HPV infection. Our objective was to assess host genetic variants related to clearance/persistence of high risk (HR-HPV) HPV in African-American HIV-1 positive women using the Human ImmunoChip which is comprised of densely spaced SNP variants and CNV markers, developed by a consortium of specialists in the fields of immunology and inflammation [17, 18]. We chose to limit our analysis to clearance of HR-HPV infections since these types are most likely to be associated with cervical cancer risk.

## Methods and Materials

### *Study Population*

Participants from the HIV Epidemiology Research Study (HERS) cohort were included in this study [19]. HERS was a multicenter, prospective study established by the CDC to examine the natural history of HIV in women. Women aged 16–55 with documented HIV status and high-risk behaviors were recruited between April 1993 and January 1995. The exclusion criteria were as follows: i) had no identified HIV risk behavior; ii) had risk only by transfusion history or vertically from HIV+ mother; iii)

were not born female (i.e., transsexual); and iv) did not consent to the full protocol, including pelvic exam, phlebotomy, and repeated HIV testing and counseling; and v) reported previously having AIDS-defining illnesses. Of the 1,987 women screened, 1,310 (66%) were enrolled in the study (871 HIV+ and 439 HIV-). After enrollment, the core visit protocol for participants included a standardized face-to-face interview; a physical examination, including a complete gynecologic exam; and specimen collection along with CD4+ and HIV viral load at 6-month intervals.

A subset of the HERS cohort was used for this analysis in order to perform the genetic analysis in a very homogenous population. Of the 1,310 women enrolled in the HERS study, the analysis was restricted to 328 HIV-1 positive African American females.

*HPV DNA Detection and Classification*

At enrollment and every 6 months thereafter, cervical samples were tested for HPV DNA. Viral DNA fragments from cervical lavage were amplified by using the consensus primers MY09/11 and HMB01 and were hybridized for a consensus probe and for 26 HPV types in the HERS cohort by using a chemiluminescent dot-blot format [19]. PCR-based HPV data were classified: as negative, as positive for the specific types, or as "positive, type unknown" if the sample was positive for the generic probe but not for a specific HPV type. PCR amplification of a human β-globin gene segment was used as an internal control for DNA quality; samples negative for this assay will be excluded from analyses. The amplification products were identified in a dot-blot format with biotinylated probes (type-specific probes for >25 HPVs, a generic HPV probe, and a β-

globin probe) by a chemiluminescence system. The 26 different HPV types were: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 66, 68, 73, 82, 83, and 84. There were 17 types that were classified as high risk (HR-HPV) HPV: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82, while the remaining 9 types were classified as low-risk (LR-HPV) HPV.

Prevalent and incident HPV infections were used in this analysis. Two consecutive HPV-negative tests were required to confirm clearance because of the possibility of false-negative test results. HPV infections that became negative at a woman's last study visit were censored at the last visit. Type-specific HPV status was assumed to remain unchanged across single missing visits.

Of the 328 African American HIV-1 females, 28 remained HPV negative throughout follow-up and 41 only had a LR-HPV infection and both of these groups were excluded from the analysis since the outcome of interest is time to clearance of HR-HPV, which left 259 individuals eligible for the study.

*Genotyping*

Genomic DNA was extracted from stored peripheral blood mononuclear cells and was used for genotyping using the the iScan system (Illumina, Inc.) and the Human ImmunoChip**,** which utilizes Infinium chemistry and is comprised of densely spaced SNP variants, developed by a consortium of specialists in the fields of immunology and inflammation [17, 18]. The chip comprises assays for 196,070 SNPs (of which 5,001 are non-synonymous coding, 1,926 are synonymous coding, and 4,065 are in the UTR). The

genotyping of the samples was processed at two different lab facilities and this was adjusted for in the statistical models.

*Statistical Analysis*

All SNPs were checked for completeness (by SNP and by subject), rare variants, and deviation from Hardy-Weinberg Equilibrium (HWE). SNP completeness by subject was set at 90% coverage and all 259 individuals meet this criterion. Kinship between the 259 individuals was also assessed in KING software and two of the individuals were determined to be monozygotic twins so one of these individuals was removed from the dataset [20]. SNP data completeness was set at 90% coverage and 5171 SNPs were removed from the dataset for not meeting this quality control (QC) threshold. There were 1234 SNPs removed for not being in HWE (p-value <0.001). The minor allele frequency (MAF) for each SNP was calculated and 71,488 rare SNPs with a frequency < 0.05 were removed from the dataset, which left 117,694 SNPs. There were also 1,523 SNPs that were not autosomal and removed from the analysis, which left 116,171 SNPs. Linkage disequilibrium (LD) between the SNPs was assessed using the K effective method, as previously described [21]. Out of the 116,171 SNPs, 27,736 were in LD with other SNPs, thus a Bonferroni corrected p-value was set at 0.05/88,435, $p= 5.65x10^{-7}$.

Cox proportional hazard model was used to assess influence of variants in immune-related genes on clearance of HPV18 using an additive model with the Wei-Lin-Weisfeld (WLW) extension [22]. All of the models were adjusted for CD4+ T lymphocyte cells (CD4+) count because all of the women were HIV+ and this served as a marker for disease status as well as a surrogate for HIV treatment. CD4+ count was

adjusted for in the model when an individual was infected with HPV, which allowed CD4+ count to serve as a time-varying covariate. LR-HPV infections were adjusted for in the model when an individual was co-infected with any HR-HPV type, which also allowed LR-HPV to serve as a time-varying covariate when infection time over lapped. Principal components analysis (PCA) for ancestry was also controlled for in the models. Hazard ratios (HR) and 95% confidence intervals were calculated. A false discovery rate (FDR) for multiple testing was calculated [23]. Quantile-quantile (Q-Q) plots of p-values were constructed to evaluate deviations from the expected p-value distribution. Genome-wide Manhattan plots were generated to visualize the results.

**Results**

There were 727 total infections among the 258 individuals and 476 of these cleared during follow up. The most common HR-HPV types were HPV18 (n=71), HPV51 (n=61), HPV58 (n=58), and HPV16 (n=57). Of the 476 infections that cleared during follow up, the average time to clearance was 437 days (median 276 days). Among the HR-HPV infections that cleared during follow-up the average CD4+ count was 407.5 cells/$mm^3$ (median 356.8 cells/$mm^3$; range 8.1-1576.9 cells/$mm^3$) and among the HR-HPV infections that persisted during follow-up the average CD4+ count was 258.9 cells/$mm^3$ (median 208.3 cells/$mm^3$; range 2.6-921.6 cells/$mm^3$). The average age at baseline was 35 years old and these HIV-1 positive women had a median baseline CD4+ count of 381 cells/$mm^3$ [interquartile range (IQR): 244-565].

Cox proportional hazard models were assessed for each SNP using an additive model for an association with time to clearance of HR-HPV, adjusting for CD4+ count,

LR-HPV infection(s), PCA for ancestry, and genotyping facility. To account for multiple testing using the Bonferroni correction, a negative-log p-value of greater than 6.2 ($p= 5.65x10^{-7}$) would be needed for the association test to reach statistical significance in our population; one of the SNPs in our analysis reached this stringent threshold (Table 1). HIV-1 positive women infected with HR-HPV that have minor allele T for SNP rs1633038 located on chromosome 6 had a HPV clearance rate that is 1.8 times (HR=1.83, $p= 6.52x10^{-7}$) higher than those with the wild type allele controlling for CD4+ count, LR-HPV infection(s), PCA for ancestry, and genotyping facility (Table 1, Figure 3).

There were several SNPs that were marginally ($p<0.00001$) associated with time to clearance of HR-HPV infection. There were two other SNPs (rs6571225 and rs2524035) located on chromosome 6 that were also associated with higher clearance rates (Figure 4). Additionally, there were three other SNPs (rs1256215, rs17781894, and rs7802321) located on chromosome 1, 19, and 7, respectively that were also associated with higher HR-HPV clearance rates. The Manhattan plot and The Q-Q plot summarize the results from the association between HR-HPV clearance and the SNPs from the ImmunoChip (Figure 1 and 2).

**Discussion**

We report several variants in immune related genes that are associated with clearance of HR-HPV infection in African-American HIV-1 positive females after accounting for the effects of CD4+ count other LR-HPV infection(s), PCA for ancestry, and genotyping facility. The most significant association with time to clearance of HR-

HPV was seen with the SNP, rs1633038, located on chromosome 6. The minor allele for SNP rs1633038 was associated with faster time (days) to clearance 1.8 times (HR=1.83, $p= 6.52x10^{-7}$) which is evident from the Kaplan-Meier survival curve in that those that were homozygous for the minor allele has faster clearance compared to heterozygous and homozygous for the major allele (Figure 3). Along with this SNP rs1633038, there was a second SNP rs2524035 that is also located in chromosome 6 and both of these are near the HLA-G gene region and both SNPs are in LD with several SNPs located in HLA-F.

HLA-G is a nonclassical HLA class Ib molecule that regulate the immune response through interaction with surface receptors on natural killer, T and antigen-presenting cells [24-27]. A Canadian cohort assessed the association between HLA-G polymorphisms and HPV infection susceptibility and persistence [27] and reported that HLA-G*01:01:02 and HLA-G*01:01:08 alleles were associated with increased risk for HPV16 and any HPV infection and HLA-G*01:01:02 and HLA-G*01:03 alleles were associated with persistent HPV16 and persistent HR-HPV infection. While HLA-G has been shown to play a role in HPV susceptibility and persistence; the association between HLA-G expression and cervical cancer tissue or progression of cervical disease has been inconsistent [26, 28-34]. In our study we report an association between two SNPs near the HLA-G region and HPV clearance; however, the specific HLA-G alleles could not be determined through our genotyping method. If HLA-G acts in immunosuppression, the lack of or increased immunosuppression may play a role in HPV clearance or persistence in the host. Further research is warranted to validate these findings and to determine the function of these SNPs.

Recently, there was a genome-wide association study (GWAS) assessing susceptibility loci for cervical cancer among the Swedish [35]. They reported three SNPs located in the major histocompatibility complex (MHC) region at 6p21.3 were associated with cervical cancer. Two of these SNPs (rs2516448 and rs9272143) also included in the ImmunoChip were evaluated in our cohort and their association with clearance of HPV; however, neither of these were significant in our population (rs2516448: HR=1.10, p=0.19 and rs9272143: HR=1.00, p=0.95). Our nonsignificant findings for these two SNPs may be explained by the fact that the Swedish GWAS is comprised of a very homogenous Caucasian population and contained family and twin cohorts while our cohort contains African-American HIV-1 positive women. A GWAS association study of HPV seropositivity was performed in a central European case-control study and they reported a significant association between HPV8 seropositivity and this rs9357152 located in MHC II region at 6p21.32 [10]. These results were replicated in a Latin-American case-control study (OR=1.35 $p=2.2x10^{-5}$). This SNP rs9357152 was also assessed in our cohort, but it was not significant (HR=1.13, p=0.14). This SNP was not associated with HR-HPV types in either study and in our analysis that SNP was not associated with HR-HPV clearance as well.

There are several limitations to this study. First, our sample was comprised of 258 women, which reduces our power to detect the significance level needed for multiple testing with genetic data using a stringent Bonferroni correction. However, we were able to observe a SNP that met this threshold and several marginally significant SNPs. Second, our cohort was also comprised of only African-American HIV-1 positive women and our study findings may not be generalizable to the general population. Although, this

admixture population of African-Americans with variability in their genetic background gives strength to the significant findings and this specific group is extremely relevant due to health disparities and the high prevalence of HPV among HIV-1 positive women. Third, the ImmunoChip was designed for use in white European populations and could be less informative for other ethnic groups if the disease-associated variants are not shared between them.

Lastly, the Q-Q plot had high deviation from expected line which might suggest true associations, population stratification due to repeated measures, the Cox proportional hazard model or may be due to a strong association with SNPs in heavily genotyped regions, like MHC on chromosome 6 [36, 37]. Women with multiple HPV infections were included in the Cox proportional hazard model with the WLW extension, which should account for the correlation between the individuals' data being used multiple times, but this could have an effect on the Q-Q plot due to the population substructure. The ImmunoChip has dense coverage of the MHC region as well as other regions so this may explain the deviation since the SNPs are close together they are in high LD and therefore result in similar p-values. Also, the majority of genetics studies model the association using logistic regression and the same assumptions of the Q-Q plot may not hold for the Cox proportional hazard model. The results could be a true association since we are assessing the association between a virus and immune related genes and therefore we would expect complex network of genes to play a role in clearance of HR-HPV.

This hypothesis generating analysis assessing SNPs in immune related genes and their association with HR-HPV clearance brought forth several significant SNPs. The

ImmunoChip is based off of a consortium of genes selected based on their previous association with autoimmune diseases, which means other important genes that may be associated with HR-HPV clearance could have been missed. We saw several SNPs in chromosome 6, located in the HLA-G region, which were consistently associated with HR-HPV clearance. The results look promising and need to be replicated in a larger dataset.

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**Table 1. Cox proportional Hazard Ratios (HR) for the SNPs associated with time to clearance of high-risk (HR-HPV) HPV infection controlling for CD4+ count, low-risk (LR-HPV) HPV infection(s), principal component analysis (PCA) for ancestry and genotyping facility.**

| snp | chr | pos | MAF | HR | 95% CI | p-value | q-value |
|---|---|---|---|---|---|---|---|
| rs1633038 | 6 | 29848016 | 0.13 | 1.89 | (1.64,2.13) | 3.48E-07 | 0.04 |
| rs12562152 | 1 | 244065966 | 0.1 | 2.04 | (1.74,2.33) | 1.83E-06 | 0.07 |
| rs17781894 | 19 | 60198938 | 0.18 | 1.68 | (1.46,1.90) | 3.45E-06 | 0.07 |
| rs2524035 | 6 | 29940311 | 0.29 | 1.46 | (1.30,1.61) | 4.05E-06 | 0.07 |
| rs7802321 | 7 | 51032682 | 0.38 | 1.41 | (1.26,1.56) | 4.13E-06 | 0.07 |
| rs16958011 | 16 | 67077947 | 0.14 | 1.74 | (1.50,1.98) | 5.25E-06 | 0.07 |
| rs2309814 | 2 | 100252424 | 0.15 | 1.52 | (1.34,1.70) | 5.76E-06 | 0.07 |
| rs2063979 | 17 | 31327679 | 0.32 | 1.52 | (1.34,1.70) | 6.34E-06 | 0.07 |
| rs10058955 | 5 | 35823630 | 0.12 | 1.59 | (1.39,1.79) | 6.4E-06 | 0.07 |
| rs73609914 | 19 | 60196414 | 0.18 | 1.66 | (1.44,1.88) | 6.49E-06 | 0.07 |
| rs6476458 | 9 | 34677876 | 0.13 | 1.52 | (1.33,1.70) | 7.1E-06 | 0.07 |
| rs6571225 | 6 | 105858243 | 0.22 | 1.48 | (1.31,1.65) | 7.53E-06 | 0.07 |

The results for each SNP using an additive model, the with major allele frequency used as the reference. Abbreviations: SNP=single nucleotide polymorphism, chr= chromosome, MAF=minor allele frequency, HR= hazard ratio, 95% CI= 95% confidence interval. q-value=False Discovery Rate

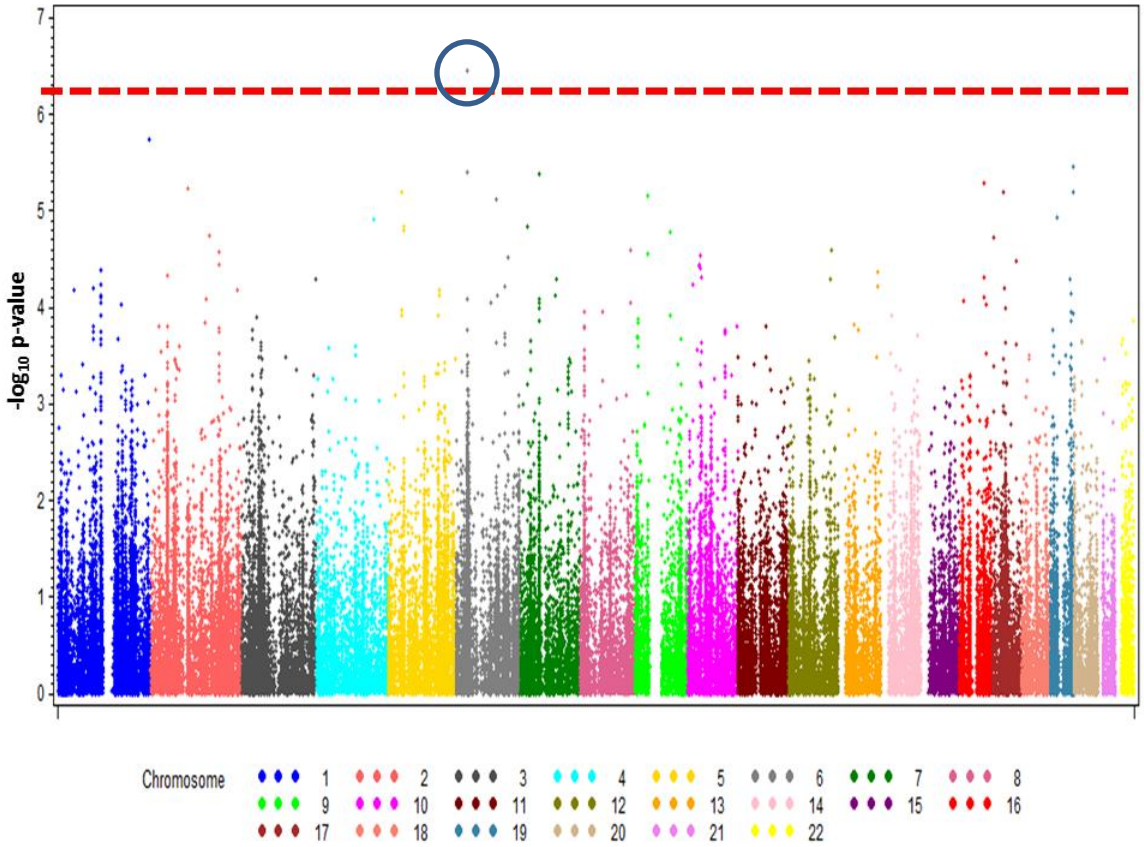


**Figure 1. Manhattan plot showing the association P-values of single nucleotide polymorphisms (SNPs) in the ImmunoChip with the time to clearance of HR-HPV. The X-axes display the chromosome on which the SNP is located, the Y-axes display −log10(P-value). The dashed red line represents a significance level needed for multiple testing using the Bonferroni correction and the blue circle is highlighting the SNP that reached that statistical significance level**

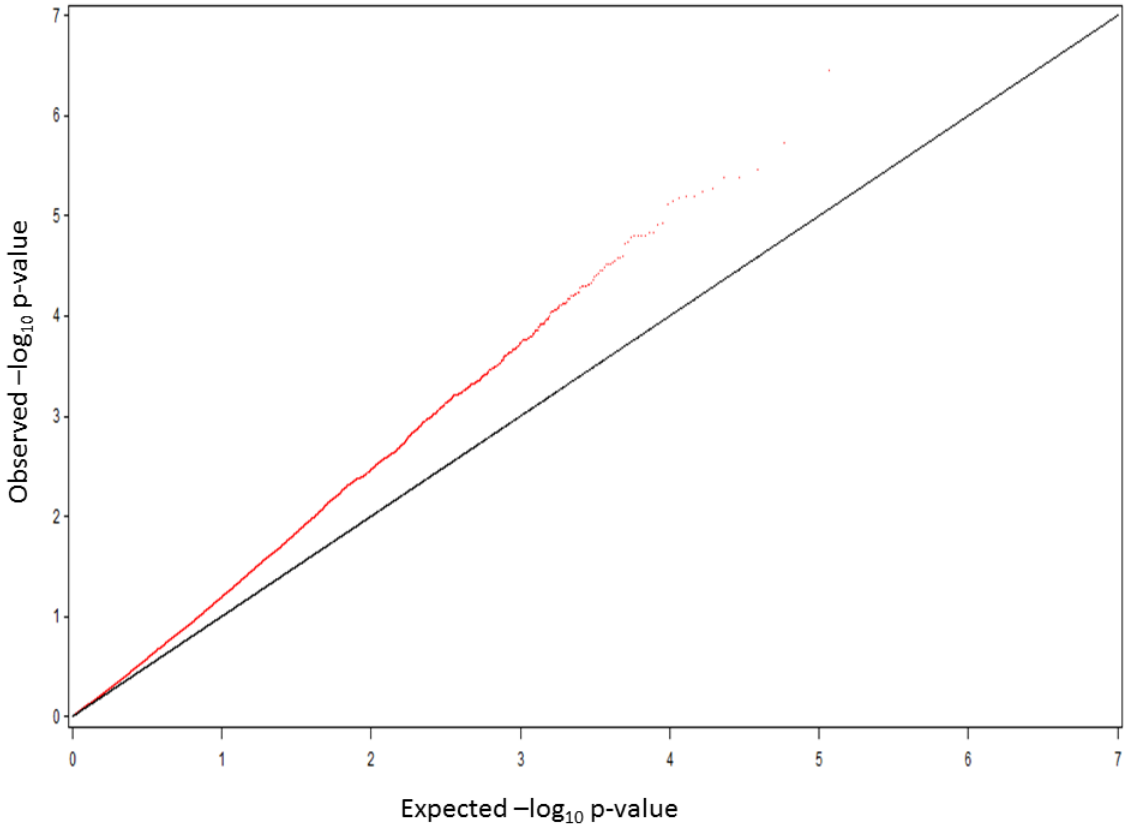


**Figure 2. Quantile-quantile (Q-Q) plot showing the association P-values of single nucleotide polymorphisms (SNPs) in the ImmunoChip with the time to clearance of HR-HPV. The X-axes display the expected $-\log_{10}$(P-value), the Y-axes display the observed $-\log_{10}$(P-value).**

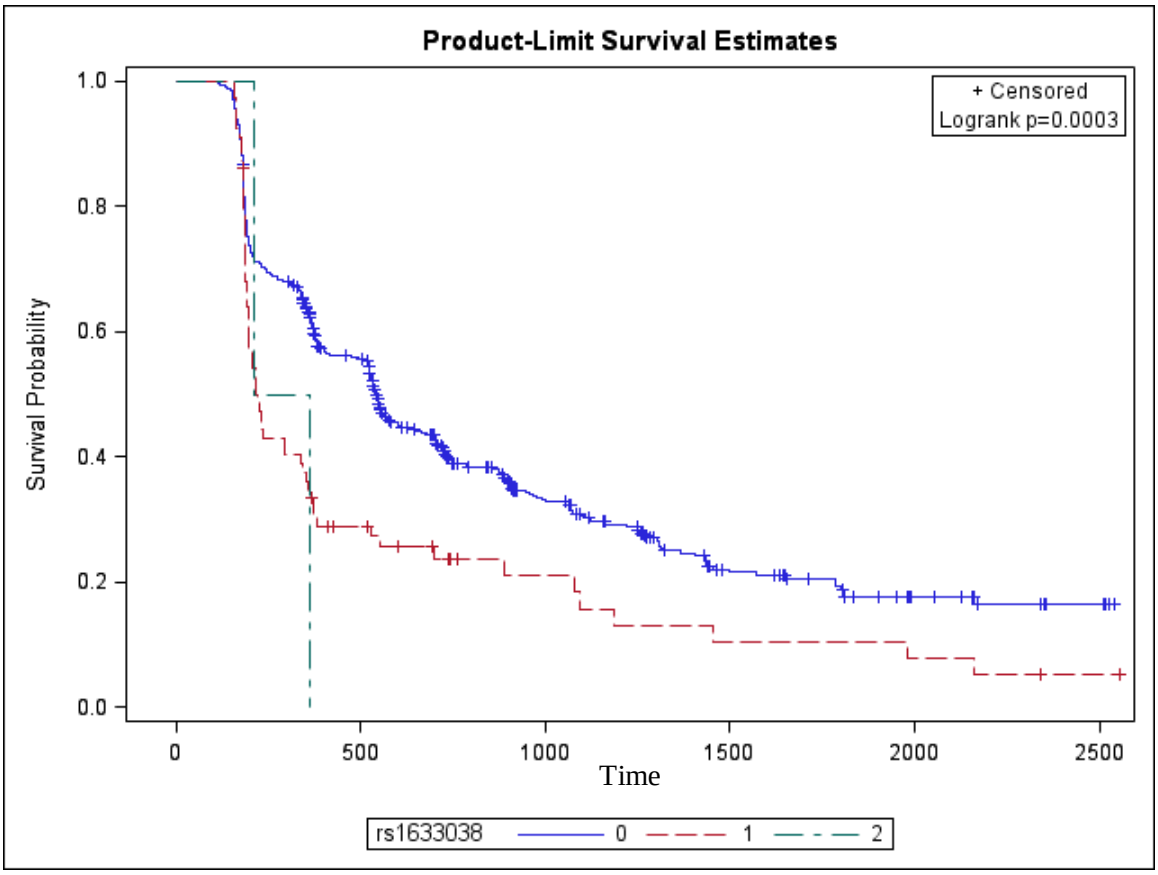


**Figure 3.Kaplan-Meier curve for the time (days) to clearance of high-risk (HR-HPV) HPV stratified by genotype for SNP rs1633038, where 0= homozygous for the major allele, 1=heterozygous, and 2= homozygous for the minor allele.**

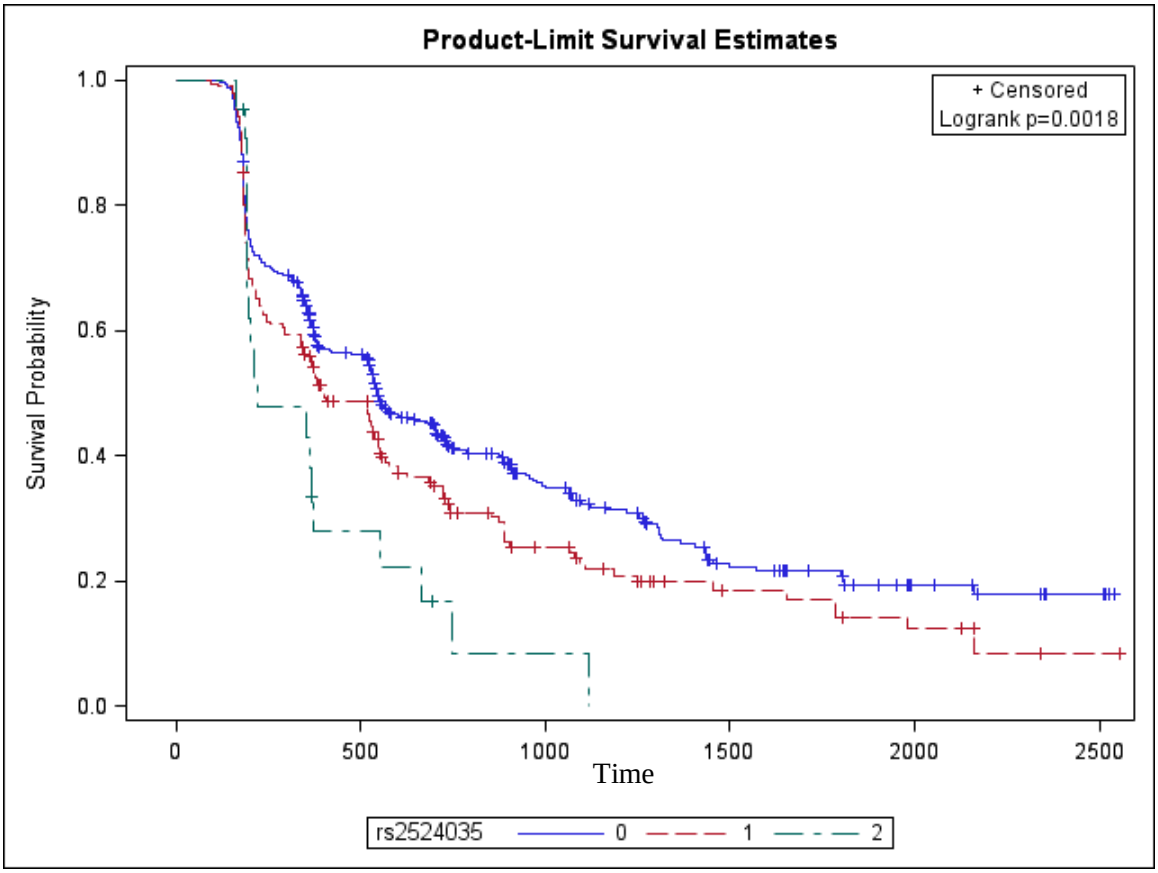


**Figure 4. Kaplan-Meier curve for the time (days) to clearance of high-risk (HR-HPV) HPV stratified by genotype for SNP rs2524035, where 0= homozygous for the major allele, 1=heterozygous, and 2= homozygous for the minor allele.**

# CHAPTER 6

# DISCUSSION

Throughout this dissertation research, I have described how variants in various host genes are associated with clearance of HPV. In this dissertation, I first examined the definition of the intermediate phenotype to cervical cancer and then I incorporated this phenotype in all my three aims by examining variations in xenobiotic metabolism genes in HIV-1 negative and immune-related genes in HIV-1 positive females and how these contribute to HPV infection outcomes. Several significant variants were associated with HPV clearance for the three aims and this chapter will focus on parsing out these findings, discussing strengths and limitations, as well as the public health significance.

Three multicenter longitudinal cohorts in the United States: ALTS, REACH, and HERS were used in the three aims of my dissertation. These three cohorts were recruited and followed during the 1990s with 2-4 years of follow-up. The ALTS cohort participants were HIV-1 negative, had a median age of 24 years, and were African-American (61%) or Caucasian (39%). The REACH cohort, for my analysis was comprised of all HIV-1 African-American adolescents (median age= 17 years). The HERS cohort was African-American HIV-1 positive females (median age of 35 years). ALTS and HERS are comparable age groups, while REACH consists of adolescents. REACH and HERS, for my analysis was restricted to HIV-1 positive, while ALTS was HIV-1 negative. The three cohorts have several similarities; however the types of

analysis and variables used for these differed therefore we have to cautious when generalizing results across cohorts.

Cervical samples were collected at enrollment and every 6 months thereafter for all three cohorts and tested for HPV DNA. For REACH and HERS, viral DNA fragments from cervical lavage were amplified by using the consensus primers MY09/11 and HMB01 and were hybridized for a consensus probe and for 30 HPV types in the REACH cohort and 26 HPV types in the HERS cohort by using a chemiluminescent dot-blot format.[16, 17] For ALTS, the viral DNA from cervical lavage were identified through L1 consensus primer PGMY09/11 polymerase chain reaction amplification and reverse line blot hybridization to detect 38 HPV genotypes.[10, 18] Both types of consensus primers (MY09/11 and PGMY09/11) are commonly used; however, PGMY09/11 was found to be significantly more sensitive (McNemar's $\chi^2$=17.2) to detect HPV than the MY09/11 primer.[18] The decreased sensitivity may have affected the ability to detect HPV types in REACH and HERS, which then the correlation between types could not have been accounted for in the model. This systemic bias for undetected types holds true in all three cohorts since new HPV types are still being discovered. All three aims assessed time to clearance of high-risk (HR-HPV) HPV types controlling for low-risk (LR-HPV) infections. HR-HPV has greater biological significance since these types are most attributable to cervical lesions and cervical cancer.

The outcome of interest for all three analyses was the association between genetic polymorphisms and time to clearance of HPV infection. The statistical method I used was the Cox proportional hazard model with the Wei-Lin-Weisfeld (WLW) extension, which accounts for the correlation between HPV subtypes within a person and has

population-level interpretations.[19] I used this method was used based on the high number of co-infections in the women and to obtain results that are more generalizable to the population. Other methods for the analysis were considered like the Cox proportional hazard model with the frailty term that also accounts for the correlation between HPV subtypes within a person, but it has individual-level interpretations, which is difficult to comprehend in epidemiological studies.[19, 20] Other methods, such as the model based on transitional probability,[21] the framework model based on the clustered longitudinal binary data structure,[22] and the discrete-time semi-Markov models[23] have also been used to account for both prevalent and incident infections. Again, given the data and scope of the aims, the Cox proportional hazard model with the WLW extension was the most appropriate.

The genotyping approaches varied across the three aims. For the ALTS cohort, I assessed functional variants within xenobiotic metabolism genes. These functional variants have measurable outcomes for the three genes assessed and all three have been previously shown to be associated with cervical cancer.[24, 25] For the REACH, I used a candidate gene approach to select SNPs within Interleukin family of cytokines and Toll-like Receptors (TLRs) that have been reported to have functional consequences or associations with cervical cancer, HPV infection or other infections. Finally for the HERS cohort, I used a global hypothesis generating approach of assessing several genetic variants in immune related genes using the ImmunoChip and their association with HPV clearance.

For the first aim, in ALTS assessing functional variants within xenobiotic metabolism genes and clearance of HR-HPV, I found a functional variant allele in

*CYP1A1* was significantly associated with lower clearance rates of HR-HPV and the *GSTM1* null variant was significantly associated with higher clearance rates of HR-HPV (Chapter 3). Many carcinogens require metabolic activation enzymes like *CYP1A1*, which are then detoxified by enzymes like *GSTT1* and *GSTM1*. Reactive metabolites that are not detoxified may result in DNA damage, which may facilitate HPV integration resulting in lower likelihood of its clearance.

In the second aim, using the REACH cohort assessing SNPs within Interleukin family of cytokines and Toll-like Receptors (TLRs) and their influence on HR-HPV clearance, I found HR-HPV clearance rates were significantly associated with five SNPs that mapped to coding and regulatory regions in three genes (*IL2RB*, *IL1RN*, and *IL7R*). One of the genes, *IL1RN* has previously been shown to be associated with cervical cancer risk.[26-28] I assessed the interaction between two of the significant SNPs in this gene and found a significant association when combining the two alleles that were associated with higher clearance rates of HR-HPV (HR=5.12, $p=3.16x10^{-20}$, 95% CI 4.77, 5.47). However, the interpretation of this interaction with smaller sample size needs caution and require replication.

Finally in the third aim, using the HERS cohort assessing SNPs within several immune related genes and their influence on HR-HPV clearance, I found several significant associations between SNPs, specifically those located on chromosome 6 in the HLA-G region that was associated with higher clearance rates. Previous literature has shown an association between HLA-G and cervical cancer risk, while my results show an association between the intermediate phenotype and HLA-G. It is possible the

association with cervical cancer may be a surrogate for a true association between HPV and HLA-G.

As with most studies, there are several limitations in my dissertation research studies. All three studies are no longer ongoing and so I was limited to the samples, data, and variables that were collected in the parent study. However, the REACH and HERS studies are among the few cohorts with follow-up data on HIV, HPV, and other cofactors and the ALTS study is a unique ancillary cohort with extensive nutritional and HPV follow-up data. The cohorts for the most part are very homogeneous in that they assessed HPV clearance among women that already had cervical lesions of undetermined stage (ALTS) or were assessed in HIV-1 positive cohorts (REACH and HERS), which warrants caution with their interpretation to the general population. However, the results of these studies are valuable in understanding the role of host genetics and HPV outcomes. Further research is needed to validate these findings in other populations.

One of the limitations specifically for the ALTS cohort in chapter 3 is the genotyping methods for *GSTT1* and *GSTM1* variants. This method can only distinguish between individuals with the null genotype versus those with a functional copy.[29, 30] While new methods using gene copy number assays (0, 1 or 2 copies of the gene), have been recently developed, my genotyping method is still commonly used including meta-analyses that have used similar analytical approach.[24, 25]

A major limitations for the REACH cohort is that my sample size was limited to 134 women (after exclusion criteria), which reduces our power to detect the significance level needed, considering multiple testing with genetic data using a stringent Bonferroni correction; however, I was able to observe associations with statistical significance using

the FDR. Second, I included prevalent HPV infections in the analysis; however, these adolescent women were likely to be infected recently with HIV and HPV due to their age and sexual risk factors that predisposed them to these infections.

There are several limitations to the HERS study. First, our sample was comprised of 258 women, which reduces our power to detect the significance level needed for multiple testing with genetic data using a stringent Bonferroni correction. However, we were able to observe a SNP that met this threshold and several marginally significant SNPs. Second, our cohort was also comprised of only African-American HIV-1 positive women and our study findings may not be generalizable to the general population. Although, this admixture population of African-Americans with variability in their genetic background gives strength to the significant findings and this specific group is extremely relevant due to health disparities and the high prevalence of HPV among HIV-1 positive women. Third, the ImmunoChip was designed for use in white European populations and could be less informative for other ethnic groups if the disease-associated variants are not shared between them. Lastly, the Q-Q plot had high deviation from expected line which might suggest true associations, population stratification due to repeated measures, the Cox proportional hazard model or may be due to a strong association with SNPs in heavily genotyped regions, like MHC on chromosome 6.

Throughout this dissertation research, I have described the importance of the intermediate phenotype to cervical cancer, HPV persistence. This intermediate phenotype is the necessary but insufficient cause of cervical cancer due to the fact the majority of women infected with HPV naturally clear the virus while only a small subset progress to cervical lesions and/or cancer. While cervical cancer rates in the United

States have dramatically decreased with effective screening, the economic and emotional burdens of treating cervical intraepitheilial neoplasias (CIN), resulting from persistent HPV infections, still exist.[31] In 2005, the U.S. spent between $2.25-$4.6 billion dollars on health care costs in HPV related conditions.[31] In 2009, Hispanic women had the highest incidence rates of cervical cancer in the United States and African-American women had the highest mortality rates for cervical cancer.[32] Cervical cancer is the third most common cancer worldwide with more than 85% of the global burden occurring in developing countries.[1] HPV related disease is a major problem economically and emotionally in the United States, but globally is responsible for over 270,000 deaths.[1]

The importance of HPV persistence as the intermediate phenotype has been acknowledged in the clinical settings, resulting in performing HPV tests into several screening programs. In a large longitudinal study, Castle, et al, recently described that while both baseline Pap and HPV tests predicted development of CIN3 within the first 2 years of follow-up, only HPV testing predicted CIN3 in 10 to 18 years.[33] Precancerous lesions and cervical cancer have often been the public health focus and recently HPV testing has been recommended in clinical screening.[4]

Host genetic factors associated with higher clearance rates of HR-HPV could serve as potential biomarkers for future HPV related disease. Also, identification of susceptibility loci may allow earlier diagnosis based on an individual's genetic constitution and may facilitate identification of disease subtypes amenable to therapeutic approaches. While the results from my dissertation need further validation in other cohorts, I believe that these genetic variants could potentially be incorporated into future screening tools or as adjuvant therapy for immune related genes. As healthcare in the

United States transitions to the incorporation of measuring HPV in cervical screening methods, I believe that this transition will also be able to incorporate genetic variants in the future. The technology for cervical cancer screening has vastly improved and eventually the technology and cost for genetic variants will be widely available. The traditional epidemiological risk factors are transitioning into epigenetics and the field of public health is transitioning with it.

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# APPENDIX A

# UAB IRB PROTOCOL FOR ALTS STUDY

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

*Institutional Review Board for Human Use*

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on January 24, 2017. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

| | |
|---|---|
| Principal Investigator: | PIYATHILAKE, CHANDRIKA J |
| Co-Investigator(s): | SUDENGA, STACI LYNN |
| Protocol Number: | **X110425002** |
| Protocol Title: | *Prospective Follow-Up Study of Gene-Nutrient Interaction Affecting the Risk of Cervical Cancer* |

The IRB reviewed and approved the above named project on 5-9-12. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 5-9-12

Date IRB Approval Issued: 5-9-12

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of
Alabama at Birmingham
Mailing Address:
AB 470
1530 3RD AVE S
BIRMINGHAM AL 35294-0104

# APPENDIX B

# UAB IRB PROTOCOL FOR REACH/HERS STUDY

OMB No. 0990-0263
Approved for use through 3/31/2015

## Protection of Human Subjects
## Assurance Identification/IRB Certification/Declaration of Exemption
### (Common Rule)

*Policy:* Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

| 1. Request Type | 2. Type of Mechanism | 3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No. |
|---|---|---|
| [X] ORIGINAL<br>[ ] CONTINUATION<br>[ ] EXEMPTION | [X] GRANT [ ] CONTRACT [ ] FELLOWSHIP<br>[ ] COOPERATIVE AGREEMENT<br>[ ] OTHER:_____ | |
| 4. Title of Application or Activity<br>Host Genetics and Outcomes of HPV Infection in HIV/AIDS | | 5. Name of Principal Investigator, Program Director, Fellow, or Other<br>SHRESTHA, SADEEP |

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 01/24/2017 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [X] Expedited Review on (date) 6-12-12
[ ] If less than one year approval, provide expiration date _____
[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
Protocol subject to Annual continuing review.

Title X100615001
Host Genetics and Outcomes of HPV Infection in HIV/AIDS

IRB Approval Issued: 6-12-12

| 9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided. | 10. Name and Address of Institution<br>University of Alabama at Birmingham<br>701 20th Street South<br>Birmingham, AL 35294 |
|---|---|
| 11. Phone No. *(with area code)* (205) 934-3789<br>12. Fax No. *(with area code)* (205) 934-1301<br>13. Email: irb@uab.edu | |
| 14. Name of Official<br>Marilyn Doss, M.A. | 15. Title<br>Vice Chair, IRB |
| 16. Signature Marilyn Doss | 17. Date 6-12-12 |

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