Human Papillomavirus Infections among Mid-Adult Women

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Abstract

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This study describes the short-term natural history of vaginal human papillomavirus (HPV) and the epidemiology of oral and fingernail HPV among mid-adult women. From 2011-2012, 409 30-50 year-old women were recruited and followed for 6 months. Women completed surveys on health and behaviors and provided self-collected vaginal samples (monthly) and oral/fingernail specimens (enrollment and exit) for type-specific PCR-based HPV DNA testing. HPV-positive vaginal samples were tested for viral load. Enrollment blood specimens were tested for type-specific HPV antibodies. In chapter 1, we describe the relationship between host and viral factors and repeat high-risk (HR) HPV detection. HPV viral load (adjusted [a]RR per one $\log_{10}$ increase in viral load=1.10; 95%CI:1.05,1.16) and current smoking (aRR=1.24; 95%CI:0.96, 1.59) were each positively associated with the proportion of type-specific HR HPV detections over follow-up, whereas incident (versus prevalent) detection status (aRR=0.28; 95%CI:0.20, 0.39) and history of pregnancy (aRR=0.83; 95%CI:0.68, 1.0) were negatively associated. Risk factors were similar to those previously identified for long-term persistence,

suggesting that short-term persistence may relate to long-term persistence. In chapter 2, we describe the incidence of, and risk factors for, newly detected type-specific HR HPV stratified by type-specific HPV serostatus. Six-month cumulative incidence of HR HPV was higher when there was serologic evidence of prior infection with the same type (2.6%) than when there was not (1.3%). Recent sexual behaviors were associated with incident HPV detection among women without serologic evidence of prior infection only, suggesting that incident detection is more likely due to reactivation or intermittent detection of persisting infection when serologic evidence of prior infection is present. In chapter 3, we evaluated the frequency of, and risk factors for oral and fingernail HPV. Prevalence of genital HPV types detected in the oral cavity (2.5%) and fingernails (3.8%) were low. Concurrent vaginal infection with the same HPV type was strongly associated with both oral (OR=10.29; 95%CI:3.34, 31.72) and fingernail (OR=104.33; 95%CI:30.44, 357.5) HPV detection. Results suggest that DNA deposition between anatomic sites is plausible, though oral and fingernail HPV detection may be unlikely to serve a key role in genital HPV transmission.

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1 Short-Term Natural History of High-Risk HPV Infection in Mid-Adult Women Sampled Monthly

1.1 INTRODUCTION

The natural history of human papillomavirus (HPV) infections among mid-adult women has been understudied.[1] Past HPV natural history studies have largely targeted women in their late teens and early 20s, the age group with the highest HPV prevalence and incidence.[2] Newly detected HPV infections among younger women are associated with reports of new sex partners, indicating new acquisition of the virus.[3] Conversely, newly detected infections among women in mid-adulthood may represent new acquisition, reactivation of a previous infection from a latent state, or intermittent persistent detection,[4] rendering the origin of newly detected infections in this age group unclear. Clarifying the source of newly detected infections may inform whether prophylactic HPV vaccination (currently targeting females 9 to 26 years of age) would be warranted in this age group.[1]

While the majority of HPV infections clear without developing clinical manifestations, repeated detection of oncogenic HPV in young women is associated with increased risk of developing cervical neoplasia.[3] In particular, long-term persistent high-risk (HR) HPV infection is necessary for developing cervical cancer.[5] Short-term persistence (e.g. for one year) also has been shown to be associated with increased cervical neoplasia risk.[6, 7] Therefore, understanding the rates of, and factors associated with, short-term persistent infections may be relevant to characterizing infections likely to have clinical significance in the long term. Closely followed cohorts of women with follow-up intervals of one to two weeks have shown that detection of HPV infection may be sporadic or intermittent even within a short period of time.[8-10] In addition, previous studies have shown positive correlations between levels of HPV DNA and risk of HPV persistence and carcinogenic progression,[11-18] but whether viral load correlates with frequency of

repeat HPV detection over short sampling intervals has not been evaluated. Characterizing the relationship between viral load and repeated HR HPV detection using frequent sampling may aid in understanding both natural history and the origin of newly detected infections in this age group. Further assessments of the relationship between HPV viral load and other factors associated with repeat HPV detection may aid in explaining why fluctuations in HPV positivity tend to be observed in frequently followed cohorts.

We therefore evaluated the relationship between various factors and repeated HR HPV detection in a cohort of mid-adult women aged 30 to 50 years, sampled monthly for up to 6 months. Factors of interest included type-specific HPV viral load and timing of HPV detection (prevalent versus incident), as well as demographic, health, and behavioral characteristics. In addition, we also identified correlates of HPV viral loads.

1.2 MATERIAL AND METHODS

1.2.1 Study Population

Between March 2011 and January 2012, we recruited mid-adult women aged 30 to 50 years to participate in a longitudinal study of HPV infections. Enrollment was limited to female students, faculty, and staff at the University of Washington (UW) for convenience of follow-up. Women who were pregnant, ever had a hysterectomy, or had any serious medical conditions that would prohibit adherence to the study procedures were ineligible to participate. Women who would not be willing to self-collect vaginal samples or would be unavailable for follow-up procedures within 6 months after enrollment were also excluded. Women were recruited through flyers, advertisements, and letters distributed to students, faculty and staff at the UW. Interested women were screened for eligibility over the telephone, and those meeting the eligibility criteria were scheduled for an enrollment visit. The study coordinator administered informed consent at the enrollment visit. The protocol was reviewed and approved by the UW Institutional Review Board.

1.2.2 Data Collection

Enrollment and 6-month exit visits took place at the Hall Health Primary Care Center (HHPCC) of the UW. The enrollment visit was comprised of multiple components, in order: (1) face-to-face interview on cervical cancer screening and HPV vaccination history administered by the study coordinator, (2) vaginal self-sampling for HPV DNA testing (oral and fingernail samples for HPV DNA testing and a blood draw for HPV antibody testing were also collected; data were not included in this analysis but included in chapters 2 and 3), (3) online self-administered survey on demographics, health status, pregnancy history, contraceptive use, hormone use, smoking habits, and sexual history, and (4) online self-administered sexual behavior diary covering the two-weeks prior to enrollment. Survey instruments were similar to those used in our previous HPV natural history studies.[19, 20] During the 6-month follow-up, women were asked to self-collect additional vaginal samples each month (resulting in a potential maximum of 7 total samples per woman) and complete online sexual behavior diaries every two weeks. The exit visit included the same components as the enrollment visit except the questionnaires were modified to capture new information occurring since enrollment. Only the enrollment visit survey data were included in this analysis.

At the enrollment visit, the study coordinator verbally outlined the procedures for self-collecting vaginal samples and also provided illustrated written instructions. Self-collection kits contained two sterile Dacron swabs (to enhance sensitivity for HPV detection),[21] a covered tube containing 1.5 mL of Specimen Transport Medium (Qiagen, Gaithersburg, MD), and nitrile gloves.[19]. Women were sent monthly reminders to self-collect vaginal samples and return mail or hand-deliver them to HHPCC, and biweekly reminders to complete the sexual behavior diaries. Each week, batched vaginal samples were transported by courier from HHPCC to the laboratory, and either stored in at -20°C or prepared for immediate testing.

1.2.3 HPV DNA Testing

Genomic DNA was isolated from self-collected vaginal specimens and HPV genotyped using the PCR-based Roche Linear Array assay (Roche Molecular Systems, Alameda, CA), which uses a β-globin control. The vaginal specimens were digested with 20 μg/mL proteinase K at 37°C for one hour, and DNA isolated from 200 μL of the digested sample using QIAamp DNA blood mini kit, following the protocol of the manufacturer (Qiagen, Cat. No.51104). Specimens negative for β-globin were considered insufficient, and specimens positive for β-globin but negative for HPV were considered negative for HPV infection. This analysis included 19 high-risk (HR) HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, IS39) considered to be carcinogenic, probably carcinogenic, or possibly carcinogenic.[22, 23]

1.2.4 HPV Viral Load Testing

Viral load testing, or quantification of HPV DNA, was conducted on samples that tested positive for type-specific HR HPV by the Roche assay. Viral load testing was conducted for 16 HR HPV types, including HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. Real-time PCR was used for simultaneous quantification of HPV DNA and cellular DNA. Optimized duplex reaction condition was used to ensure no interference between the detection of HPV and cellular DNA. Each sample was assayed in triplicate. HPV type-specific primers/probes for the E7 gene were designed using Primer Express (Applied Biosystems) (Table 1.1). The primers and probe for β-actin gene (used for cellular DNA estimation) are commercially available (Applied Biosystems, Foster City, CA). The assay was set up in a reaction volume of 5 μl with the TaqMan® Universal PCR Master Mix kit (Applied Biosystems). Amplification was carried out on Applied Biosystems 7900 HT Sequence Detection System with a cycling program of holding at 50°C for 2 minutes and then at 95°C for 10 minutes followed by a two-step cycle of 15 seconds at 95°C and 1 minute at 60°C for 40 cycles. The known copy number of type-specific HPV genome and the known amount of cellular DNA were used as standards for

absolute quantification. The number of viral copies was normalized according to the input amount of cellular DNA and expressed as the number of HPV copies per nanogram of cellular DNA. These corrected copy numbers were $\log_{10}$ transformed, and a mean of the three measures was used for analysis. If one of the three measures was negative or differed by more than two standard deviations, the mean of the two remaining measures was used. Samples positive for β-actin but negative for HPV in two or more runs were assigned a value of 1 copy number per nanogram of cellular DNA if they tested positive by the Roche assay (n = 133/773 type-specific positive samples; 17.2%). For all statistical analyses using viral load data, sensitivity analyses were conducted excluding samples with undetectable viral load results.

1.2.5 HPV Variant Characterization

Samples with the same HPV type redetected after at least one intercurrent HPV negative sample were targeted for HPV variant characterization. In general, the first sample of the initially detected infection and the last sample of the subsequently redetected infection were selected and sequenced. If any selected sample had weak PCR genotyping results, it was replaced with the chronologically closest sample of the same HPV type; if no such redetected sample (with at least one HPV negative sample in between) was available, then the original selected sample was sequenced.

HPV DNA fragments covering the 3' part of the long control region and the entire E6 and E7 region were generated by PCR with a set of type-specific external primers (*Table 1.1)*.[24] PCR products were then sequenced with a pair of external primers and a pair of internal primers using BigDye™ Sequencing kit (Applied Biosystems, Foster City, CA).[25] The sequencing reaction was run from both directions. DNA sequences were analyzed using Sequencher™ package (Gene Codes Corp., Ann Arbor, MI). A viral isolate was defined as a distinct variant if, as compared to the prototype and other isolates detected in the study, there was one or more nucleotide alterations in the region analyzed.

1.2.6 Statistical Analyses

Samples were numbered according to the sample window during which the samples were received (*Figure 1.1*). A sample window was defined as 14 to 45 days after the previous sample was received or a 30-day period after the end of the previous sample window if no sample was received. The sample window numbering system ensured that samples were spaced at least 14 days apart and accounts for the fact that samples were not always submitted on a monthly schedule. Additional samples that were received during the same sample window were dropped at random (n = 123/2755 samples dropped; 4.5%). Samples received ≥ 8 sample windows after the enrollment sample were excluded (n = 153/2755 samples excluded; 5.6%). As a result of these exclusions (and loss to follow-up), not all women analyzed had 7 available samples. Samples from women who provided less than 4 self-collected vaginal samples (n = 47/2755; 1.7%) were excluded from all analyses. Furthermore, to assess the potential effect of the missing data, sensitivity analyses were conducted restricting to a subset of women with at least 6 available samples.

Type-specific detections were classified as prevalent if detected at enrollment and incident if detected during follow-up but negative at enrollment. Mean type-specific HPV viral loads (pooled across all types) were calculated separately for prevalent, incident, and redetected infections.

We evaluated factors associated with type-specific HR HPV viral load at the first positive detection by the Roche assay using linear regression with robust variance estimates to account for correlation within subjects. Analyses were adjusted for HPV type. Timing of detection (incident vs. prevalent) was considered as a factor. Potential factors assessed at enrollment included demographic characteristics, women's health (abnormal Pap test history, prior pregnancies, hormonal contraceptive use history, and STD history), general immunosuppression (defined as HIV positivity or currently taking immunosuppressive medications), smoking history, lifetime number of male sex partners, and recent sex with male

partners. Variables for which $p < 0.10$ by Wald test in the univariate analyses were included in a multivariate analysis.

To evaluate factors associated with repeat type-specific HR HPV detection over follow-up, we used Poisson regression to model the proportion of follow-up samples that were repeatedly positive for the same HR HPV type. The number of type-specific HR HPV detections per woman was used as the outcome of interest, and the number of available samples per woman used as the offset variable. Robust variance estimates were used to account for correlation within subjects and deviations from the Poisson assumption. All models were adjusted for HPV type. Incident detections at the last available sample were excluded from this analysis because subsequent repeat detection could not be assessed. The risk factors assessed were similar to those described previously with the addition of type-specific HPV viral load at the first positive sample by the Roche assay. Factors for which $p < 0.10$ by Wald test in univariate analyses were included in multivariate models. Two multivariate models were constructed, including and excluding HPV viral load at first positive detection. Comparing the associations between potential health or sexual behavior factors and repeat HPV detection in the two models enabled an informal assessment of whether these associations are mediated by viral load.

1.3 RESULTS

1.3.1 Characteristics of study population

Of the 409 women enrolled, 387 (94.6%) who returned at least 4 self-collected vaginal samples were included in this analysis (*Figure 1.2*). (Three hundred and forty-five (89.1%) returned ≥6 samples for HPV testing). Their mean age at enrollment was 38.4 (SD = 6.1) years. The majority was white (79.1%), and approximately half were married (48.1%). The median lifetime number of male sex partners reported was 7 (IQR = 3 – 15), and 30.5% reported sex with at least one new male sex partner within the year prior to enrollment (*Table 1.2*). Out of 2,432 vaginal samples collected within a valid sample window, 2 (0.1%) were insufficient for HPV DNA testing.

1.3.2 HR HPV detections at enrollment and over follow-up

At enrollment and over follow-up, 139 (35.9%) women tested positive for at least one HR HPV type. Overall, a total of 243 type-specific HR HPV infections were observed, with HPV types 53 (16.1%), 51 (14.4%), and 16 (8.6%) most commonly detected (*Table 1.3*). Of these infections, 132 (54.3%) were prevalently detected and 111 (45.7%) were incidently detected. Eighty-four type-specific infections (34.6%) were transiently detected (i.e. single-time positive), 62 (25.5%) were intermittently detected (i.e., at least one HPV-negative sample between two HPV-positive samples), and 97 (39.9%) were repeatedly detected without any intercurrent negative samples. To determine whether redetected infections following a period of intercurrent negativity were of the same variant as the initial detection, we sequenced 90 vaginal samples, 13 (14.4%) of which tested HPV-negative during PCR sequencing steps. Of the 62 intermittently detected infections, variant characterization was conducted for 45 paired type-specific infections that were of the 12 HPV types of which variant sequencing methods were available. Thirty-five (77.8%) paired type-specific infections had two samples that were not HPV-negative and available for variant comparison. One (2.9%) infection (HPV-16) displayed different HPV variants in the pre- and post-intercurrent negative samples. This HPV-16 infection (with a detection pattern of +/−/−/−/−/−/+) was therefore reclassified as transient (with a pattern of +/−/−/−/−/−/−) for all remaining analyses described below.

1.3.3 Quantification of HR HPV viral load in type-specific prevalent, incident, and redetected infection

The mean $\log_{10}$ HPV viral load (HPV DNA copies per nanogram of cellular DNA) measured at the time of first positive detection by the Roche assay was 2.12 (SD = 1.67) for prevalent detections and 0.70 (SD = 1.14) for incident detections. The mean viral load for incident detections was similar when restricting to first positive detections preceded by ≥2 (versus ≥1) negative samples (data not shown). For infections that were intermittently detected,

the mean $\log_{10}$ viral load per nanogram of cellular DNA measured at the time of first type-specific redetection was 0.90 (SD = 1.06).

1.3.4 Factors associated with HR HPV viral load

In univariate analyses adjusted for HPV type, incident detection status, being married, and ever having been pregnant were negatively associated with HPV viral load while current smoking was positively associated with HPV viral load (*Table 1.4*). In multivariate analysis, incident detection status and current smoking remained independent factors associated with HPV viral load. Incident detection status (adjusted β = -1.30; 95% CI: -1.76, -0.84) was associated with lower HPV viral load while current smoking was associated with higher HPV viral load (adjusted β = 0.70; 95% CI: -0.03, 1.43). A sensitivity analysis excluding samples with undetectable viral load results generated similar findings, with slightly attenuated results observed for smoking status (data not shown).

1.3.5 Factors associated with repeatedly detected HR HPV infections

Prevalently detected infections were more likely than incidently detected infections to be repeatedly detected. On average, the likelihood of a prevalent infection being repeatedly detected in any given follow-up sample was 67% (range: 60 – 75%) compared to 27% (range: 16 – 36%) for incident infections, with little variation over time (*Figure 1.3*). In univariate analyses, type-specific HR HPV viral load (at first positive detection by the Roche assay) and both former and current smoking were positively associated with the proportion of follow-up samples that were repeated positive for the same HR HPV type among women with HPV detected during follow-up (*Table 1.5*). Incident detection status and ever having been pregnant were negatively associated with the proportion of HR HPV detections. In the multivariate analysis without HPV viral load, incident detection status, pregnancy, and smoking status remained statistically significantly associated with the proportion of HR HPV detections (*Table 1.6*). Incident detection status was associated with a 76% reduction in the proportion of HR HPV detections

compared to prevalent detection status (RR = 0.24; 95% CI: 0.17, 0.34). History of pregnancy relative to nulliparity was associated with a 24% reduction in the proportion of HR HPV detections over the course of follow-up (RR = 0.76; 95% CI: 0.63, 0.93). Compared to never smoking, former and current smoking were associated with 19% (95% CI: 0.99, 1.42) and 37% (95% CI: 1.04, 1.79) increase in the proportion of HR HPV detections, respectively. After adding HPV viral load at first positive detection to the model (to explore associations with repeat detection independent of potential effects on viral load), the effects of incident detection, pregnancy, and smoking were only slightly attenuated. In the same model, type-specific HPV viral load remained positively associated with the proportion of HR HPV detections after adjustment for other factors (RR = 1.10; 95% CI: 1.05, 1.16; per one $\log_{10}$ increase in viral load). Neither lifetime number of male sex partners nor sexual activity with male partners within one year prior to enrollment were significantly associated with repeat detection of HR HPV. Results were similar when restricting to women with at least 6 available vaginal samples (data not shown). Similar findings were also found when excluding samples with undetectable viral load, with attenuated results for both former and current smoking (data not shown).

1.4 DISCUSSION

In this study of mid-adult women, we utilized monthly sampling intervals to characterize the short-term fluctuations in HPV detection over a 6-month follow-up. We sought to characterize differences between prevalent versus incident detections and to explore the relationships between repeat HR HPV detection, type-specific viral load, and demographic, health, and behavioral characteristics.

During follow-up, we found that the majority of type-specific HR HPV infections were detected either transiently (35%) or intermittently (25%). Temporal detection of different type-specific variants within a woman was rare, suggesting that type-specific redetection after a short period of intercurrent negativity most likely represents intermittent persistent detection rather

than new infection. In longitudinal studies, the reported frequency of intermittent type-specific HPV detection varies, and tends to increase with more frequent sampling and longer follow-up.[8-10, 26-28] In two prior small studies using weekly sampling intervals, intermittent infections were detected in 10% of 20 women aged 22 to 43 years followed for one month in the Netherlands,[10] and in 55% of 72 women aged 18 to 35 years followed for 10-12 weeks in New Mexico.[8] We may infer that more frequent sampling enhances the likelihood of capturing brief periods of HPV negativity and contributes to the varying rates of HPV intermittence observed across studies.

Past research has hypothesized fluctuations in detecting HPV infections could be caused by changes in HPV viral load or HPV DNA replication over time.[8, 10, 29] Positive associations between increased HPV viral load and persistent infection have been reported in some,[14-18] but not all[30, 31] previous studies. However, to our knowledge HPV viral load has not been measured in studies using monthly or more frequent follow-up. In our study, we found that initial type-specific HPV viral load was positively associated with repeat detection of HR HPV with monthly sampling for 6 months, which is similar to the associations observed in studies with longer sampling intervals.[14-18, 32] These findings suggest that the relationship between HPV viral load and repeat detection may be similar for short-term and long-term persistent detection.

We observed notable differences between HR HPV infections that were first detected at enrollment (prevalent) versus at a follow-up visit (incident). First, prevalently detected infections were more likely than incidently detected infections to be repeatedly positive at subsequent follow-up visits and did not decline over time. Second, significantly higher HPV viral load at first detection was observed among prevalent versus incident detections. This finding is consistent with our previous study that followed a cohort of mid-adult female online daters with triannual sampling for one year.[18] As noted in that study, this result is consistent with the theory that prevalent detections represent a mix of incident and persistent infections (and that persistent infections tend to be of greater viral load),[14-16, 32] whereas newly detected infections

among mid-adult women may represent a mix of new acquisition, reactivation of previous infections, or intermittently detected infections.[18] We also observed that HPV viral loads in infections that were redetected after a period of intercurrent negativity (measured at the time of redetection) were lower than in prevalent detections but slightly higher than in incident detections. In contrast, in our previous high-risk cohort of mid-adult women, HPV viral load was significantly higher for newly detected infections versus redetected infections.[18] Although sampling intervals differed between these two studies, the results nonetheless suggest that the majority of incident detections in this lower-risk cohort likely represented reactivation of previous infection or intermittent persistent detection, whereas a higher proportion of incident detections in the previous higher-risk cohort were newly acquired infections.

Both former and current smoking were positively associated with repeated HR HPV detection in our study. Results from past studies on the relationship between smoking and the persistence of HR HPV infections have been equivocal.[30, 33-37] Possible mechanisms through which smoking may facilitate acquisition or persistence of HPV may be the reduction of Langerhans cells and CD4 lymphocytes, as well as the inactivation of natural killer cells.[38, 39] Decreased immune responses in conjunction with higher HPV viral load may lead to increased risk of viral acquisition or persistence. We also observed that current smoking, but not former smoking, was positively associated with HPV viral load. These findings are consistent with a previous study measuring HPV-16 and HPV-18 viral load, where the enrollment viral load was found to be greater among current smokers when compared with never smokers,[40, 41] but inconsistent with our previous study among high-risk mid-adult women, where viral load was higher in never or former smokers compared with current smokers.[18] A case control study conducted in Sweden also found a synergistic effect between current smoking, HPV-16 DNA positivity, and HPV-16 viral load on cervical cancer *in situ* development.[41] The inconsistency in findings between studies may be due in part to residual confounding by sexual behavioral factors.[18] Our study measured factors related to both cumulative and more recent sexual

behavior, including lifetime number of male sex partners and sexual activity within the year prior to enrollment, but these characteristics were unassociated with HPV viral load. In addition to smoking, other factors that were associated with viral load included timing of initial detection (incident versus prevalent) and prior pregnancies. While we are unaware of any mediation analysis methods for correlated data, we did an informal comparison of models adjusted versus unadjusted for viral load. The point estimate for the association between current smoking and repeat HPV detection was only slightly attenuated after adjusting for viral load, suggesting that at least part of the effect of smoking on repeat HPV detection is via a pathway independent of an effect on HPV viral load.

A history of pregnancy was negatively associated with repeat HR HPV detection in our study. Studies among HPV-positive women have found an increased association between increasing number of pregnancies and cervical abnormalities,[42] however, studies on prior pregnancies and HPV infection have been scarce. One study among 2,110 Colombian women aged 14 to 69 years showed that women with no prior pregnancies had increased risk of HPV infection compared to women with at least five prior full-term pregnancies.[43] The effect of prior pregnancies may also be associated with parity, which has been a fairly well-characterized co-factor for cervical cancer progression among HPV-positive women,[44, 45] though studies on the relationship between parity and HPV infection suggest only weak associations.[46-48] The direction of association in our study, however, was the opposite. Our findings were comparable to prior studies that found an inverse association between increasing parity and HR HPV detection of multiple HPV types.[19, 49, 50] One potential explanation for why prior pregnancies or high parity could lead to decreased risk of repeat HPV infection is that nulliparous women may be more prone to high-risk sexual behavior and new sexual exposures than parous women.[19, 51] Infections among women with prior pregnancies may therefore be more likely to represent reactivation of previous infections, which tend to have lower viral load and are less likely to be repeatedly

detected.[18, 19] We adjusted for age and sexual behavior in our study, but residual confounding may still have been present.

Several limitations of our study should be considered. First, our study population is a convenience sample of generally well educated mid-adult women affiliated with the University of Washington, and therefore may not be representative of women in the general population or higher-risk groups of women. Within one year prior to enrollment, 15% were not sexually active and 55% did not engage in sexual activity with new sex partners. For a comparison, population based data from the 2006-2008 National Survey of Family Growth indicated that 8% of 25 to 44 year old women in the US were not sexually active in the past year.[52] However, the median lifetime number of male sex partners reported by the women in our cohort (7 partners) was actually higher than that reported by 25 to 44 year old women in the same national survey the3.6 partners).[52] Second, the duration of follow-up in this study was only 6 months. Our study provides a snapshot of frequent sampling during a short period of time but may not inform long-term persistence of HR HPV. In addition, all behaviors were self-reported and may be prone to biases. However, we utilized a computer-assisted self-administered questionnaire for the majority of questions, which has been shown to minimize social desirability bias.[53] Finally, our Poisson regression analysis only accounts for the proportion of HPV positive detections but does not account for the patterns of infection. Our choice of analysis methods was supported by a previous study which found that long-term persistent infection was associated with the number of sporadic HPV detections rather than consecutive detections.[54]

In conclusion, our study utilized frequent sampling to investigate the role of HPV viral load and other characteristics in the short-term natural history of HPV among mid-adult women. Our data suggest that while incident HPV detection likely represented a mixture of new acquisition, reactivation, and intermittent persistent detection, new acquisition was likely less common than reactivation or intermittent persistence in this mid-adult cohort. Therefore, prophylactic HPV vaccines may have limited benefit among average-risk mid-adult women due

to the dominance of pre-existing infections. Furthermore, clinical counseling for this group of women should reflect the possibility of multiple sources of newly detected infection, with new acquisition only one of several possibilities. Future studies are needed to clarify the clinical significance of these different infection patterns.

Table 1.1 Type-specific primers and probes for HR HPV viral load testing and variant characterization

(A1) Viral load testing primers		
Types	*Forward Primer (5' – 3')*	*Reverse Primer (5' – 3')*
HPV-16	TTCGGTTGTGCGTACAAAGC	TGCCCATTAACAGGTCTTCCA
HPV-18	AAGGCAACATTGCAAGACATTG	AG(A/G)AGGTCAACCGGAATTTCATT
HPV-31	AATGGGCTCATTTGGAATCG	TGGATCAGCCATTGTAGTTACAGTCT
HPV-35	ACAGCTCAGAGGAGGAGGAAGA	TGGAGGTGTCTGGTTTTGCTT
HPV-39	CGGGAGGACCGCAGACTAA	GGTCCA(C/T)GCATATCTG(A/G)TGTTATACT
HPV-45	GCGAGTCAGAGGAGGAAAACG	CGGGCTGGTAGTTGTGCAT
HPV-51	GCTCCGTGTTGCAGGTGTT	GGGTGTCTCCACTGCTTTCC
HPV-52	GACAGCTCAGATGAGGAGGATACA	TGGCTTGTTCTGCTTGTCCAT
HPV-53	GCAGTTGGCTGTTCAGAGTTCA	TGTGCCCATAAGCATTTGTTG
HPV-56	CGGAGGAAAAGCAATTGCA	GGTCCAACCATGTGCTATTAGATG
HPV-58	GACAGCTCAGACGAGGATGAAA	TGGCCGGTTGTGCTTGT
HPV-59	CGACTCCGAGAATGAAAAAGATG	GGTTCAGCTCGTCTAGCTAGTAGCA
HPV-66	AAGGTGCTACCGATGTCAATGTC	CGTCTTTTATGTTCACAGTGCAATT
HPV-68	CCCGACCATGCAGTTAATCA	ATT(G/C)TGTGACGCTGTTGTTCG
HPV-73	ACAAGCTGAACGAGAGTGTTACAGA	GCAAGGCATACTGTGCACTGA
HPV-82	CAGCTCGCAGTGGAAAGCA	GGTCGCCCAGTAACATTTGC

Table 1.1 Type-specific primers and probes for HR HPV viral load testing and variant characterization (cont.)

(A2) Viral load testing probes	
Types	*Probe (5' – 3')*
HPV-16	VIC-CACACGTAGACAT(T/C)CG-MGB
HPV-18	VIC-ATTGCATTTAGAGCC(C/T)C-MGB
HPV-31	VIC-TGCCCCAACTGTTCTA-MGB
HPV-35	VIC-TTGACGGTCCAGCTGG-MGB
HPV-39	VIC-ACGAAGAGAAACCC-MGB
HPV-45	VIC-TGAAGCAGATGG(A/C)GTTAGT-MGB
HPV-51	VIC-AAGTGTAGTACAACTGGC-MGB
HPV-52	VIC-ATGGTGTGGACCGGC-MGB
HPV-53	VIC-AAGAGCTGCGTATTTT-MGB
HPV-56	VIC-TGTGACAGAAAAAGAC-MGB
HPV-58	VIC-AGGCTTGGAC(G/A)GGC-MGB
HPV-59	VIC-ACCAGATGGAGTTAATC-MGB
HPV-66	VIC-TAACACCGGAGGAAAA-MGB
HPV-68	VIC-CACCAACATC(A/T)ACTACTAG-MGB
HPV-73	VIC-TAGTTACTGACTGCACGAAG-MGB
HPV-82	VIC-AGACAGCCTTCGCATAT-MGB

Table 1.1 Type-specific primers and probes for HR HPV viral load testing and variant characterization (cont.)

(B) Variant characterization			
Types	*Source*	*Forward Primer (5' – 3')*	*Reverse Primer (5' – 3')*
HPV-16	External	ACGCCTTACATACCGCTGTT	TGCCCATTAACAGGTCTTCC
	Internal	AGAATGTGTGTACTGCAAGCAAC	GCATAAATCCCGAAAAGCAA
HPV-18	External	CATGTCCAACATTCTGTCTACCC	AGCCATTGTTGCTTACTGCTG
	Internal	TGTATGGAGACACATTGGAAAAAC	AGTTTTTCTGCTGGATTCAACG
HPV-31	External	TGTTTAAACTGCCAAGGTTGTG	CATAAAACCAACCATTGCATCC
	Internal	TGGAACAACATTAGAAAAATTGACA	TCTTCTGGACACAACGGTCTTT
HPV-35	External	TTCTAAAGGGCTTTAATTGCACA	CCATTGTAGATTATGCTCTCTGTGA
	Internal	ATGACTTTGCATGCTATGATTTGT	TTTGTTGCATTGTTTTTCTAACG
HPV-39	External	TTTTGATTCAGGAATGTGTCTTACA	CACACAAATCCTAGTGAGTCCATAA
	Internal	TATAGGGACGGGGAACCACTA	TCTTCGTTTGCTATTTAGGTGTCTT
HPV-45	External	GCGTGTAGAACCACTTTCTTATCC	CCGCCATTGTAGATTATTGGTT
	Internal	TCTGTATATGGAGAGACACTGGAAA	CGTTTGTCCTTAAGGTGTCTACG
HPV-51	External	CACTGTTTTCCGCCCTATAATAA	GCTTAGTTCGCCCATTAACATC
	Internal	AAGCTTTGAACGTTTCTATGCAC	CACCAATTTTTGCTTTTCTTCAG
HPV-52	External	CACAAGTACATCCTACGCCAAA	CATTGCAGGGTTGTTTATAGCC
	Internal	GCGTGTGTATTATGTGCCTACG	TCTTCAGGACATAATGGCGTTT
HPV-56	External	ACTTTGGTGTTTTGGCTTGC	ACGCCATTGCAGTTAGTTACTTG
	Internal	CCTTATGCAGTGTGCAGAGTATG	CGGAGTTAACGGACTTTGACA
HPV-58	External	TTGGCTTGCACAATAGTTTGTTAT	TCGTTCTATTAC(C/T)GCTTCTACCTC
	Internal	CGCTATATGGAGACACATTAGAACA	AACCTTTTGTTTAAATCCACATGC
HPV-59	External	GAAAAAGGTCGGGCAAGTACA	CGTCATCTGAAATTTTGTCACCT
	Internal	GCCTAAAACCTCTATGTCCAACAG	TGTCGTTGCTGTCTTAGGTGTC
HPV-68	External	GTTGGGCACACATACCAATACTT	(G/A)TCCTCATCCTCTGAGACTGTGT
	Internal	GCAGGAAACTTTACAGGACAGTG	GGTCCTCTCG(C/T)TTACTGGTC

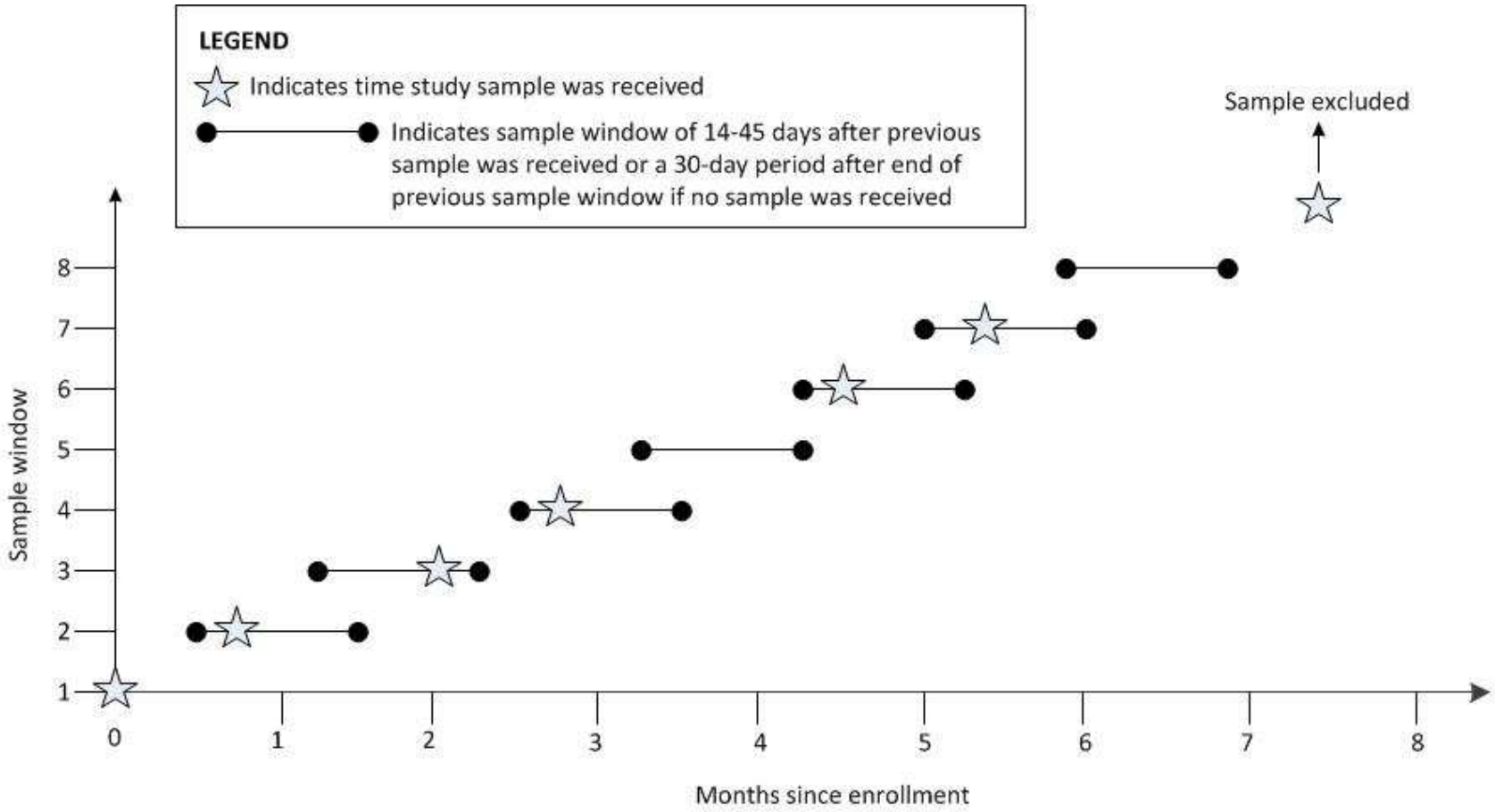

Figure 1.1 Example of how study samples were numbered according to sample windows

This hypothetical subject provides a total of 7 samples. Sample 1 indicates the sample collected at enrollment (time 0). Samples 2, 3, and 4 are received during the corresponding sample windows. No sample is received during the 5th sample window. The next two samples are received during the 6th and 7th sample windows. The last sample is excluded because it is received after the 8th sample window.

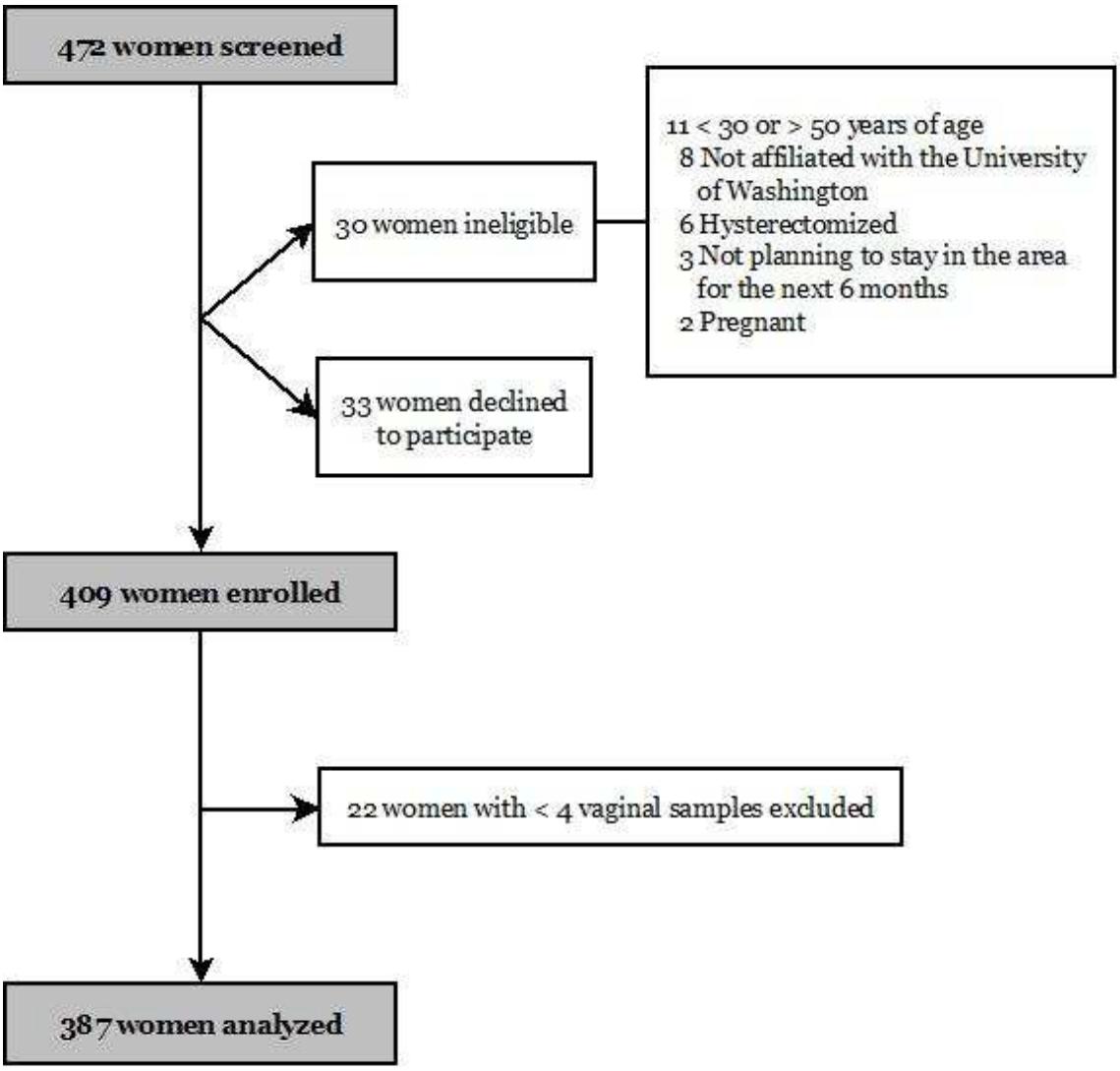

Figure 1.2 Enrollment and follow-up status of mid-adult women in Seattle, WA (2011-201)

Table 1.2 Enrollment characteristics of 387[a] mid-adult women in Seattle, WA (2011-2012)

Characteristics	*Mean*	*(SD)*
Age (years)	38.4	(6.1)
	Median	*(IQR)*
Age at first sexual intercourse (years)[b]	18	(16 – 21)
Lifetime number of male sex partners	7	(3 – 15)
	n[c]	*(%)*
Race		
African American	9	(2.3)
Asian	45	(11.6)
White	306	(79.1)
Other[d]	27	(7.0)
Current marital status		
Unmarried, not living with partner	139	(36.1)
Unmarried, living with a partner	54	(14.0)
Married	185	(48.1)
Separated	7	(1.8)
Education		
Some college or less	65	(16.8)
College bachelor's degree	145	(37.5)
College master's or doctoral degree	177	(45.7)
Ever had a non-HPV-related sexually transmitted disease[e]		
No	308	(80.0)
Yes	77	(20.0)
Ever had genital warts		
No	343	(88.6)
Yes	44	(11.4)
Ever had an abnormal Pap test		
No	218	(56.3)
Yes	169	(43.7)
Ever received ≥1 dose of HPV vaccine		
No	357	(92.5)
Yes	29	(7.5)
Ever been pregnant		
No	158	(40.8)
Yes	229	(59.2)
Currently using hormonal birth control methods[f]		
No	255	(65.9)
Yes	132	(34.1)
Currently have an immunosuppressive condition[g]		
No	379	(97.9)
Yes	8	(2.1)
Smoking status[h]		
Never	287	(74.4)
Former	83	(21.5)
Current	16	(4.2)

Table 1.2 Enrollment characteristics of 387 mid-adult women in Seattle, WA (2011-2012) (cont.)

Characteristics	*n*[c]	*(%)*
Sexual activity within one year prior to enrollment		
No sexual activity	58	(15.0)
Sex with non-new male partners only	211	(54.5)
Sex with ≥ 1 new male partner(s)	118	(30.5)

[a]Twenty-two women with fewer than 4 samples were excluded.
[b]Restricted to 376 women who have ever had sex with a male partner.
[c]Numbers may not add up to total due to missing data.
[d]Includes individuals indicating the following: American Indian/Alaska Native, Native Hawaiian/other Pacific Islander, other race, or multiple races.
[e]Includes chlamydia, gonorrhea, genital herpes, and HIV.
[f]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.
[g]Includes HIV positivity (n=1) or currently taking immunosuppressive medications (n=7).
[h]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smokers reported currently smoking.

Table 1.3 High-risk (HR) HPV detection at enrollment and during follow-up among mid-adult women in Seattle, WA (2011 – 2012) (N = 387)[a]

	Type-specific HR HPV infections *n (%)*	Prevalent detections[b] *n (%)*	Incident detections[c] *n (%)*
All HR HPV types	243[d]	132	111
HPV-16	21 (8.6)	15 (11.4)	6 (5.4)
HPV-18	9 (3.7)	5 (3.8)	4 (3.6)
HPV-26	0 (0.0)	0 (0.0)	0 (0.0)
HPV-31	13 (5.4)	5 (3.8)	8 (7.2)
HPV-33	1 (0.4)	1 (0.8)	0 (0.0)
HPV-35	6 (2.5)	3 (2.3)	3 (2.7)
HPV-39	15 (6.2)	8 (6.1)	7 (6.3)
HPV-45	10 (4.1)	4 (3.0)	6 (5.4)
HPV-51	35 (14.4)	16 (12.1)	19 (17.1)
HPV-52	13 (5.4)	8 (6.1)	5 (4.5)
HPV-53	39 (16.1)	27 (20.5)	12 (10.8)
HPV-56	14 (5.8)	8 (6.1)	6 (5.4)
HPV-58	12 (4.9)	8 (6.1)	4 (3.6)
HPV-59	12 (4.9)	6 (4.6)	6 (5.4)
HPV-66	19 (7.8)	9 (6.8)	10 (9.0)
HPV-68	7 (2.9)	3 (2.3)	4 (3.6)
HPV-73	10 (4.1)	4 (3.0)	6 (5.4)
HPV-82	6 (2.5)	1 (0.8)	5 (4.5)
HPV-IS39	1 (0.4)	1 (0.8)	0 (0.0)

[a]Twenty-two women with fewer than 4 samples were excluded.
[b]Type-specific HR HPV DNA detected at enrollment.
[c]Type-specific HR HPV DNA detected during follow-up but not at enrollment.
[d]Among 139 women with HR HPV detected.

Table 1.4 Associations between various factors and type-specific HR HPV viral load at 1st positive detection among mid-adult women in Seattle, WA (2011 – 2012) (N = 241)[a]

		Univariate Analysis[b]			Multivariate Analysis[b]		
Model Term	*n*	*β*	*(95% CI)*	*p-value*	*β*	*(95% CI)*	*p-value*
Intercept	--	--	--	--	2.06	(1.26, 2.85)	<0.001**
HPV detection status							
Prevalent	130	0.00	--	--	0.00	--	--
Incident	105	-1.42	(-1.88, -0.97)	<0.001**	-1.30	(-1.76, -0.84)	<0.001**
Age							
≤ 40 years	162	0.00	--	--	--	--	--
> 40 years	73	-0.09	(-0.63, 0.45)	0.736	--	--	--
Marital status							
Unmarried or separated	136	0.00	--	--	0.00	--	--
Married or living with partner	98	-0.46	(-0.99, 0.08)	0.095*	-0.20	(-0.68, 0.27)	0.401
Abnormal Pap before enrollment							
No	104	0.00	--	--	--	--	--
Yes	131	-0.06	(-0.58, 0.47)	0.826	--	--	--
Ever had genital warts							
No	213	0.00	--	--	--	--	--
Yes	22	-0.08	(-0.77, 0.60)	0.807	--	--	--
Ever been pregnant							
No	121	0.00	--	--	0.00	--	--
Yes	114	-0.63	(-1.14, -0.12)	0.015**	-0.31	(-0.78, 0.15)	0.186
Currently using hormonal birth control[c]							
No	120	0.00	--	--	--	--	--
Yes	100	-0.17	(-0.68, 0.34)	0.504	--	--	--
Current immunosuppressive condition[d]							
No	230	0.00	--	--	--	--	--
Yes	5	-0.27	(-1.05, 0.52)	0.506	--	--	--
Smoking status[e]							
Never	160	0.00	--	--	0.00	--	--
Former	63	0.22	(-0.35, 0.79)	0.450	-0.08	(-0.58, 0.42)	0.747
Current	12	1.01	(0.23, 1.79)	0.012**	0.70	(-0.03, 1.43)	0.060*

Table 1.4 Associations between various factors and type-specific HR HPV viral load at 1st positive detection among mid-adult women in Seattle, WA (2011 – 2012) (N = 241) (cont.)

		Univariate Analysis[b]			Multivariate Analysis[b]		
Model Term	*n*	*β*	*(95% CI)*	*p-value*	*β*	*(95% CI)*	*p-value*
Lifetime no. of male sex partners[f]							
0-2	10	0.00	--	--	--	--	--
3-6	45	-0.25	(-1.83, 1.33)	0.756	--	--	--
7-14	77	-0.30	(-1.82, 1.23)	0.700	--	--	--
15+	100	-0.54	(-2.02, 0.94)	0.472	--	--	--
Sexual activity within 1 year prior to enrollment							
No sexual activity	19	0.00	--	--	--	--	--
Sex with non-new male partners only	131	0.23	(-0.49, 0.95)	0.530	--	--	--
Sex with ≥ 1 new male partner(s)	85	0.32	(-0.41, 1.05)	0.390	--	--	--

*p-value < 0.1; **p-value < 0.05

[a]HR HPV types tested for viral load include HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82. Infections of HPV types 26, 33, and IS39 were excluded (n=2).

[b]All models were HPV type-adjusted.

[c]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.

[d]Includes HIV positivity or currently taking immunosuppressive medications.

[e]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smoker reported currently smoking.

[f]Lifetime number of male sex partners categorized based on approximate quartiles.

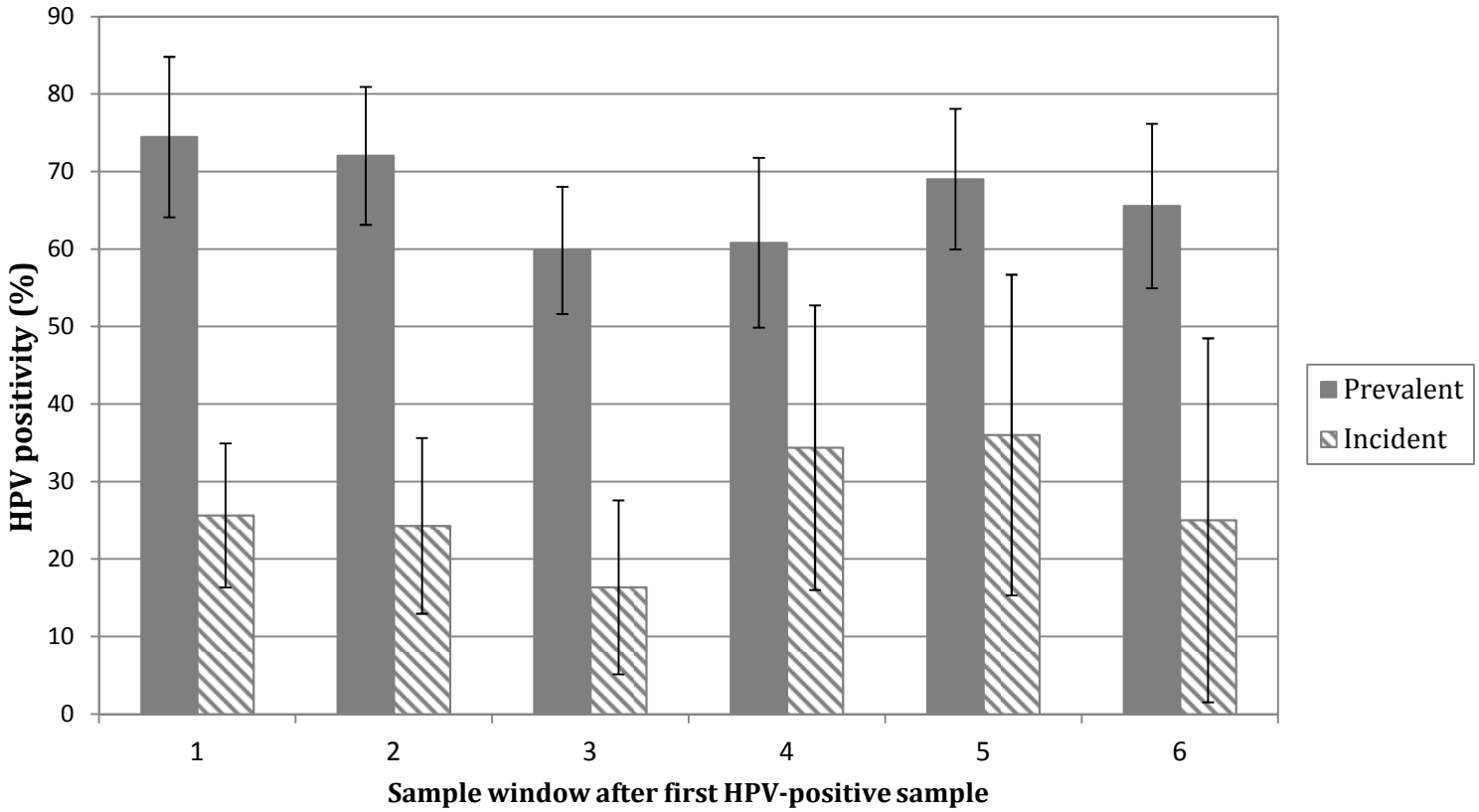

Figure 1.3 HR HPV positivity over time for prevalent and incident infections detected among mid-adult women in Seattle, WA (2011 – 2012)

The histogram depicts HPV positivity over time separately for prevalent and incident infections, and the error bars represent 95% confidence intervals of each estimate. This figure includes HPV infections with 19 HR types (HPV-16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82 and IS39). The x-axis denotes the sample window after the first HPV-positive sample, with the sample window defined as 14-45 days after the previous sample was received or a 30-day period after end of previous sample window if no sample was received. HPV positivity at each sample window was not conditioned on testing HPV positive in the prior sample window but on the first HPV positive sample.

Table 1.5 Univariate association between various characteristics and number of HR HPV detections over follow-up among HPV-positive mid-adult women in Seattle, WA (2011 – 2012) (N = 222)[a]

		Univariate Analysis[b]		
Characteristics	*n*	*RR*	*(95%CI)*	*p-value*
HPV viral load at 1st positive sample[c]	--	1.26	(1.19, 1.33)	<0.001*
HPV detection status				
Prevalent	130	1.00	--	--
Incident	92	0.21	(0.15, 0.29)	<0.001**
Age				
≤ 40 years	151	1.00	--	--
> 40 years	71	1.00	(0.75, 1.35)	0.963
Marital status				
Unmarried or separated	130	1.00	--	--
Married or living with a partner	91	0.80	(0.60, 1.08)	0.149
Abnormal Pap before enrollment				
No	94	1.00	--	--
Yes	128	1.07	(0.82, 1.40)	0.625
Ever had genital warts				
No	200	1.00	--	--
Yes	22	0.77	(0.56, 1.05)	0.104
Ever been pregnant				
No	114	1.00	--	--
Yes	108	0.59	(0.45, 0.77)	<0.001*
Currently using hormonal birth control[d]				
No	127	1.00	--	--
Yes	95	0.99	(0.76, 1.29)	0.966
Current immunosuppressive condition[e]				
No	215	1.00	--	--
Yes	7	0.67	(0.29, 1.53)	0.337
Smoking status[f]				
Never	150	1.00	--	--
Former	61	1.50	(1.17, 1.92)	0.002*
Current	11	1.74	(1.25, 2.42)	0.001*

Table 1.5 Univariate association between various characteristics and number of HR HPV detections over follow-up among HPV-positive mid-adult women in Seattle, WA (2011 – 2012) (N = 222) (cont.)

		Univariate Analysis[b]		
Characteristics	*n*	*RR[b]*	*(95%CI)*	*p-value*
Lifetime no. of male sex partners				
0-2	8	1.00	--	--
3-6	45	0.57	(0.26, 1.24)	0.157
7-14	71	0.82	(0.40, 1.65)	0.570
15+	96	0.68	(0.33, 1.37)	0.278
Sexual activity within 1 year prior to enrollment				
No sexual activity	18	1.00	--	--
Sex with non-new male partners only	119	0.99	(0.64, 1.53)	0.969
Sex with ≥ 1 new male partner(s)	79	0.90	(0.57, 1.43)	0.656

*p-value < 0.05

[a]Nineteen single-time positives detected in the last available sample were excluded from this analysis. HPV types 26, 33, and IS39 (representing 2 infections) were dropped due to low numbers.

[b]RR = risk ratios and were HPV type-adjusted.

[c]HPV types tested for HPV viral load include HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.

[d]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, or hormonal intrauterine devices.

[e]Includes HIV positivity or currently taking immunosuppressive medications.

[f]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smoker reported currently smoking.

Table 1.6 Multivariate association between various characteristics and number of HR HPV detections over follow-up among HPV-positive mid-adult women in Seattle, WA (2011 – 2012) (N = 217)[a]

		Multivariate Analysis 1[b]			Multivariate Analysis 2[b]		
Characteristics	*n*	*RR*[c]	*(95%CI)*	*p-value*	*RR*[c]	*(95%CI)*	*p-value*
HPV viral load at 1st positive sample[d]	--	--	--	--	1.10	(1.05, 1.16)	<0.001**
Incident HPV detection	87	0.24	(0.17, 0.34)	<0.001**	0.28	(0.20, 0.39)	<0.001**
Ever been pregnant	104	0.76	(0.63, 0.93)	0.006**	0.83	(0.68, 1.00)	0.049**
Smoking status[e]							
Never	145	1.00	--	--	1.00	--	--
Former	61	1.19	(0.99, 1.42)	0.058*	1.21	(1.01, 1.43)	0.034**
Current	11	1.37	(1.04, 1.79)	0.023**	1.24	(0.96, 1.59)	0.100

*p-value < 0.1; **p-value < 0.05

[a]Nineteen single-time positives detected in the last available sample were excluded from this analysis. HPV types 26, 33, and IS39 (representing 2 infections) were dropped due to low numbers. Five infections with missing viral load measurements were also excluded this analysis.

[b]Multivariate analyses included variables with p < 0.1 in the univariate analyses; model 1 did not include viral load as a covariate while model 2 did. Both models were HPV type-adjusted

[c]RR = risk ratios and were HPV type-adjusted.

[d]HPV types tested for HPV viral load include HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.

[e]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smokers reported currently smoking.

2 Re-infection versus Intermittent Detection versus New Acquisition of High-Risk HPV in Mid-Adult Women

2.1 INTRODUCTION

The natural history of HPV infections has been fairly well-characterized among young women after sexual debut, but major gaps exist in the understanding of HPV natural history in mid-adult women.[1] Whereas incident HPV infections detected in newly sexually active young women are likely to represent new acquisition (and show strong associations with recent sexual behavior),[47, 55-58] newly detected HPV in mid-adult women may represent reactivation of a prior infection, intermittent detection of a persisting infection, re-infection, or new acquisition. Furthermore, the origin of a given infection is usually unknown.[59] Understanding the relative frequencies of these infection states have important implications for both HPV vaccination recommendations and clinical counseling.[1, 55] Current HPV vaccines are effective for preventing new infections, but are not therapeutic.[60] Therefore, elucidating the frequency of new HPV acquisition in mid-adulthood may help inform potential vaccine effectiveness estimations and recommendations for this age group. Furthermore, information on the relative frequencies of different infection states may be useful for clinicians in counseling women who test positive for HPV during routine cervical cancer screening.

Differentiating between new acquisition and re-infection or reactivation of a prior or latent infection is methodologically challenging without a reliable indicator of prior infection. HPV serology testing has limitations, including limited sensitivity, lack of a standardized approach,[61] and the fact that antibody responses are not uniformly detected nor lifelong.[62-65] However, augmenting serology testing with HPV DNA testing for current infection and self-reported sexual behavior data may be a useful research strategy to better characterize the likelihood that a newly detected infection is new acquired. Using data from a cohort of mid-adult women followed for up to 6 months with HPV serology testing at enrollment, monthly

vaginal self-sampling for HPV DNA testing, and detailed reporting of demographics, health history, and sexual behaviors, we assessed whether the incidence of, and risk factors for, newly detected type-specific HR HPV infections varied depending on whether or not there was serologic evidence of prior infection with the same HPV type. We hypothesized that new sexual exposures would only be associated with newly detected infections in the absence of serologic evidence of prior infection.

2.2 METHODS

2.2.1 Study Population

From March 2011 to January 2012, we enrolled women into a longitudinal study of HPV natural history. A total of 409 female staff, faculty, and students affiliated with the University of Washington (UW) were followed for up to six months. Women were recruited through flyers, advertisements, and letters distributed at the UW and subsequently screened over the telephone. Women were eligible to participate if they: were aged 30 to 50 years, were not currently pregnant, had never had a hysterectomy, did not have serious medical conditions that would preclude participation in the study, and were willing to provide monthly self-collected vaginal samples. Informed consent was administered by the study coordinator at the in-clinic enrollment visit. The protocol was reviewed and approved by the UW Institutional Review Board.

2.2.2 Data Collection

In-clinic enrollment and six-month exit visits were held at the on-campus Hall Health Primary Care Center (HHPCC) of the UW. At enrollment, women provided a venous blood specimen, self-collected a vaginal sample in 1.5 mL STM, and filled out an online questionnaire covering basic demographic characteristics, women's health history (prior pregnancies, use of hormonal contraceptives, abnormal Pap results, sexually transmitted diseases[STDs]), general immunosuppression, and recent and cumulative sexual behaviors. Similar data components

were gathered at the exit visit, with the online questionnaire covering health status and behaviors since enrollment. During follow-up, email reminders were sent to study subjects asking them to self-collect vaginal samples at home every month (up to 7 samples per woman) and complete online sexual behavior diaries every two weeks. Sexual behavior diaries instructed women to report daily male partner-specific information on frequency of vaginal intercourse and condom use, and partner characteristics (e.g. whether the partner was new, ever had STDs, had other concurrent partners, or whether the partner was a casual or regular sex partner). Survey instruments and self-collection materials were similar to those used in our previous studies.[20] Women had the option to return samples through campus mail or in-person delivery to HHPCC. Batched specimens were transported to the laboratory weekly and either stored at -20°C or prepared for immediate testing.

2.2.3 HPV Genotyping

From self-collected vaginal samples, we isolated genomic DNA and conducted HPV genotyping using the Roche Linear Array assay.[20] Briefly, the vaginal samples were digested with 20 μg/mL proteinase K at 37°C for one hour, and DNA isolated from 200 μL of the digested sample using QIAamp DNA blood mini kit, following the protocol of the manufacturer (Qiagen, Cat. No.51104). Specimens were directly genotyped using the Roche Linear Array assay for 37 alpha-genus HPV types. Specimens testing negative for β-globin were considered insufficient (n=2).

2.2.4 HPV Serology

Antibody testing of enrollment sera samples was performed using a Luminex-based assay described in detail previously.[66, 67] In brief, HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 67, and 68 L1 proteins and BKPyV VP1 proteins (a positive control)[68] were expressed from a modified pGex4T vector[69] and bacterial lysates were prepared. Up to 100 polystyrene microspheres (beads) containing a unique combination of fluorescent dyes (MiraiBio, South San

Francisco, CA) covalently coupled with glutathione-linked casein. Each protein preparation was bound to a different bead set. After incubation with protein, bead sets were washed and combined. Human sera were diluted 1:50 in blocking buffer (0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone, 0.025% CBS-K superblock [Chemicon International, Temecula, CA], and GST-containing bacterial lysate at 2 mg/mL in PBS–casein) in polypropylene plates. After blocking for 1 hour at room temperature with shaking, 50 µl of diluted sera was mixed with an equal volume of bead mixture in a 96-well filter plate. Sera were incubated with beads overnight at 4°C followed by 1 hour at room temperature with shaking. After washing, bound antibodies were detected by reaction with biotinylated anti-human IgG and streptavidin-phychoerythrin. Plates were read on a BioPlex 200 (Luminex) Instrument (BioRad Laboratories, Hercules, CA). For each sample, the median fluorescent intensity (MFI) for GST-tag (empty vector control) was subtracted from the MFI of the other antigens. Cut points for antibody positivity were selected by visual inspection of the distribution of HPV-16 values and informed by previous studies using this technique.[70-72] The cut point for HPV-16 antibodies was MFI = 1,000. Cut points for other types were adjusted if their distribution was significantly different than HPV-16 values. We selected the following cut points: 2,000 for HPV-11, 35; 1,000 for HPV-18, 31, 51, 52, 56, 58, 59, 67; and 500 for HPV-33, 39, 45, 68. No cut point was selected for HPV-6 because the shape of the distribution did not allow for reasonable comparison with HPV-16. It was determined *a priori* that samples negative for the BKPyV control would be excluded from analysis, but all samples were positive for BKPyV. We also made a *post hoc* decision to exclude samples seropositive for all 15 HPV types with valid cut points due to a high likelihood that cross-reactivity interfered with type-specific determination of seropositivity. Serologic controls included the HPV-16 international standard (10 U/ml) that was consistently positive (average[a]MFI = 2458; 95% CI: 2178, 2738)[73] (National Institute for Biological Standards and Controls). This serum was consistently positive for HPV-58 as well (aMFI = 1613; 95% CI: 1452, 1774). A second control was a serum being evaluated by the World Health Organization as a

potential HPV-18 standard and was HPV-18 positive in 7 of 8 tests (aMFI = 1193; 95% CI: 994, 1441). This serum was also consistently positive for HPV-45 (aMFI = 15207; 95% CI: 12631, 17784) and HPV-58 (aMFI = 3754; 95% CI: 2826, 4683).

2.2.5 Statistical Analyses

Our analysis was restricted to 13 high-risk (HR) HPV types with both HPV genotyping and serology results (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68).[22] Women who reported a history of HPV vaccination or had a serum sample that had insufficient beads for serology testing were excluded from the analysis. Each self-collected vaginal sample was numbered according to the sample window during which it was received, defined as a period of 14 to 45 days after the previous sample was received or within 30 days after the end of the previous sample window if no sample was received. The sample window numbering system was described in detail in chapter 1 (*Figure 1.1*). Samples received ≥ 8 sample windows after the enrollment sample were excluded. At enrollment, each HPV type within a woman, or "woman-type" was classified as negative or positive by HPV DNA and by serology (*Table 2.1*). Women-types that were DNA-positive in the enrollment vaginal sample (i.e., prevalent) were excluded from all analyses. For women-types not detected in the enrollment vaginal sample, type-specific incident detection was defined as the first DNA-positive in a monthly vaginal sample.

Cumulative incidence of detecting type-specific HR HPV at 6 months was calculated using Kaplan-Meier methods, with estimates stratified by type-specific HPV serostatus at enrollment. Bootstrapping was used to compute 95% confidence intervals to account for within-women correlation due to the assessment of multiple HPV types per woman. Each woman could contribute up to 13 women-types to the analysis, and the same woman could contribute to both the seropositive and seronegative analysis. Using the example in *Table 2.1*, this woman contributed 8 women-types (HPV-16, 33, 39, 45, 51, 56, 58, 68) to the seropositive analysis and 3 women-types (HPV-18, 52, 58) to the seronegative analysis. Time at risk was represented by

the discrete number of sample windows (an approximation of months) until type-specific incident detection. Women-types were censored after incident detection or at the last follow-up visit if infection was not detected.

Generalized estimating equations (GEE) logistic regression was used to estimate odds ratios for the association between potential risk factors and type-specific incident HR HPV detection, with separate models for women-types that were seropositive versus seronegative at enrollment (*Table 1*). Robust variance estimates were used to account for correlation within women due to multiple measurements and multiple HPV types. Sample window numbers, a surrogate for time, were included in the models as indicator variables. Both time-fixed and time-dependent variables were included in univariate models *(Figure 2.1)*. Time-fixed factors from the enrollment survey that were explored included age (years, continuous), body mass index (BMI) (<25 underweight or normal/25 – 29.9 overweight/≥30 obese), marital status (unmarried or separated/married or living with a partner), history of pregnancy (yes/no), history of non-HPV STDs (yes/no), history of genital warts (yes/no), current use of hormonal birth control (yes/no), current immunosuppressive condition (yes/no), smoking status (never/former/current), lifetime number of male sex partners (0-3/4-9/10+; approximate tertiles), and age at first sexual intercourse with a male partner (years, continuous). Time-dependent sexual behaviors during the 6 months prior to each collected sample were also assessed by pooling sexual behavior data from enrollment and exit surveys as well as bi-weekly behavior diaries. Sexual behavior data in enrollment and exit surveys were collected separately for each male sex partner reported (up to 6 partners). Partner-specific data were included for a specific sexual behavior variable if the dates of first or last sex with the relevant partner fell within the 6-month interval prior to each sample. Online diaries collected daily information on sexual behaviors, and data were used if the corresponding date fell within the 6-month interval prior to each sample (*Figure 2.1*). The two data sources were merged to form time-dependent sexual behavior variables. Sexual behaviors that were reported in any data source were

considered present in the merged variables and absent if the subject reported sexual activity in the past 6 months but did not report the particular sexual behavior. Time dependent variables included the average number of sex acts per week and condom use, restricted to women who have had sex in the past 6 months. The average number of sex acts per week was estimated using only data from the enrollment survey and bi-weekly diaries. The enrollment survey asked women to report the average weekly number of sex acts for each male sex partner. When multiple partners were reported, averages were summed. Actual numbers of daily sex acts reported in diaries were summed over the time period of interest and divided by the total time in weeks to calculate the average number of sex acts per week. The two data sources were weighted according to the time period covered. For example, at four months follow-up, if a woman reported an average of 2 sex acts per week at enrollment and a total of 48 sex acts over the 4-month (16-week) follow-up period, then the combined average number of sex acts per week would be calculated by: $[2*(2/6)] + [(48/16)*(4/6)] = 2.67$. Condom use with all reported male sex partners was combined, with "always" indicating always using a condom with every male partner and "not always" indicating not always using a condom with at least one male partner. Condom use was categorized as "not always" if condom use with any male partner reported through surveys was "never", "less than half the time", or "at least half the time", or through diaries not reported for any sex act during the specified time period. In addition, to assess the overall impact of recent sexual behaviors, a composite variable was constructed ranging from not sexually active with male partners to sexually active with male partners with 0, 1, 2, or ≥ 3 risk factors (from among the following risk factors: >1 sex partner, ≥1 new sex partner, ≥1 casual partner, ≥1 partner with other concurrent partners, and ≥1 partner with STDs), with the variable coded to missing only if information on all factors were missing. Model terms with an associated p-values of $p<0.10$ by the Wald test in the univariate analyses were included in the multivariate analysis. P-values for multi-level model terms were assessed using the joint Wald test. To test the robustness of the associations with sexual behavior variables, we conducted sensitivity

analyses for associations with incident HR HPV detection considering sexual behaviors one month rather than 6 months prior to each collected sample, using only diary data. Additional sensitivity analyses were also done defining incident detection as requiring at least two prior DNA-negative samples instead of one.

Since increased risk on the multiplicative scale does not necessarily translate to an appreciable risk difference on the additive scale, the effect of type-specific HPV seropositivity and recent sexual behaviors on incident HR HPV detection was assessed on the additive scale by calculating the attributable risk percent (%AR) and population attributable risk percent (%PAR).[74] To estimate the different sources of infection, our exposure of interest was a variable combining HPV serostatus and composite sexual behavior risk factors (seropositive, seronegative and either not sexually active or sexually active with no risk factors, or seronegative and sexually active with ≥1 risk factor). Potential variables for adjustment were assessed in GEE logistic regression models with robust variances estimating odds ratios associated with the effect of HPV seropositivity on incident HR HPV detection. Model terms with an associated p-value of $p<0.10$ in the univariate analyses were included in the multivariate model to estimate odds ratios used to calculate %AR and %PAR.

2.3 RESULTS

2.3.1 Characteristics of the study population

Of the 409 mid-adult women enrolled, 30 women were excluded due to reporting a history of HPV vaccination, one woman was excluded due to a sample with insufficient beads for HPV serology testing, and six women were excluded due to seropositivity of 15 HPV types. Therefore, we included 372 women (91%) in our analysis. Their mean age at enrollment was 38.6 (standard deviation [SD]=6.1) years, and their mean age at first sexual intercourse was 18.7 (SD=4.0) years (*Table 2.2*). The majority was white (79%) and either married or living with a partner (61%), and less than half (45%) were either overweight or obese, with a BMI of ≥ 25 kg/m^2. The median lifetime number of male sex partners reported was 7 (IQR: 3 – 15). Two

hundred and eighty-eight women (78%) reported sexual activity with a male partner within 6 months prior to enrollment. One hundred and fifty-six (54%) of these women reported more than partner in the prior 6 months or at least one "risky" partner (i.e., new, casual, or reported to have other concurrent partners, or a history of STDs). At enrollment, 68% of women were HPV-seropositive for at least one HPV type and 15% were HPV DNA-positive for at least one HPV type. The mean follow-up time for the 372 women in our analysis was 6.7 months (SD=1.8). Of the women included in our analysis, 347 women (93%) completed follow-up with an in-clinic exit visit and provided a total of seven self-collected vaginal samples.

2.3.2 Prevalent and incident type-specific HR HPV DNA detection

Out of a total of 4,837 possible women-types evaluated, 120 prevalent type-specific HPV infections were detected at enrollment and excluded from further analyses. Of the remaining 4,717 women-types, 4,065 (84%) were seronegative at enrollment, and 772 (16%) were seropositive at enrollment. Among the women-type seropositive at enrollment, the most common serotypes detected were HPV-31 (11.8%), HPV-59 (11.8%), HPV-16 (11.7%), and HPV-51 (11.7%). One hundred and fifty incident type-specific HPV infections were detected during the course of follow-up, and 124 (83%) were preceded by at least two type-specific HPV-negative samples. One hundred and eight incident detections (72% of all incident detections) were observed when there was no serologic evidence of prior infection with the same type. Forty-two incident detections (28% of all incident detections) were observed when there was serologic evidence of prior infection with the same type.

2.3.3 Six-month cumulative incidence of HR HPV detection

The 6-month cumulative incidence of type-specific HR HPV detection was higher when there was serologic evidence of prior infection with the same type (2.6%; 95% CI: 1.7, 4.1) that when there was not serologic evidence of prior infection with the same type (1.3%; 95% CI: 0.9, 1.7) (OR=2.26; 95% CI: 1.31, 3.88).

2.3.4 Risk factors associated with incident HR HPV detection

In univariate analyses, among women with serologic evidence of prior type-specific infection, being overweight (OR=2.39; 95% CI: 0.89, 6.43) or obese (OR=3.03; 95% CI: 0.94, 9.79) was associated with an increased likelihood of incident HR HPV detection relative to being normal or underweight (*Table 2.3*). The observed dose-response relationship between higher BMI categories increased likelihood of HR HPV detection was not statistically significant (joint Wald test p-value = 0.120). Current use of hormonal birth control methods was also associated with an increased likelihood of incident HR HPV detection (OR=2.26; 95% CI: 0.96 5.34). No other demographic, health or sexual behavior factors were associated with incident HPV detection among the seropositive group.

In the absence of serologic evidence of prior infection with the same type, being married or living with a partner (OR=0.55; 95% CI: 0.28, 1.08) and older age at first sexual intercourse (OR=0.91; 95% CI: 0.85, 0.99) were each negatively associated with incident HR HPV detection. Ever having had a non-HPV-related STD (OR=2.23; 95% CI: 1.17, 4.25) and reporting a lifetime number of male partners greater than or equal to 10 (OR=4.06; 95% CI: 1.66, 9.94 relative to 0 – 3 lifetime partners) were each associated with increased likelihood of incident HPV detection. Having at least three sexual behavior risk factors within the prior 6 months was associated with significantly increased risk of incident HR HPV detection relative to not being sexually active (OR: 6.27; 95% CI: 2.40, 16.39).

A multivariate model was constructed separately for the seropositive and seronegative group. Among women with serologic evidence of prior type-specific infection, the effect of BMI or current use of hormonal birth control methods on HR HPV detection were both attenuated and no longer statistically significant (*Table 2.4*). In the absence of serologic evidence of prior type-specific infection, recent sexual behavior remained independently associated with an increased likelihood of HR HPV detection (sexually active and one factor OR-4.43; 95% CI: 0.94, 20.80; sexually active and two risk factors OR=4.02; 95% CI: 0.79, 20.61; sexually active and at

least three risk factors OR=9.88; 95% CI: 2.45, 39.77; each relative to not being sexually active) after adjusting for marital status, non-HPV-related STD history, lifetime number of male sex partners, and age at first sexual intercourse (*Table 2.5*). A dose-response relationship was observed with increasing number of sexual behavior risk factors and increased risk of HPV detection (joint Wald test p-value = 0.003). Other measures of cumulative sexual behavior were attenuated in the multivariate model for the seronegative group.

Sensitivity analysis conducted using sexual behaviors one month prior to each sample generated associations in the same directions as those observed in the models with the 6-month variables, but with wider confidence intervals (data not shown). Sensitivity analysis restricting to incident detections preceded by at least two HPV-negative samples also generated similar results (data not shown), suggesting that potential bias due to misclassification of HPV DNA detection when defining incident detection with only one prior HPV-negative sample was likely minimal.

2.3.5 Risk of incident HR HPV detection attributable to type-specific seropositivity and sexual behaviors

While the risk of incident detection was 5.7 fold higher when there was serologic evidence of prior infection than when there was no serologic evidence of prior infection and no evidence of new sexual exposures (*Table 2.6*), only 29% of women-types with incident HPV detected were seropositive at enrollment. Therefore, only 32% of incident detections in our cohort were attributable to serologic evidence of prior infection with the same type. 44% of incident detections were attributable to being sexually active in the past 6 months with having at least one risk factor (new sex partners, multiple partners, casual partners, partners with other concurrent partners, or partners with STDs) and no serologic evidence of prior infection with the same type.

2.4 DISCUSSION

In this longitudinal study of mid-adult women, we estimated the incidence of HR HPV detection over 6 months of follow-up based on whether or not there was serologic evidence of prior infection. Risk factors were also assessed separately by type-specific HPV serostatus and attributable risk estimates due to prior infection and recent sexual behaviors were calculated.

In our study, cumulative incidence of HR HPV DNA detection was higher when there was serologic evidence of prior infection with the same type compared to when there was not. Possible interpretations for this result may be that antibodies present after clearance of natural infection are not protective against re-infection and/or that reactivation and intermittent detection are more common than newly acquired infection in this age group. Previous studies have found mixed results regarding the protective effect of HPV antibodies against re-infection with the same type.[28, 62, 75-81] Studies have consistently found a protective effect of HPV antibodies against re-infection among younger women under 25-years-old[28, 75, 79, 80] but among older cohorts, results were inconsistent.[62, 76, 81] The consistent reduction in HPV detection observed with serologic evidence of prior infection among younger women suggests that HPV antibodies do play some role in preventing re-infection, but the fact that this same protective effect has not been observed in older cohorts may be due to increased likelihood of a newly detected infection in an older woman representing reactivation of a prior infection or intermitted detection of a persisting infection. Our results are somewhat consistent with a study conducted by Merck using data from the placebo arm of the Gardasil vaccine trial, which found that in women aged 35 to 45 years, incidence of HPV detection (types 6, 11, 16, or 18) was higher among seropositive women (2.8 per 100 person-years) than among seronegative women (2.1 per 100 person-years).[55, 59] Furthermore, among seronegative women, lower HPV incidence was observed in older women aged 35-45 years (2.1 per 100 person-years) versus younger women aged 24-34 years (5.7 per 100 person-years), suggesting a lower likelihood of new sexual exposures among older women. Therefore, one interpretation of the Merck results is that

observed lack of protection associated with HPV antibodies was likely caused by a high proportion of reactivations and intermittent persistent detections in the seropositive group.[59] We conducted a post-hoc analysis comparing cumulative incidence of HR HPV between women aged 30-39 years and 40-50 years. Though we did not observe a statistically significant association of HPV incidence between age groups, we did observe a similar trend that HPV incidence was slightly lower in the 40-50 year-old women compared to 30-39 year-old women (data not shown). In our study, we estimated that approximately 56% of the incident HPV detections in our cohort were likely attributable to non-recent sexual exposures. Incident HPV detections associated with seropositivity (24% of all detections), an indicator of prior infection, likely represented reactivation of previous infections or intermittent detection of persistent infections. Even when there was no serologic evidence of prior infection, new detection in the absence of recent sexual activity or high-risk sexual behaviors was more likely due to reactivation or intermittent persistent detection than to new acquisition (especially given the limitations of serology testing). Even among the seronegative group with recent high-risk behaviors (e.g. new sex partners, multiple partners), we would expect a proportion of the HPV detections (representing 44% of all detections) not to be new acquisitions, despite the association with recent new sexual exposures. Studies have had the tendency to attribute HPV detections associated with recent new partners to new acquisitions of the virus. However, recent sexual behavior could be indicative of past behavior as well, and challenges exist regarding methods to disentangle the effects of recent and past sexual behaviors.[19]

Among women without serologic evidence of prior HPV infection, we observed an independent association between recent sexual behaviors and incident HR HPV detection. Several other studies have reported a link between new sexual exposures and newly detected HPV among mid-adult women, but did not include serology testing.[47, 82-84] However, Trottier et al. observed a statistically significant association between new sexual partners and incident HPV-16 detection among women within medium and high tertiles of HPV seroreactivity but not

among those with low seroreactivity.[81] This inconsistency with our results could be due to differences in the HPV types evaluated or to differences in characterizing recent sexual behaviors between the two studies. In our study, in addition to new sex partners, we included other risk factors, such as multiple partners, partners with other concurrent partners, and partners with a history of STDs. We constructed a composite variable to assess whether recent sexual behavior as a whole has an effect on incident HPV detection. Rositch et al. observed that among women aged 35-60 years of age, both past and recent sexual partnerships were sources of newly detected HPV in older women.[85] If we assume that true new acquisition would be strongly associated with new sexual exposures whereas reactivation or intermittent persistent detection would be associated with cumulative sexual exposures, our detected associations between recent sexual behaviors and incident HPV detection among the seronegative group support a role for new acquisition among mid-adult women without serologic evidence of prior infection. After adjusting for recent sexual behaviors, cumulative sexual behavior risk factors (i.e. non-HPV-related STDs, lifetime number of sex partners, and age at first sexual intercourse) were attenuated and no longer statistically significantly associated with incident HPV detection in the seronegative group. However, a strong relative association does not necessarily translate to a increase in the number of affected cases if the frequency of the risk factor in the population is low. Rositch et al. found a 5.6-fold increase in incident HPV detection associated with new sex partners, but only 13% of the detected cases could be attributable to new partners due to low prevalence of new partners in the population.[85] In our cohort, prevalence of recent high risk sexual behaviors was higher, and we observed that 44% of incident detections were attributable to being recently sexually active with at least one high-risk behavior and not having serologic evidence of prior infection. While a majority of new detections in this seronegative subgroup likely did represent new acquisitions, some likely represented re-detection of prior infection given the limitations of HPV serology measurements.

Among women with serologic evidence of prior infection, we observed a weak positive association between obesity and incident HR HPV as well as current use of hormonal contraceptives and incident HPV. Newly detection HR HPV was not associated with sexual behaviors. Obesity has been shown to be associated with increased risk for other viral pathogens such as herpes simplex virus-1 and -2, adenoviruses, enteroviruses, and influenza.[86, 87] Prior studies proposed that the increased susceptibility to viral pathogens was due to changes in adipokine production, especially leptin, which may be involved in activating inflammation.[87, 88] For HPV, Baker et al. detected increased levels of adipokines among older women with persistent infection,[89] but Liu et al. did not find an association between obesity and incident HPV infection among a cohort of perimenopausal women.[90] Future studies with more power and more detailed measurements of adipokines are needed to further explore the relationship between obesity, immune response, and reactivated HPV infections. The relationship between hormonal contraceptive use and HPV infections has been inconsistent across studies.[91] Current oral contraception use was associated with increased HPV prevalence in some studies[92-95] but not in others.[96, 97] In smaller studies involving college-aged women or younger, no association was found between current hormonal contraceptive use and viral persistence.[54, 98, 99] However, in a population-based study of 1,256 Thai women aged 20-37 years, hormonal contraception was associated with HPV persistence.[100] Another larger study among 1,166 women in Denmark (20-29 years of age) reported an association of current oral contraception and reduced likelihood of viral clearance.[30] Possible biological mechanisms for the relationship between hormonal contraception and HPV may be that female sex hormones are involved in the up-regulation of HPV oncogenes,[101, 102] the modulation of the host immune response through anti-inflammatory or regulatory markers,[103] or the maintenance of cervical ectopy which influences viral susceptibility.[104, 105] Such influences on host-related factors may also lead to increased likelihood of detecting reactivated infections among women with serologic evidence of prior infection. However, prior studies looking at the effect of hormonal contraceptives and HPV redetection

have generated inconsistent results.[28, 106] While assessment of risk factors for incident HPV detection among women with evidence of prior infection may have been limited in power due to smaller sample size, the fact that no statistically significant associations were observed between sexual behaviors and incident HR HPV detection in seropositive women was consistent with our hypothesis that re-infection is uncommon and that most of the incident detections in this group represented reactivation or intermittent detection of persistent infection.

Several limitations to our study should be noted. First, the length of follow-up was fairly short, only six months, with at most 7 samples collected per woman. Second, there may also be potential misclassification of prior infection history due to the limitations of HPV serology, including the potential for positive serology to reflect infection at non-genital anatomic sites.[107] While HPV serology measurements are traditionally used for evidence of prior exposure, studies suggest that only about 60% of women with cervical HPV infections will develop type-specific detectable antibodies[62, 63] and that antibodies may wane with aging.[64, 65] HPV serology methods are also limited in sensitivity and not very well-standardized,[61] all of which might lead to potential misclassification of women with prior infection in the seronegative group and limit comparison of results across studies. There is also potential for misclassification of our incident HR HPV outcome. Though we used HPV DNA positivity as an indicator for current infection, it is unclear whether a DNA-negative result represents complete clearance of the virus or latent infection below detectable levels of available assays. Our sensitivity analysis restricting to incident detections with two prior HPV-negative results generated results similar to when we only required one prior HPV-negative result, minimizing the likelihood of biased results due to misclassification of HPV DNA detection. Also, cumulative and recent sexual exposure measurements relied on participant memory. Furthermore, selecting an appropriate time interval for assessing risk factors for incident HPV detection is subjective. We selected an at-risk time period of 6 months prior to each collected sample, and our sensitivity analysis using one-month at-risk intervals suggests that the likelihood of biased results due to incorrect selection of

affected timeframes was minimal. Finally, our group of HPV-seropositive women was fairly small, limiting our power to make inferences regarding risk factors for re-infection or reactivation. Future studies assessing a larger sample size of HPV-infected women with longer follow-up, using both serology methods and detailed measures of sexual behavior, are needed to identify risk factors for reactivation and re-infection.

In conclusion, among mid-adult women, the likelihood of newly detected HR HPV infections over 6 months of follow-up was higher when there was serologic evidence of prior infection than when there was not. Risk factors for HR HPV detection differed by enrollment HPV serology status. When there was serologic evidence of prior infection, obesity was the only factor associated with newly detected HR HPV. When there was not serologic evidence of prior infection, recent sexual behaviors were associated with HR HPV infection. Our interpretations of these results are that among women with serologic evidence of prior infection, HR HPV detection is more likely due to reactivation or intermittent detection of persisting infection than to re-infection from new partners. In comparison, among women without serologic evidence of prior infection, incident HR HPV is likely due to a combination of new acquisition and intermittent detection of persisting infection. Future studies are needed to further characterize the source of infections detected in mid-adult women, which is key to determining age-specific HPV vaccine strategies and cost-effectiveness of implementing the vaccine in this age group.[1] Better estimates of the absolute risk of new acquisition among HPV infections detected in this age group would be needed to truly estimate the benefit of vaccine implementation. In addition, understanding of the nature of these infections serves as strong basis for HPV counseling of risks associated with new acquisition and methods of prevention. Our study demonstrates that a significant proportion of infections detected in 30-50 year-old women may be due to non-recent sexual exposures and may imply reactivation or intermittent detection of persistent infection instead of new acquisition. A deeper understanding of the frequency of reactivated infections

and their related risk factors would serve as reassurance for women in monogamous relationships regarding reactivation of prior infections.

Table 2.1 Example of infection-level analysis of type-specific incident high-risk HPV DNA stratified by enrollment serostatus

ID no.	*HPV type*	*Baseline serostatus*	*HPV DNA by month*						
			0	1	2	3	4	5	6
1	16	+	–	–	+				
1	39	+	–	–	–	–	–	–	–
1	33	+	–	–	–	–	–	–	–
1	45	+	–	–	–	–	–	–	–
1	51	+	–	–	–	–	–	–	–
1	56	+	–	–	–	–	–	–	–
1	59	+	–	–	–	–	–	–	–
1	68	+	–	–	–	–	–	–	–
1	18	–	–	–	–	–	–	–	–
1	52	–	–	–	–	–	+		
1	58	–	–	–	–	–	–	–	–

Note: This woman contributed 11 HPV types, or "woman-types", to the analysis: 8 seropositive (HPV-16, 33, 39, 45, 51, 56, 58, 68) and 3 seronegative (HPV-18, 52, 58). We observed a total of 2 incident HPV detections over follow-up (shaded in gray), 1 among seropositive woman-types (HPV-16, in month 2) and 1 among seronegative woman-types (HPV-52, in month 4). Two woman-types (HPV-31, 35) were excluded from the analysis due to prevalent HPV DNA detection at enrollment and are not shown in this figure.

Figure 2.1 Assessment of time-fixed and time-dependent risk factors for incident high-risk HPV detection

Risk factors assessed for incident high-risk HPV infection included both time-fixed variables collected at enrollment (month 0) on demographics, general and women's health history, and cumulative sexual behavior (e.g. age at first sexual intercourse) as well as time-dependent variables covering recent sexual behaviors in the 6 months prior to each collected sample (e.g. new sex partners, multiple sex partners), as indicated by timeframes $T_1 - T_6$. For example, if a woman reported a new sex partner 2 months before enrollment (month -2), at month 1 we would classify her as having a new partner, but at month 5, we would not.

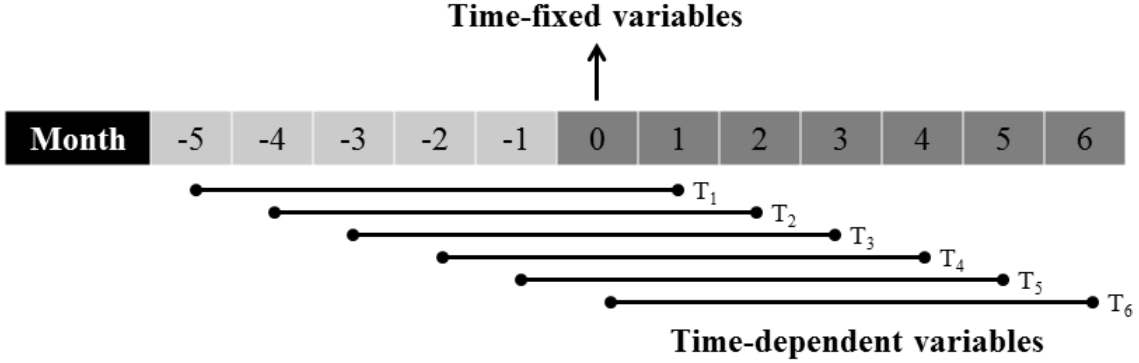

Table 2.2 Enrollment characteristics of mid-adult women in Seattle, Washington, 2011-2012 (N = 372[a])

Characteristics	*Mean*	*(SD)*
Age (years)	38.6	(6.1)
Age at first sexual intercourse (years)[b]	18.7	(4.0)
	Median	*(IQR)*
Lifetime number of male sex partners	7	(3 – 15)
	n[c]	*(%)*
Body Mass Index (kg/m^2)		
< 25 (underweight or normal)	204	(55.0)
25 – 29.9 (overweight)	101	(27.2)
≥ 30 (obese)	66	(17.8)
Race		
African American	11	(3.0)
Asian	42	(11.3)
White	293	(78.8)
Other[d]	26	(7.0)
Education		
Some college or less	63	(16.9)
College bachelor's degree	132	(35.5)
College master's or doctoral degree	177	(47.6)
Marital status		
Unmarried or separated	144	(38.8)
Married or living with a partner	227	(61.2)
Ever had a non-HPV-related sexually transmitted disease[e]		
No	296	(80.0)
Yes	74	(20.0)
Ever had genital warts		
No	330	(88.7)
Yes	42	(11.3)
Ever had an abnormal Pap test		
No	219	(58.9)
Yes	153	(41.1)
Ever been pregnant		
No	146	(39.3)
Yes	226	(60.8)
Currently using hormonal birth control methods[f]		
No	245	(65.9)
Yes	127	(34.1)
Currently have an immunosuppressive condition[g]		
No	364	(97.9)
Yes	8	(2.2)
Smoking status[h]		
Never	273	(73.6)
Former	79	(21.3)
Current	19	(5.1)
Sexual activity with male partners within 6 months prior to enrollment		
Not sexually active	81	(22.0)
Sexually active – no risk factors[i]	132	(35.8)
at least 1 risk factor[i]	156	(42.3)

Table 2.2 Enrollment characteristics of mid-adult women in Seattle, Washington, 2011-2012 (N = 372[a]) (cont.)

Characteristics	*n*[c]	*(%)*
Seropositive for at least one HR HPV type[j]		
No	121	(32.5)
Yes	251	(67.5)
DNA-positive for at least one HR HPV type[j]		
No	318	(85.5)
Yes	54	(14.5)

[a]Of the 409 mid-adult women recruited, 30 women were excluded due to previous HPV vaccination history, 1 woman was excluded due to an insufficient blood samples for HPV serology testing, and 6 women were excluded due to seropositivity to 15 HPV types.
[b]Restricted to 360 women who reported ever having had sex with a male partner.
[c]Numbers may not add up to total due to missing data.
[d]Includes individuals indicating any of the following: American Indian/Alaska Native, Native Hawaiian/Other Pacific Islander, other race, or multiple races.
[e]Includes chlamydia, gonorrhea, genital herpes, and HIV.
[f]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.
[g]Includes HIV positivity (n=1) or currently taking immunosuppressive medications (n=7).
[h]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smoker reported currently smoking.
[i]Risk factors include ≥1 new sex partner, multiple partners (>1 sex partners), any casual partner, any partner with other concurrent partners, and any partner with STDs.
[j]HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68.

Table 2.3 Univariate analysis of factors associated with incident type-specific HR HPV infection among mid-adult women by enrollment type-specific HPV-serostatus[a], Seattle, WA, 2011-2102

	HPV-seropositive women-types (n = 737[b])			HPV-seronegative women-types (n = 3,948[b])		
Characteristics	*OR*	*(95% CI)*	*p-value*	*OR*	*(95% CI)*	*p-value*
Time-fixed variables from enrollment survey						
Age (years)	0.95	(0.89, 1.01)	0.112	0.96	(0.91, 1.01)	0.101
Body Mass Index (kg/m²)[c]						
< 25	1.00	--	--	1.00	--	--
25 – 29.9	2.39	(0.89, 6.43)	0.084*	1.03	(0.48, 2.21)	0.949
≥ 30	3.03	(0.94, 9.79)	0.063*	0.96	(0.29, 3.17)	0.949
Marital status						
Unmarried or separated	1.00	--	--	1.00	--	--
Married or living with a partner	0.64	(0.27, 1.51)	0.307	0.55	(0.28, 1.08)	0.085*
Ever been pregnant	1.54	(0.63, 3.74)	0.339	0.69	(0.35, 1.35)	0.279
Ever had non-HPV-related STDs[d]	0.95	(0.39, 2.30)	0.905	2.23	(1.17, 4.25)	0.015**
Ever had genital warts	0.81	(0.19, 3.49)	0.781	1.65	(0.64, 4.25)	0.300
Current use of hormonal birth control[e]	2.26	(0.96, 5.34)	0.063*	0.90	(0.46, 1.75)	0.748
Current immunosuppressive condition[f]	1.74	(0.21, 14.36)	0.607	1.88	(0.49, 7.29)	0.360
Smoking status[g]						
Never	1.00	--	--	1.00	--	--
Former	0.75	(0.22, 2.57)	0.650	0.85	(0.36, 2.01)	0.714
Current	0.85	(0.11, 6.37)	0.878	0.93	(0.23, 3.76)	0.916
Lifetime number of sex partners[h]						
0 – 3	1.00	--	--	1.00	--	--
4 – 9	1.66	(0.45, 6.07)	0.447	2.00	(0.66, 6.10)	0.219
10+	0.92	(0.25, 3.45)	0.902	4.06	(1.66, 9.94)	0.002**
Age at first sexual intercourse (years)	1.04	(0.93, 1.17)	0.474	0.91	(0.85, 0.99)	0.020**

Table 2.3 Univariate analysis of factors associated with incident type-specific HR HPV infection among mid-adult women by enrollment type-specific HPV-serostatus, Seattle, WA, 2011-2102 (cont.)

	HPV-seropositive women-types (n = 737[b])			HPV-seronegative women-types (n = 3,948[b])		
Characteristics	*OR*	*(95% CI)*	*p-value*	*OR*	*(95% CI)*	*p-value*
Time-dependent variables from enrollment/exit surveys and diaries (within six months before each sample)						
Average weekly number of sex acts[i]	1.06	(0.57, 1.97)	0.850	1.18	(0.87, 1.61)	0.278
Condom use[i]						
Not always	1.00	--	--	1.00	--	--
Always	0.89	(0.12, 6.35)	0.906	0.60	(0.15, 2.42)	0.474
Composite variable for additive sexual behavior risk factors[j]						
Not sexually active with male partners	1.00	--	--	1.00	--	--
Sexually active – no risk factors	0.40	(0.07, 2.43)	0.322	0.64	(0.18, 2.31)	0.493
1 risk factor	0.91	(0.22, 3.70)	0.896	2.17	(0.77, 6.11)	0.141
2 risk factors	0.76	(0.16, 3.67)	0.736	2.46	(0.80, 7.56)	0.117
≥ 3 risk factors	1.20	(0.29, 4.89)	0.802	6.27	(2.40, 16.39)	<0.001**

*p-value < 0.01; **p-value < 0.05

[a]At enrollment, each woman-type was classified as either positive or negative by HPV DNA and serology. Incident type-specific HR HPV detection was defined as the first DNA-positive sample for a HPV type not detected at enrollment; samples after the first type-specific positive detection were considered censored. Analyses were conducted separately by enrollment type-specific HPV serostatus.

[b]243 women contributed a total of 737 women-types to the seropositive analysis, and 366 women contributed 3,948 women-types to the seronegative analysis.

[c]Joint Wald test p-value for HPV-seropositives = 0.120.

[d]Includes chlamydia, gonorrhea, genital herpes, and HIV.

[e]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.

[f]Includes HIV positivity or currently taking immunosuppressive medications.

[g]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smoker reported currently smoking.

[h]Lifetime number of sex partners categorized based on approximate tertiles.

[i]Restricted to women who reported sexual activity within the past 6 months.

[j]Risk factors include ≥1 new sex partner, multiple partners (>1 sex partner), ≥1 casual partner, ≥1 partner with other concurrent partners, and ≥1 partner with a history of STDs.

Table 2.4 Multivariate analysis of factors associated with incident type-specific HR HPV infections among type-specific HPV-seropositive mid-adult women, Seattle, WA, 2011-2012

Characteristics	*aOR*[c]	HPV-seronegative (n = 737 women types[b]) *(95% CI)*	*p-value*
Time-fixed variables from enrollment survey			
Body Mass Index (kg/m²)[cd]			
< 25	1.00	--	--
25 – 29.9	2.27	(0.84, 6.16)	0.106
≥ 30	2.71	(0.85, 8.67)	0.092*
Current use of hormonal birth control[e]	2.04	(0.87, 4.77)	0.100

*p-value < 0.01

[a]Incident type-specific HR HPV detection was defined as the first DNA-positive sample for a HPV type not detected at enrollment; samples after each type-specific first positive detection were considered censored.

[b]243 women contributed 737 women-types to the seropositive analysis.

[c]Adjusted odds ratio adjusted for all other variables in the table.

[d]Joint Wald test p-value = 0.1715.

[e]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.

Table 2.5 Multivariate analysis of factors associated with incident type-specific HR HPV infection among type-specific HPV-seronegative mid-adult women, Seattle, WA, 2011-2102[a]

		HPV-seronegative (n = 3,736 women types[b])	
Characteristics	*aOR[c]*	*(95% CI)*	*p-value*
Time-fixed variables from enrollment survey			
Marital status			
Unmarried or separated	1.00	--	--
Married or living with a partner	0.89	(0.36, 2.18)	0.794
Ever had non-HPV-related STDs[d]	1.60	(0.82, 3.11)	0.167
Lifetime number of sex partners[e]			
0 – 3	1.00	--	--
4 – 9	1.48	(0.40, 5.53)	0.556
10+	2.39	(0.67, 8.49)	0.179
Age at first sexual intercourse (years)	0.99	(0.91, 1.09)	0.885
Time-dependent variables from enrollment/exit surveys and diaries (within six months before each sample)[f]			
Composite variable for additive sexual behavior risk factors[gh]			
Not sexually active with male partners	1.00	--	--
Sexually active – no risk factors	1.42	(0.26, 7.80)	0.687
1 risk factor	4.43	(0.94, 20.80)	0.059*
2 risk factors	4.02	(0.79, 20.61)	0.095*
≥ 3 risk factors	9.88	(2.45, 39.77)	0.001*

*p-value < 0.05

[a]Incident type-specific HR HPV detection was defined as the first DNA-positive sample for a HPV type not detected at enrollment; samples after each type-specific first positive detection were considered censored.

[b]347 women contributed 3,736 women-types to the seronegative analysis.

[c]Adjusted odds ratio adjusted for all other variables in the table.

[d]Includes chlamydia, gonorrhea, genital herpes, and HIV.

[e]Lifetime number of sex partners categorized based on approximate tertiles.

[f]Sexual behavior data from enrollment and exit surveys and diaries were combined into summary variables. For each monthly HPV assessment, partner data from enrollment and exit surveys were included if the dates of first or last sex with the partners fell within 6 months prior to the sample collection date. Daily sexual behavior data in diaries were included for days falling within the 6-month period before each sample. The two data sources were merged to form the combined time-dependent sexual behavior variables used in our analysis. Sexual behaviors that were reported in any of the data sources were considered present in the merged variables.

[g]Risk factors include ≥1 new sex partner, multiple partners (>1 sex partners), ≥1 casual partner, ≥1 partner with other concurrent partners, and ≥1 partner with a history of STDs.

[h]Joint Wald test p-value = 0.003.

Table 2.6 Attributable risk of incident HR HPV infection due to type-specific HPV serostatus and cumulative and recent sexual behavior among mid-adult women, Seattle, WA, 2011-2102

Characteristics	*N*[a]	*n*[b]	*aOR*[c]	*(95% CI)*	*%AR*[d]	*%PAR*[e]
Seronegative, not sexually active OR sexually active and no risk factors[f]	9,792	9	1.00	--		32.16
Seronegative, sexually active and ≥ 1 risk factor[f]	10,287	39	4.17	(1.90, 9.15)	76.01	43.59
Seropositive	3,728	20	5.70	(2.40, 13.57)	82.46	24.25

[a]Indicates the total number of women-types * number of samples included in each stratum.
[b]Indicates the number of women-types * number of samples with incident HPV detected.
[c]Adjusted for age, marital status, ever had non-HPV-related STDs, and lifetime number of sex partners in tertiles,.
[d]Attributable risk percent calculated as [(OR – 1) / OR] * 100.
[e]Population attributable risk percent calculated as ^{108} * 100 where p_d is the proportion of cases exposed to the risk factor.
[f]Risk factors include ≥1 new sex partner, multiple partners (>1 sex partners), ≥1 casual partner, ≥1 partner with other concurrent partners, and ≥1 partner with a history of STDs.

3 Oral and Fingernail HPV Infections and Concordance across Anatomic Sites in a Cohort of Mid-Adult Women

3.1 INTRODUCTION

Though alpha HPV infections are most commonly detected in the genital areas, infections of the oropharynx or fingernails may be of importance because of the risk of developing HPV-related carcinomas at non-genital sites and the potential to foster transmission of the virus to the genitals.[109] Oral HPV infection, particularly HPV-16, is an established etiological factor for oropharyngeal cancers.[110-113] Although oropharyngeal cancers are considered rare, the incidence of these malignancies has increased significantly in the United States from 1973 to 2004 (overall annual percent change=0.8)[114] with most of the increase due to a doubling of rates in 25 to 49-year-old men.[115] This increase in oropharyngeal cancers was substantially caused by HPV-related cancers, with the proportion of oropharyngeal cancers attributable to HPV increasing from 16.3% in the 1980s to 72.7% in the 2000s.[116] Given the link between HPV and oral cancers, characterizing the frequency of and risk factors for oral HPV infection is a priority. A systematic review of 18 studies comprising 4,581 healthy adults reported that the prevalence of oral HPV was 4.5% for all HPV types and 3.5% for high-risk HPV types.[117] A population-based study using data from the National Health and Nutrition Examination Survey (NHANES) 2009-2010 in the United States reported that the prevalence of oral HPV infections for all types and high-risk types was 6.9% and 3.7%, respectively.[118] Risk factor studies on oral HPV have found that mouth-to-mouth contact and oral sex are associated with oropharyngeal HPV infections, suggesting a direct oral transmission route.[119] Other risk factors for prevalent oropharyngeal HPV infections that have been identified in previous studies include increasing age, male gender, cigarette smoking, immunosuppression, number of partners or frequency of oral or vaginal sex, concurrent HPV infections at genital sites, and oral mucosal abnormalities.[108, 118-121] Detection of genital HPV types in the palms, fingertips, or

fingernails are even rarer than oral infections,[122-124] but studying these infections may aid in understanding the modes of transmission between and within individuals. HPV detection at multiple anatomic sites within an individual has been documented, and is likely due to a single source of infection (e.g. a sexual partner), autoinoculation (i.e. a secondary infection originating from an infection already present in the body), or DNA deposition between sites (i.e. the transfer of DNA via contact with another anatomic site).[122, 123, 125-131] Concordance between oral and genital samples for detecting HPV has generally been low, though results are inconsistent.[125-130, 132] Studies on genital HPV types detected in fingernails and its concordance with other anatomic sites have been scarce. Though concordance between fingernails and genital areas also seems low, fingernail infections were strongly associated with a concurrent genital infection of the same HPV type.[123, 133] Utilizing data from a 6-month longitudinal study among mid-adult women, we evaluated the frequency of and risk factors for oral and fingernail HPV infections in this cohort and in addition, described the concordance of HPV detection across the three anatomic sites.

3.2 METHODS

3.2.1 Study Design and Population

Between March 2011 and January 2012, we enrolled a convenience sample of 409 mid-adult women aged 30 to 50 years affiliated with the University of Washington into a longitudinal study. Detailed inclusion criteria were described previously in chapter 1. Women provided self-collected oral, fingernail, and vaginal samples for HPV genotyping at the in-clinic enrollment visit and 6-month exit visit. During follow-up, women were also asked to self-collect vaginal samples every month. Information on demographics, general health status, HPV vaccination history, pregnancy history, contraceptive use, alcohol and smoking habits, and sexual history were collected through online surveys at enrollment and exit. In addition, supplemental questions were added to the exit survey in February 2012 to capture additional potential risk factors for oral HPV, including open mouth kissing and oral sex. Women who had

already completed follow-up before the supplemental questions were incorporated (n=135) were invited to complete an online supplemental survey with the additional questions post exit, and 81 women (60% of those invited) submitted a supplemental survey. Data on open mouth kissing and oral sex and the number of male and female partners for each behavior were collected separately for the time period before enrollment as well as the time period between enrollment and exit. Three hundred and eighty one women had an exit visit, of which 54 (14%) completed an exit survey without additional kissing and oral sex questions, 81 (21%) completed a supplemental survey after exit, and 242 (64%) women completed an updated exit survey with the additional questions incorporated.

3.2.2 Sample Collection

Women provided samples from three different anatomic sites: oral, fingernail and vaginal samples. Fingernail sample collection was not implemented until after the start of the study (June 2011); therefore, enrollment fingernail samples were not collected for 139 (34%) women. All samples were obtained through subjects' self-collection, which has been shown in previous studies to be feasible and acceptable for HPV testing at all three anatomic sites.[26, 123, 134, 135] Two types of oral specimens were collected: an oral rinse sample and two oral swabs. Results from the two types of oral samples were combined to maximize sensitivity for HPV detection.[136] For the oral rinse sample, women were asked to gargle 10 mL of Scope mouthwash for 30 seconds and then expectorate into a collection cup. The mouthwash sample was then centrifuged for 5 minutes and the remaining pellet stored in 1 mL of Qiagen cell lysis solution. For the oral swabs, the woman was instructed and supervised in mirror-assisted collection, with the first swab swabbing the tonsils or palatine arches and the second swab swabbing the back of the tongue and pharynx. The swabs were stored in a separate vial from the mouthwash sample in 1.5 mL specimen transport medium (STM). For the fingernail sample, women were asked to rub their fingertips and the underside of the tip of their fingernails on both hands with a

cytology brush. The brush was then stored in a vial with 1 mL STM. For vaginal samples, women were asked to self-collect a vaginal specimen in a private room after receiving illustrated written and verbal instructions. Women were provided with a kit containing two sterile Dacron swabs (to enhance sensitivity for HPV detection),[21] a covered tube containing 1.5 mL of STM, a plastic cup, and nitrile gloves.

3.2.3 HPV Genotyping

Genomic DNA was isolated from self-collected oral, fingernail, and vaginal specimens and HPV genotyped using PCR-based methods. Specimens were digested with 20 μg/mL proteinase K at 37°C for one hour. For the oral swab, fingernail, and vaginal specimens, DNA was isolated from 200 μL of the digested sample using the QIAamp DNA blood mini kit, following the protocol of the manufacturer (Qiagen, Cat. No. 51104). Genomic DNA from the oral lavage cell pellet was isolated using the Gentra Puregene Buccal Cell kit, following the protocol of the manufacturer (Qiagen, Cat. 158845). Both HPV and host gene β-globin were PCR amplified using the MY09/11 system and detected by dot blot hybridization, probed with either HPV generic probe or β-globin probe. Specimens negative for β-globin were considered insufficient, and specimens positive for β-globin but negative for HPV were considered negative for HPV infection. Specimens positive by both probes were subsequently genotyped for 37 alpha-genus HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, CP6108, IS39) via the Roche Linear Array assay. Samples that were HPV-positive by dot blot but HPV-negative by the Roche assay (1 oral swab, 5 oral rinse, and 3 fingernail samples) were considered HPV-negative in the analyses. Samples that were positive by dot blot but negative for β-globin by the Roche assay (2 fingernail samples) were considered insufficient and excluded from the analysis. Vaginal samples bypassed the dot blot hybridization step and were tested with the Roche Linear Array assay directly.

To further examine oral and fingernail insufficient samples, we determined the amount of human genomic DNA present in the samples by a real-time PCR Alu(q) assay. Samples were also tested by real time PCR HPV-16 and HPV-18 assays. A sample was considered insufficient if the Ct on the Alu(q) assay was above 30, and a sample was considered negative for HPV-16 or HPV-18 if the Ct on HPV assays were above 40. In addition, we determined whether there was PCR inhibition by performing the Alu(q) assay on serial diluted samples (1:10 and 1:100 dilutions). A sample was considered inhibited if the quantification from the 1:10 dilution sample was higher than the undiluted sample, or if quantification from the 1:100 dilution sample was higher than the 1:10 dilution sample. We re-tested all 20 oral insufficient samples and a subset of 40 fingernail insufficient samples. All oral samples were sufficient by the Alu(q) assay, but 15 of 20 oral samples showed PCR inhibition. Therefore, we concluded that oral sample insufficiency by dot blot analysis was likely due to PCR inhibition. No oral sample that was insufficient by dot blot tested positive for HPV-16 or HPV-18. Of 40 insufficient fingernail samples, only 4 were insufficient by the Alu(q) assay with high Ct values, and no inhibition was detected. Therefore, we concluded that fingernail sample insufficiency by dot blot analysis was likely due to low amount of genomic DNA present in the samples. None of the fingernail samples that were insufficient by dot blot were positive for HPV-16 or HPV-18.

3.2.4 HPV Viral Load Testing

Viral load testing, or quantitative HPV DNA testing, was conducted on vaginal samples only using real-time PCR, described in detail previously.[18] We tested vaginal samples DNA-positive for type-specific high-risk HPV by the Roche assay. Testing was conducted for 16 high-risk types, including HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82. A reaction volume of 5 µl was used for DNA amplification at 50°C for 2 minutes, 95°C for 10 minutes, and a two-step cycle of 95°C for 15 seconds and 60°C for 1 minute (40 cycles). HPV type-specific primers and probes were used, and the copy numbers of type-specific HPV genome and cellular

DNA were used as standards for absolute quantification. Viral load was normalized and expressed as the number of HPV viral copies per nanogram of cellular DNA. Samples were analyzed in triplicate, and the log-transformed viral copy numbers of the three measures were averaged and used for analysis. When one of the three measures was HPV-negative or differed by more than two standard deviations, the average value was calculated using the two remaining measures. Samples that tested HPV-negative on two or more runs but were positive for β-actin were assigned a value of 1 copy number per nanogram of cellular DNA if they tested positive by the Roche assay.

3.2.5 HPV Variant Sequencing

Type-specific HPV variant sequencing was conducted on samples positive for the same HPV type in different anatomic sites within a woman at the same visit or for paired type-specific positive vaginal samples within a woman that were separated by an observed period of DNA negativity. Variant testing was available for 12 types: HPV-16, 18, 31, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

DNA extraction was conducted on an aliquot of 100 µl Specimen Transport Medium per sample using QIAamp DNA blood mini kit (Qiagen, Valencia, CA). PCR with type-specific external primers (*Table 1.1*) were used to generate HPV DNA fragments covering the 3' part of the long control region (LCR) and the entire E6 and E7 region. PCR products were then sequenced with both external and internal primers using BigDyeTM Sequencing kit (Applied Biosystems, Foster City, CA). Due to the size of the DNA fragment, the sequencing reaction was run from both directions. SequencerTM package (Gene Codes Corp., Ann Arbor, MI) was used to analyze DNA sequences. If one or more nucleotide alterations were detected in the region analyzed compared to the prototype or the other isolates detected in our study, then we consider the isolate to be a distinct variant.

3.2.6 Statistical Analysis

Prevalence and 6-month cumulative incidence (with 95% confidence of intervals [CIs]) of HPV at different anatomic sites were conducted as woman-level analyses. Women were considered to have oral HPV infection if either the rinse sample or the swab sample tested positive for HPV DNA. Insufficient or inhibited samples were excluded from the denominators in all calculations. Among women DNA-negative for all HPV types at enrollment, we calculated the 6-month cumulative incidence and 95% CI for each anatomic site by dividing the number of women positive for any HPV type at the exit visit divided by the number of women with sufficient exit samples. Six-month persistence and clearance were calculated on the type-specific infection-level. Among infections prevalent at enrollment, persistence was defined as the proportion of infections that were DNA-positive at the exit visit for the same HPV type, and clearance was defined as the proportion of infections that were DNA-negative at exit for the same type.

We used proportion positive agreement (PPA) and kappa statistics to describe the concordance of type-specific HPV detection between oral, fingernail, and vaginal samples, combining the enrollment and exit samples. PPA between oral/vaginal, oral/fingernail, and fingernail/vaginal samples was calculated by dividing the number that tested DNA-positive in both samples by the number that tested DNA-positive in either sample. To adjust for the PPA caused by chance, the un-weighted kappa statistic was calculated for each pair of the different types of samples using the following formula:

$$\kappa = (\text{observed PPA} - \text{expected PPA}) / (1 - \text{expected PPA})$$

where the expected PPA is calculated assuming independence of the assay results. 95% CIs for the kappa statistic were estimated using percentile bootstrap methods with 1,000 repetitions to account for correlation due to multiple HPV types and multiple visits within women. The kappa statistics were interpreted based on Landis and Koch's guidelines.[137]

Generalized estimating equations (GEE) logistic regression was utilized to separately measure the association of various risk factors with type-specific oral and fingernail HPV detection. Both enrollment and exit visit samples were included in the analysis, and time-varying covariates collected at each visit were assessed as risk factors. Robust variance estimates were used to account for correlation within women due to dual measurements and multiple HPV types. Potential risk factors that were assessed included age (years; continuous), marital status (unmarried or separated/married or living with a partner), history of non-HPV-related sexually transmitted diseases (yes/no), history of genital warts (yes/no), history of an abnormal Pap test (yes/no), history of prior pregnancies (yes/no), current hormonal contraceptive use (yes/no), cigarette smoking (never/former/current), current alcohol use (yes/no), type of vaginal sex partners (male only/female only or both male and female), age at first sexual intercourse with a male partner (years; continuous), lifetime number of male sex partners (0-3/4-9/10+; approximate tertiles), lifetime number of open-mouth kissing partners (0-3/4-19/20+; approximate tertiles), type of oral sex partners (male only/female only or both male and female), lifetime number of oral sex partners (0-1/2-6/7+; approximate tertiles), concurrent vaginal HPV infection with the same type (yes/no), and type-specific vaginal viral load (copy numbers per nanogram of cellular DNA; $\log_{10}$-transformed, continuous; restricted to those with type-specific vaginal HPV infections). History of prophylactic HPV vaccination (yes/no) was also evaluated as a risk factor for oral HPV in an analysis restricting to vaccine types 6, 11, 16, and 18 (we did not conduct a similar analysis for fingernail HPV because no fingernail samples were positive for vaccine types). We considered variables statistically significant if p-values were less than 0.05. Exact logistic regression was used to estimate 95% CI's for variables with zero cases of HPV detection in either risk factor category.

3.3 RESULTS

3.3.1 Characteristics of study population

Of the 409 women in the study, the majority were White (78%), received a bachelor's degree or higher (84%), and were either married or living with a partner (61%). Most women only had sex with male partners during their lifetime (80%), 2% had sex with female partners only, and 17% had sex with both male and female partners. The median lifetime number of male sex partners for the women in our cohort was 7 (IQR: 3 – 15), and 342 (84%) women reported engaging in sexual activity with male partners within one year prior to enrollment. Of the 323 (85%) women who answered supplemental questions regarding additional potential risk factors for oral HPV, 99% reported having ever open-mouth kissed either male or female partners. For oral sex, 77% reported ever having performed oral sex on male partners only, 2% on female partners only, and 17% on both male and females partners (*Table 3.1*).

3.3.2 Prevalence, incidence, and persistence of HPV DNA detection

Insufficient samples for HPV genotyping at each anatomic site were excluded from analyses (4 [0.5%] oral swab samples; 16 [2.0%] oral rinse samples; 140 [21.8%] fingernail samples; and 2 [0.1%] vaginal samples). Oral or fingernail HPV was detected among 22 (5%) women either at enrollment or exit, and their HPV infection status across anatomic sites is presented in *Table 3.2*. Only one pair of oral samples tested positive for HPV (type 16) in both the rinse and the swab (this infection also represented one of three persistent oral infections detected in the study); in all 17 other cases, oral HPV was detected in only one sample of a pair. Variant testing was conducted on 14 samples representing 6 infections with the same HPV type detected across anatomic sites (*Table 3.2*). Of the 6 infections tested, variant characterization was not completed for 2 paired type-specific infections due to samples testing HPV-negative during PCR sequencing steps. Samples from 3 out of 4 (75%) sets of infections tested positive for the same type-specific variant within a woman, and one (25%) set of HPV-16 infections had a

different variant detected in the enrollment oral sample versus the enrollment and exit vaginal samples.

On the woman-level, the prevalence of detecting any HPV DNA at enrollment was 2.5%, 3.8%, and 33.1% among oral, fingernail, and vaginal samples, respectively (*Table 3.3*). The prevalence of high-risk HPV DNA at enrollment was 2.2%, 2.4%, and 21.8% for oral, fingernail, and vaginal samples, respectively; the prevalence of HPV-16 at enrollment was 0.7%, 0.0%, and 3.7% for oral, fingernail, and vaginal samples, respectively. The 6-month cumulative incidence of detecting a HPV type not detected at enrollment was 0.3% for oral samples, 0.5% for fingernail samples, and 15.0% for vaginal samples. On the infection-level, persistent detection was low among both oral (23%) and fingernail (0%) infections after 6 months follow-up. In contrast, 67% of prevalent infections detected in vaginal samples were persistently detected at 6 months.

3.3.3 Concordance of HPV DNA detection between different anatomic sites

Proportion positive agreement (PPA) was lowest between oral and vaginal samples (0.6%) and highest between fingernail and oral samples (9.5%), though PPA was low across all sites in general (*Table 3.4*). To account for chance, we estimated kappa statistics for all three pairs of comparisons across anatomic sites and found that concordance was poor (kappa < 0.20) for all site comparisons.

3.3.4 Risk factors for oral and fingernail HPV detection

We conducted univariate analyses to evaluate potential risk factors separately for oral and fingernail infections at both enrollment and exit visits. For oral HPV infections, ever having had an abnormal Pap test (OR=11.10; 95% CI: 2.35, 52.40), having a lifetime number of male sex partners greater than 10 (OR=5.15; 95% CI: 1.07, 24.93; relative to 0 – 3 partners), and having concurrent vaginal infection with the same HPV type (OR=10.29; 95% CI: 3.34, 31.72) were each positively associated with oral HPV detection (*Table 3.5*). For fingernail HPV

infections, ever having had a non-HPV-related STD (OR=3.48; 95% CI: 1.12, 10.78), having concurrent vaginal HPV infection with the same type (OR=104.33; 95% CI: 30.44, 357.5), and vaginal HPV viral load (OR per one $\log_{10}$ increase in viral load=2.35; 95% CI: 1.42, 3.87) were each positively associated with fingernail HPV detection (*Table 3.6*).

3.4 DISCUSSION

In our study of mid-adult women aged 30-50 years, prevalence estimates for oral HPV detection were 2.5% for any HPV, 2.2% for high-risk HPV, and 0.7% for HPV-16. Prior studies in immunocompetent populations have reported substantial variation in oral HPV prevalence according to different populations and geographic areas, ranging from 0.6% in 668 healthy volunteers in Japan[138] to 25% in 317 HIV-negative older men in the United States.[135, 139-144] A systematic review including 18 studies worldwide estimated a pooled oral HPV prevalence of 4.5% for any HPV, 3.5% for high-risk HPV, and 1.3% for HPV-16 among 4,581 healthy individuals, with similar prevalence estimates between men and women.[117] Another large study using the NHANES data in the United States found an oral HPV prevalence of 6.9% for any HPV, 3.7% for high-risk HPV, and 1.0% for HPV-16 among 5,501 men and women aged 14 to 69 years, but prevalence of any HPV in men (10.1%) was significantly greater than in women (3.6%).[118] Oral HPV prevalence in the United States demonstrated a bimodal pattern with age, and our prevalence estimates are comparable with the modeled female oral prevalence estimates of the same age range in the NHANES study.[118] Other differences in prevalence estimates may be attributable to different study populations, sampling methods, laboratory assays and protocols, and birth cohort effects.[145]

There are limited data on the natural history of oral HPV incidence and persistence in immunocompetent cohorts. Cumulative incidence and persistence of any oral HPV over 6 months was estimated to be 0.3% and 23.1%, respectively, in our cohort of mid-adult women. The Finnish HPV Family Study reported an oral HPV cumulative incidence of approximately 10%

at 24 months follow-up for 331 women, and none of the 40 women with high-risk HPV infections detected at enrollment cleared their infections at 24 months.[146] D'Souza et al. reported a 60% persistence of oral HPV at 6 months among 63 HIV-negative women.[147] In general, incidence of oral HPV has been observed to be higher in male cohorts than among women. In a study of young men aged 18 to 25 years in Washington State, the 12-month cumulative incidence of oral HPV infection was 12.3%, and 28.6% of prevalent infections were repeatedly detected within 4 months to 1.5 years.[135]

Differences in specimen collection methods may affect oral HPV detection. There is currently no standardized way to collect oral HPV specimens;[148] therefore, we used both oral rinse and oral swab samples to increase sensitivity.[136] Our methods for detecting oral HPV were identical to a previous study looking at oral HPV infection among young men, which also detected discordance between oral swab and oral rinse samples.[135] We observed in our study that HPV genotyping results between the two different oral samples are mainly discordant, with only one woman HPV-positive for type 16 in both oral rinse and oral swab samples. Differences in oral rinse versus oral swab samples may also reflect different infection sites within the oral cavity.[135]

The prevalence of HPV in fingernail samples in our study was 3.8%. HPV was not redetected in any fingernail sample at 6 months, but only 9 of 12 samples with prevalent HPV had a paired exit sample that was sufficient for testing. [124] A previous study conducted among 128 women aged 18-22 years found an HPV prevalence of 14.3% in fingertip samples and a redetection rate of 14.5% at the subsequent visit approximately four months after.[123] The low prevalence and transient nature of HPV detected in fingernails suggests that detection of HPV DNA in the fingernails is unlikely to represent true infection. The incidence of HPV detection in the fingernails was also low in our study (0.5%). Partridge et al. detected a cumulative incidence of fingernail infections of 30% for any HPV type and 26% for any high-risk type at 24 months follow-up among young college men aged 18 to 20 years.[133] Studies of HPV infections occurring

on the fingernails/fingertips have been scarce, but an understanding of these infections could potentially inform about HPV transmission from other anatomic sites to fingernails (and vice versa), both between and within individuals.

We observed poor concordance of type-specific HPV detection between oral, fingernail, and vaginal sites. Of the few studies that have addressed concurrent oral and genital HPV infections, findings have been mixed, reporting a wide range of concordance estimates (ranging from 0% to 60%).[128, 129, 131, 147, 149-157] A meta-analysis of women with cervical infection from 10 published studies estimated a type-specific HPV concordance of 27% between oral and genital sites.[158] Another study among 1,812 women in the United States using the NHANES data found a low type-specific concordance (6.6%) between oral and cervical sites, which may suggest differences in the natural history of infection at the two different sites.[157] Concurrent oral and genital HPV infections may represent immune vulnerability, overlap in sexual behaviors between oral and genital HPV, or acquisition of the virus from an infected sex partner during vaginal sex and/or oral sex.[145] Winer et al. previously reported low type-specific concordance between fingertips and genital sites (kappa = 0.17) among young women aged 18-22 years.[123] Fair concordance between fingernails and genital HPV has been observed in a cohort of young heterosexual men aged 18-20 years (kappa = 0.26).[159] Concordance across anatomic sites was low but nonetheless suggests that HPV detection at multiple sites may be due to a single source of infection (e.g. a sexual partner), autoinoculation, or DNA deposition between sites.[122, 123, 125-131] A previous study on HPV transmission between heterosexual couples provides evidence that hands may serve as reservoirs of HPV infection in both males and females, especially between male genitals and female hands as well as autoinoculation for both men and women.[122] Though these mechanisms for transmission may be theoretically possible, however, our results suggest that they are not commonly occurring.

Among the risk factors assessed in our study, we found that concurrent vaginal HPV infection of the same type was strongly associated with concurrent detection of oral HPV

infection. A history of abnormal Pap results was also associated with oral HPV infection. We did not observe statistically significant associations between any other risk factors and oral infections. Strong associations between HPV infections across sites further suggest the possibility of autoinoculation, though our analysis of concordance across sites suggests that autoinoculation was infrequently occurring. These results were consistent with a prior study conducted among US women, which found that prevalence of oral infections was higher among women with cervical infection than those without cervical infection (prevalence ratio=4.9; 95% CI: 2.8, 8.7).[157] A study conducted among young men aged 18-20 years also found increased risk of oral infection associated with current infection of the same HPV type in the genitals (hazard ratio=6.2; 95% CI: 2.4, 16.4).[135]

The primary mode of HPV transmission to the oral cavity and oropharynx is hypothesized to be sexual contact.[110, 128, 147] We observed that a lifetime number of male vaginal sex partners ≥10 (compared to 0 to 3) was associated with oral HPV detection in our cohort, consistent with prior studies that observed a link between greater lifetime number of vaginal sex partners and increased likelihood of oral HPV.[120, 121] We did not observe notable associations between any other measured sexual behaviors and oral HPV. Risk factor studies have found that mouth-to-mouth contact and oral sex are associated with oropharyngeal HPV infections,[119, 135, 160, 161] suggesting a direct oral transmission route. However, the majority of our cohort (96%) engaged in prior oral sex, therefore, we did not have a large enough comparison group to assess the effect of history of oral sex on oral HPV. One prior study observed that performing oral sex on a woman may be of higher risk than performing oral sex on a man (offering one possible mechanism by which oral HPV is more common in men than in women), but the study was underpowered to make such inference.[160] We did observe a positive association between oral sex with female partners only or both male and female partners relative to male partners only, but the association was not statistically significant. Other risk factors for prevalent oropharyngeal HPV infections that have been identified in previous studies include increasing age, cigarette

smoking, alcohol use, immunosuppression, and oral mucosal abnormalities.[110, 118, 119, 121, 132, 147, 161-163] We observed positive associations between smoking and alcohol use with oral HPV detection, but the results were not statistically significant.

Plausibility for a finger-genital route of HPV transmission was first suggested by a previous report that genital HPV types were found on the fingernails of 12 of 22 (54.5%) patients with genital warts..[124] Along these lines, we observed that risk factors for fingernail HPV infection included concurrent vaginal HPV infection with the same HPV type and viral load in concurrent vaginal infections. These results are consistent with a study among young men aged 18-20 years which found increased risk of concurrent genital infection with fingernail infections of the same HPV type (hazard ratio=11.8; 95% CI: 4.1, 34.2).[135] Winer et al. found a higher likelihood of detecting concurrent infections between genital sites and fingernails if both cervical and vaginal/vulvar sites were HPV-positive, and hypothesized that infection at multiple genital sites may reflect infections of higher viral load that in turn increase the likelihood of DNA deposition or transmission between sites.[123] Our results further support this hypothesis with the observation that higher HPV viral load was associated with fingernail infection. We did not observe notable associations between any other risk factors and fingernail infections, although we did observe a non-significant positive association between lifetime number of male vaginal sex partners and fingernail HPV.

Several limitations to our study should be noted. First, the protocol for testing vaginal samples was different and more sensitive than that for oral and fingernail samples, since vaginal samples were tested with the Roche Linear Array assay directly (oral and fingernail samples were first tested by less costly dot blot hybridization, given the expected low prevalence of oral and fingernail versus vaginal HPV infections). Therefore, concordance between vaginal and oral or fingernail samples for detecting HPV DNA may have been underestimated. Another limitation of our study is the small number of oral and fingernail infections detected. Our ability to draw inferences was therefore limited due to low power, and we were unable to examine risk

factors for persistent or incident oral or fingernail infections. Furthermore, a few of our main exposures of interest, such as open-mouth kissing and oral sex, were too common in the study population to adequately assess. Due to the design of the questionnaires, we did not gather information on recent or new oral sex behaviors. Finally, many of the fingernail samples collected were insufficient, with a higher insufficiency rate (21.8%) compared to previous studies using the same laboratory methods. The insufficiency rate for fingernail samples was 1.4% in a previous study among young men[133] and 6.2% in a study among young women.[123] It is unclear why the insufficiency rate was higher in our study. We re-tested 40 insufficient fingernail samples with real-time PCR and found that they were truly insufficient with low detectable values. The large number of fingernail insufficient samples also limited our ability to assess concordance across anatomic sites due to fewer paired samples for comparison. In addition, fingernail samples were not collected until after the start of the study, so there were fewer enrollment fingernail samples available for analysis, further limiting power.

In conclusion, detection of HPV in the oral cavity/oropharynx and fingernails was uncommon among this cohort of mid-adult women, and persistent detection of HPV at these non-genital sites was rare. Though concordance between anatomic sites was poor, concurrent vaginal infection of the same HPV type was strongly associated with oral or fingernail infection. These results suggest that deposition between anatomic sites is plausible, though both oral and fingernail infections may be unlikely to serve a key role in the process of HPV transmission in this age group of women.

Table 3.1 Enrollment characteristics of mid-adult women in Seattle, Washington, 2011-2012 (N = 409)

Characteristics	*Mean*	*(SD)*
Age (years)	38.3	(6.1)
Age at first sexual intercourse with male partner (years)[a]	18.8	(4.1)
	Median	*(IQR)*
Lifetime number of male sex partners	7	(3 – 15)
	n[b]	*(%)*
Race		
African American	10	(2.4)
Asian	46	(11.2)
White	318	(77.8)
Other[c]	35	(8.6)
Education		
Some college or less	68	(16.6)
College bachelor's degree	152	(37.2)
College master's or doctoral degree	189	(46.2)
Marital status		
Unmarried or separated	157	(38.6)
Married or living with a partner	250	(61.4)
Ever had a non-HPV-related sexually transmitted disease[d]		
No	327	(80.3)
Yes	80	(19.7)
Ever had genital warts		
No	363	(88.8)
Yes	46	(11.3)
Ever had an abnormal Pap test		
No	234	(57.2)
Yes	175	(42.8)
Ever had ≥ 1 dose of HPV vaccine		
No	378	(92.7)
Yes	30	(7.4)
Ever been pregnant		
No	168	(41.1)
Yes	241	(58.9)
Currently using hormonal birth control methods[e]		
No	270	(66.0)
Yes	139	(34.0)
Currently have an immunosuppressive condition[f]		
No	401	(98.0)
Yes	8	(2.0)
Smoking status[g]		
Never	301	(73.8)
Former	88	(21.6)
Current	19	(4.7)
Currently consumes alcoholic beverages		
No	63	(15.4)
Yes	346	(84.6)

Table 3.1 Baseline Enrollment characteristics of mid-adult women in Seattle, Washington, 2011-2012 (N = 409) (cont.)

Characteristics	*n*[b]	*(%)*
Type of vaginal sex partners (lifetime)		
Male only	327	(80.0)
Female only	8	(2.0)
Both male and female	70	(17.1)
No sex partners	4	(1.0)
Sex with male partners within 12 months before enrollment		
No sexual activity	64	(15.8)
Sex with non-new male partners only	218	(53.7)
Sex with ≥1 new male partner	124	(30.5)
Ever open-mouth or tongue kissed[h]		
Never	4	(1.3)
Male partners only	207	(68.1)
Both male and female partners	93	(30.6)
Ever performed oral sex[h]		
Never	12	(3.9)
Male partners only	234	(77.0)
Female partners only	6	(2.0)
Both male and female partners	52	(17.1)

[a]Restricted to 397 women (97.1%) who reported ever having had sex with a male partner.
[b]Numbers may not add up to total due to missing data.
[c]Includes individuals indicating the following: American Indian/Alaska Native, Native Hawaiian/Other Pacific Islander, other race, or multiple races.
[d]Includes chlamydia, gonorrhea, genital herpes, and HIV.
[e]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.
[f]Includes HIV positivity (n=1) or currently taking immunosuppressive medications (n=7).
[g]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smokers reported currently smoking.
[h]Restricted to 323 women who provided information on these additional potential risk factors for oral HPV infections. Additional questions were added to the exit survey after the start of the study; 81 completed a supplemental survey after exit, and 242 women completed an updated exit survey with the additional questions incorporated.

Table 3.2 Type-specific HPV DNA status across anatomic sites in mid-adult women with oral or fingernail HPV detected at enrollment or 6-month exit, Seattle, Washington, 2011-2012

ID no.	Enrollment			Exit		
	Oral	Fingernail	Vaginal[a]	Oral	Fingernail	Vaginal[a]
1	–		–	–	6	6
2	–		–	–	58	–
3[b]	**16**[s]		**16**	–	**16**	**16**
4	–		66	–	66	66
5	16[s]		–	16[rs]	–	–
6	45[r]		–	–	–	–
7	18[r], 39[r]		–			
8	39[r]		39	–	–	–
9	31[r]		–	–	–	–
10	–	51, 66	–	–	–	–
11	18[r], 35[r], 39[r], 52[r]		35	–	–	–
12	–	**35**, 53	**35**, 53	–	–	53
13	–	83	83	–	–	83
14	**51**[s], 66[s]	**51**, 66	–	–	–	–
15	–	58	–	–		–
16	–	54, 61	54, 61	–	–	54, 61
17	–	72	72	–	–	72
18	–	–	–	61[r]	–	–
19	–	**56**	**56**	–	–	**56**
20	–	–	62	–	62	62
21	61[r]	–	–	61[r]		–
22	16[s]	–	–	16[s]	–	–

Note: Bolded HPV types indicate that variant testing was completed on the woman-type. Cells shaded black indicate samples not collected; cells shaded gray indicate insufficient samples for HPV DNA testing.

[a]Only HPV types detected in oral or fingernail samples at either enrollment or exit are included.

[b]Different HPV-16 variants were detected in the oral and vaginal samples (the same variant was detected in the 2 vaginal samples); the fingernail sample tested negative for HPV-16 by PCR during variant sequencing.

[r]HPV DNA positive in oral rinse sample.

[s]HPV DNA positive in oral swab sample.

Table 3.3 Prevalence, incidence, and persistence of oral, fingernail, and vaginal HPV DNA among mid-adult women in Seattle, Washington, 2011-2012

	Oral[a]			Fingernail			Vaginal		
	N	*n*	*(%)*	*N*	*n*	*(%)*	*N*	*n*	*(%)*
Woman-level									
Prevalence at enrollment	408[b]			210[c]			408[d]		
Any HPV[e]		10[f]	(2.5)		8[g]	(3.8)		135[h]	(33.1)
High-risk HPV[i]		9	(2.2)		5	(2.4)		89	(21.8)
HPV-16		3	(0.7)		0	(0.0)		15	(3.7)
6-month cumulative incidence[j]	408	1[k]	(0.3)	210	1[l]	(0.5)	408	61[m]	(15.0)
Infection-level (type-specific)									
6-month persistence[n]	13	3[o]	(23.1)	9	0	(0.0)	249	167	(67.1)
6-month clearance	13	10	(76.9)	9	9	(100.0)	249	82	(32.9)

[a]Detection of HPV in either oral swab or rinse sample.
[b]Women with a sufficient oral sample either through swab or rinse were considered sufficient. One sample was insufficient on both oral rinse and oral swab; 10 additional samples were insufficient on oral rinse only.
[c]56 fingernail samples were insufficient; we did not add fingernail sampling to the study protocol until June 2011, therefore fingernail samples were not collected at enrollment for 139 women. There were 286 sufficient fingernail samples at exit, of which 5 samples tested positive for any HPV type, translating to a prevalence estimate of 1.8%.
[d]One vaginal sample was insufficient.
[e]Any of the following 37 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, CP6108, and IS-39.
[f]Represents 15 type-specific oral infections.
[g]Represents 12 type-specific fingernail infections.
[h]Represents 274 type-specific vaginal infections.
[i]Any of the following 19 high-risk HPV types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, and IS-39.
[j]Incident infection defined as testing HPV DNA positive for a HPV type not detected at enrollment.
[k]Represents 1 type-specific oral infection.
[l]Represents 1 type-specific fingernail infection.
[m]Represents 33 type-specific vaginal infections.
[n]Persistent infection defined as testing HPV DNA positive at the exit visit for the same HPV type detected at enrollment, among prevalent infections at enrollment with a sufficient exit sample for comparison.
[o]Persistent oral infections consisted of two HPV-16 and one HPV-61 infections.

Table 3.4 Concordance among oral, fingernail, and vaginal samples[a] for HPV DNA detection among mid-adult women in Seattle, Washington, 2011-2012

	No. of pairs[b]				PPA (%)[c]	Kappa[d]	(95% CI)[e]
	+ / +	+ / –	– /+	– / –			
Oral / vaginal	3	16	520	28,543	0.56	0.01	(0.00, 0.01)
Vaginal / fingernail	11	316	6	17,982	3.30	0.03	(0.01, 0.06)
Fingernail / oral	2	15	4	18,331	9.52	0.09	(0.00, 0.29)

[a]Enrollment and exit samples combined to calculated concordance between anatomic sites.
[b]Represents women * HPV types * number of samples.
[c]PPA is proportion positive agreement (number DNA-positive in both samples) / (number DNA-positive in either sample).
[d]Kappa is calculated by (observed PPA – expected PPA) / (1 – expected PPA).
[e]Confidence intervals estimated using percentile bootstrap methods with 1,000 repetitions to account for correlation due to multiple HPV types and multiple visits within women.

Table 3.5 Univariate analysis of factors associated with oral HPV detection among mid-adult women in Seattle, WA, 2011-2102, using GEE logistic regression[a]

	Oral HPV detection (N_{Total} = 29,156[b])				
Characteristics	*N*[c]	*n*[d]	*OR*	*(95% CI)*	*p-value*
Age (years)	29,137	19	1.04	(0.96, 1.14)	0.348
Marital status					
Unmarried or separated	10,767	7	1.00	--	--
Married or living with a partner	18,167	12	1.02	(0.24, 4.24)	0.983
Ever had a non-HPV related STD[e]					
No	23,347	15	1.00	--	--
Yes	5,809	4	1.07	(0.26, 4.38)	0.923
Ever had genital warts[f]					
No	25,659	19	1.00	--	--
Yes	3,330	0	0.00	(0.00, 1.65)	0.1970
Ever had an abnormal Pap test					
No	16,502	2	1.00	--	--
Yes	12,654	17	11.10	(2.35, 52.40)	0.002*
Ever had ≥ 1 dose of HPV vaccine[g]					
No	2,906	6	1.00	--	--
Yes	235	1	2.06	(0.23, 18.48)	0.518
Ever been pregnant					
No	12,062	5	1.00	--	--
Yes	16,909	14	2.00	(0.54, 7.41)	0.301
Currently using hormonal contraceptives[h]					
No	22,977	11	1.00	--	--
Yes	6,179	8	2.71	(0.72, 10.21)	0.142
Smoking status[i]					
Never	21,608	10	1.00	--	--
Former	6,105	7	2.48	(0.55, 11.23)	0.239
Current	1,295	2	3.34	(0.67, 16.55)	0.140
Currently consumes alcoholic beverages					
No	4,662	1	1.00	--	--
Yes	24,346	18	3.45	(0.44, 27.09)	0.239

Table 3.5 Univariate analysis of factors associated with oral HPV detection among mid-adult women in Seattle, WA, 2011-2102, using GEE logistic regression (cont.)

	Oral HPV detection (N_{Total} = 29,156[b])				
Characteristics	*N*[c]	*n*[d]	*OR*	*(95% CI)*	*p-value*
Type of vaginal sex partners (lifetime)					
Male only	22,088	16	1.00	--	--
Female only or both male and female	5,513	3	0.79	(0.16, 3.96)	0.770
Age at first sexual intercourse with a male partner (yrs)[j]	27,972	18	0.85	(0.71, 1.01)	0.071
Lifetime number of male sex partners[k]					
0 – 3	8,103	2	1.00	--	--
4 – 9	8,769	2	0.92	(0.23, 3.69)	0.911
10+	11,803	15	5.15	(1.07, 24.93)	0.041*
Lifetime number of open-mouth kissing partners[klm]					
0 – 3	10,249	8	1.00	--	--
4 – 19	8,991	1	0.14	(0.01, 1.37)	0.091
20+	9,916	10	1.29	(0.32, 5.26)	0.720
Lifetime number of oral sex partners[klm]					
0 – 1	10,656	9	1.00	--	--
2 – 6	9,028	4	0.52	(0.09, 2.94)	0.463
7+	9,472	6	0.75	(0.18, 3.21)	0.698
Type of oral sex partners[ln]					
Male only	17,353	8	1.00	--	--
Female only or both male and female	4,292	3	1.52	(0.28, 8.34)	0.632
Vaginal HPV infection of the same type					
No	28,559	16	1.00	--	--
Yes	523	3	10.29	(3.34, 31.72)	<0.001*
Vaginal HPV viral load of the same type (continuous)[op]	363	3	1.44	(0.83, 2.51)	0.200

*p-value < 0.05
[a]Both enrollment and exit samples were included.
[b]409 women contributed a total of 29,156 women-types to the oral HPV analysis.
[c]Indicates the total number of women * HPV types * samples.
[d]Indicates the number of women * HPV types * samples positive for oral HPV.
[e]Includes chlamydia, gonorrhea, genital herpes, and HIV.
[f]Analyzed using exact logistic regression due to 0 in cells; cannot account for correlation within women.
[g]Restricted to HPV types 6, 11, 16, and 18 included in the vaccine. One women who received 3 doses of the Gardasil HPV vaccine tested positive for HPV-18 on her oral rinse sample collected at enrollment
[h]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.
[i]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smokers reported currently smoking.
[j]Restricted to 393 women who reported ever having had sex with a male partner.
[k]Categorized by approximate tertiles.
[l]Restricted to 323 women who filled out either a modified exit survey or a supplemental exit survey with additional questions on potential risk factors for oral HPV infection.
[m]Included both male and female partners.
[n]Twelve women reported no history of oral sex.
[o]Restricted to 16 high-risk HPV types with available viral load results, including HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82.
[p]Restricted to samples with a vaginal infection of the same HPV type detected.

Table 3.6 Univariate analysis of factors associated with fingernail HPV detection among mid-adult women in Seattle, WA, 2011-2102, using GEE logistic regression[a]

	Fingernail HPV detection (N_{Total} = 18,352[b])				
Characteristics	*N*[c]	*n*[d]	*OR*	*(95% CI)*	*p-value*
Age (years)	18,335	17	1.01	(0.92, 1.10)	0.831
Marital status					
Unmarried or separated	6,697	6	1.00	--	--
Married or living with a partner	11,544	11	1.06	(0.33, 3.40)	0.917
Ever had a non-HPV related STD[e]					
No	14,615	9	1.00	--	--
Yes	3,737	8	3.48	(1.12, 10.78)	0.030*
Ever had genital warts[f]					
No	16,078	17	1.00	--	--
Yes	2,183	0	0.00	(0.00, 1.65)	0.197
Ever had an abnormal Pap test					
No	9,879	8	1.00	--	--
Yes	8,473	9	1.31	(0.42, 4.09)	0.640
Ever been pregnant					
No	7,326	6	1.00	--	--
Yes	10,952	11	1.23	(0.41, 3.69)	0.717
Currently using hormonal contraceptives[g]					
No	14,615	14	1.00	--	--
Yes	3,737	3	0.84	(0.17, 4.08)	0.827
Smoking status[h]					
Never	13,727	13	1.00	--	--
Former	3,700	4	1.14	(0.35, 3.67)	0.824
Current	851	0			
Currently consumes alcoholic beverages					
No	3,071	1	1.00	--	--
Yes	15,207	16	3.23	(0.43, 24.41)	0.255

Table 3.6 Univariate analysis of factors associated with fingernail HPV detection among mid-adult women in Seattle, WA, 2011-2102, using GEE logistic regression (cont.)

	Fingernail HPV detection (N_{Total} = 18,352[b])				
Characteristics	*N*[c]	*n*[d]	*OR*	*(95% CI)*	*p-value*
Type of vaginal sex partners (lifetime)					
Male only	14,652	15	1.00	--	--
Female only or both male and female	3,330	2	0.59	(0.13, 2.63)	0.485
Age at first sexual intercourse with a male partner (yrs)[i]	17,707	16	0.94	(0.83, 1.06)	0.299
Lifetime number of male sex partners[j]					
0 – 3	4,921	2	1.00	--	--
4 – 9	5,624	4	1.75	(0.28, 10.78)	0.546
10+	7,548	11	3.59	(0.76, 17.03)	0.108
Lifetime number of open-mouth kissing partners[jkl]					
0 – 3	5,920	5	1.00	--	--
4 – 19	5,550	2	0.43	(0.08, 2.38)	0.331
20+	6,882	10	1.72	(0.48, 6.19)	0.405
Lifetime number of oral sex partners[jkl]					
0 – 1	5,957	4	1.00	--	--
2 – 6	6,068	5	1.23	(0.26, 5.86)	0.797
7+	6,327	8	1.88	(0.44, 8.04)	0.392
Type of oral sex partners[lm]					
Male only	11,914	12	1.00	--	--
Female only or both male and female	2,553	1	0.39	(0.05, 3.09)	0.372
Vaginal HPV infection of the same type					
No	17,988	6	1.00	--	--
Yes	327	11	104.33	(30.44, 357.5)	<0.001*
Vaginal HPV viral load of the same type (continuous)[no]	221	5	2.35	(1.42, 3.87)	0.001*

*p-value < 0.05
[a]Both enrollment and exit samples were included.
[b]342 women contributed a total of 18,352 women-types to the fingernail HPV analysis.
[c]Indicates the total number of women * HPV types * samples.
[d]Indicates the number of women * HPV types * samples positive for fingernail HPV.
[e]Includes chlamydia, gonorrhea, genital herpes, and HIV.
[f]Analyzed using exact logistic regression due to 0 in cells; cannot account for correlation within women.[g]Includes birth control pills, hormonal patches, vaginal rings, implanted contraception, injectable contraception, and hormonal intrauterine devices.
[h]Smoking was defined as smoking at least one cigarette a day for one month or longer; former smokers reported ever smoking but not currently smoking, and current smokers reported currently smoking.
[i]Restricted to 393 women who reported ever having had sex with a male partner.
[j]Categorized by approximate tertiles.
[k]Restricted to 323 women who filled out either a modified exit survey or a supplemental exit survey with additional questions on potential risk factors for oral HPV infection.
[l]Included both male and female partners.
[m]Twelve women reported no history of oral sex.
[n]Restricted to 16 high-risk HPV types with available viral load results, including HPV-16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82.
[o]Restricted to samples with a vaginal infection of the same HPV type detected.

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