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# Viral load of human papillomavirus types 16/18/31/33/45 as a predictor of cervical intraepithelial neoplasia and cancer by age

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## HIGHLIGHTS

- In HPV16/18/31 positive women, higher viral loads predict precancerous lesions.
- The largest viral load difference is between cytology normal and low-grade precancer.
- The viral load has a lower diagnostic accuracy than other HPV triage tests.
- The viral load has better diagnostic accuracy in women aged 30 y and over.

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## ABSTRACT

*Objective:* We assessed whether human papillomavirus (HPV) viral load is an independent predictor of underlying cervical disease and its diagnostic accuracy by age.

*Methods:* The Biomarkers of Cervical Cancer Risk study was a case-control study from 2001 to 2010 in Montréal, Canada. Cases were histologically-confirmed cervical intraepithelial neoplasia (CIN), adenocarcinoma in situ (AIS), or cervical cancer cases. Controls were women presenting for routine screening with normal cytology results. We quantified HPV16/18/31/33/45 viral load from exfoliated cervical cells using a real-time PCR assay. Diagnostic accuracy of viral load was assessed using the area under the receiver operating characteristic curve (AUC). We restricted the analysis to the 632 cases and controls who were HPV16/18/31/33/45 positive.

*Results:* Geometric mean HPV16/18/31/33/45 viral load increased with severity of lesion grade, ranging from 0.7, 3.1, 4.8, 7.2, and 12.4 copies/cell in normal, CIN1, CIN2, CIN3&AIS, and cervical cancer respectively. The adjusted odds ratio of CIN1+ and CIN2+ increased respectively by 1.3 (95%CI 1.1−1.4) and 1.2 (95%CI 1.1−1.3) per log-transformed viral copy/cell increase of HPV16/18/31/33/45. This association was mainly driven by HPV16, 18, and 31 viral loads. The AUC of HPV16/18/31/33/45 viral load for discriminating between normal and CIN1+ women was 0.70 (95%CI 0.64−0.76) in HPV-positive women, and was 0.76 (95%CI 0.66−0.86) for women ≥30 years and 0.66 (95%CI 0.58−0.74) for women under 30 years.

*Conclusions:* HPV viral load has lower diagnostic accuracy than has been reported for other HPV screening triage tests. However, it may be useful for triaging HPV tests in settings without cytology results such as HPV self-sampling.



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## 1. Introduction

Infection with human papillomavirus (HPV) is a necessary cause of cervical cancer [1]. Many countries worldwide have now adopted HPV testing for primary cervical cancer screening [2,3]. Clinical trials have shown that screening with HPV tests has higher sensitivity for detecting high-grade lesions than cytology tests [4]. However, many HPV-positive women do not have underlying cervical lesions. Triage of HPV-positive women with a second test is needed in order not to overwhelm colposcopy services and reduce harms from over-referral. Potential triage tests include cytology, HPV genotyping, p16/Ki-67 dual staining, methylation makers, and HPV E6 protein assays [5]. Viral load is another potential biomarker that could be used for triaging of HPV-positive screen tests, though it has received relatively less attention than other triage biomarkers [6–8]. This may in part be because the association between viral load and lesion grade has been inconsistent, and the methods for measuring viral load have varied across studies [7–16]. However, because measuring HPV viral load does not require cytology, it may be useful for triage in the context of triaging of self-sampled HPV tests in hard to reach populations. The diagnostic ability of screening and triage tests could also vary by age [17]. The diagnostic accuracy by age is important to consider because in some countries primary HPV screening may be restricted to women aged 30 years and over [2,3], while in others HPV testing is also done in women under 30 [18].

The objective of this analysis was to assess whether the HPV viral load is an independent predictor of underlying cervical disease and its diagnostic accuracy in HPV-positive women by age.

## 2. Methods

### 2.1. Study design and population

The Biomarkers of Cervical Cancer Risk (BCCR) study was a case-control study designed to investigate the role of biomarkers as predictors of cervical intraepithelial neoplasia (CIN) and cancer. Details of the study and its methods have previously been published [19–21]. Recruitment occurred between February 2001 and November 2010 in Montréal, Canada. Women were excluded from the study if they were pregnant, had a history of cervical disease or cancer other than non-melanoma skin cancer, or had undergone a previous hysterectomy or conization.

Cases included women referred on the basis of a Pap result of high-grade squamous intraepithelial neoplasia (HSIL) or atypical squamous cells of undetermined significance (ASCUS) to one of five colposcopy clinics of the collaborating hospitals of the McGill University Health Centre (MUHC) and the Centre Hospitalier de l'Université de Montréal (CHUM). Only women who underwent a colposcopy-guided cervical biopsy were invited to participate as cases in the study. Women recruited as cases remained in the case series if they had a histologically confirmed CIN 1, 2, 3, adenocarcinoma in situ (AIS) or cervical cancer (invasive adenocarcinoma and squamous cell).

Controls were recruited during the same period from among women presenting for a routine cytology screening test at family medicine and gynecology clinics that typically refer women to the collaborating hospitals. Controls were eligible if their enrollment cytology test was within normal limits (WNL) or consistent with benign cellular changes (BCC) and reported no history of cervical abnormalities. Controls were eligible to become cases if, during the study period, they had abnormal test results and were referred to a participating colposcopy clinic and met the case definition.

Cases and controls were frequency-matched by age. Participants filled out a self-administered questionnaire on their demographic and behavioral characteristics. The questionnaire did not ask whether women were vaccinated because the study was implemented before HPV vaccine licensure in Canada in 2006. However, it is unlikely many of the participants recruited after 2006 would have received the HPV vaccine as the vaccine at the time was only approved for women 26 and under and represented a significant out-of-pocket expense. All participants provided written informed consent. The BCCR study protocol was approved by the local research ethics boards of all participating hospitals, the McGill Institutional Review Board, and the Comité d'éthique de la recherche du CHUM.

### 2.2. HPV typing, viral load, and cellular quantification

Exfoliated cervical cells were collected for HPV testing by the study nurses using an Accelon biosample (Medscand, Inc., Hollywood, FL) and re-suspended in Preservcyt (Hologic, Marlborough, MA). Extracted DNA was amplified for HPV detection using L1 consensus primers PGMY09/11 and typed with the Linear array assay (Roche Molecular Systems, CA). Samples were screened for the presence of PCR inhibitors by amplification of internal controls for HPV16, 18, 31, 33, 45, and β-globin DNA, as described previously [22,23]. The presence of PCR inhibitors was suspected if 1000 copies of at least one internal control generated a signal corresponding to <700 copies [24]. All samples were free of inhibitors. Two μL of the processed sample was tested in duplicate for quantification of β-globin DNA to estimate the cell content of samples [22,23]. HPV16 E6 and HPV18 E7 DNA were quantified using a standard protocol [25]. HPV31 L1 DNA was measured with the assay described by Weissenborn et al. [26] HPV33 and HPV-45 E6 DNA was amplified in a Light Cycler PCR and detection system (Roche Molecular Systems, CA) in a 20-μL reaction mixture containing 1× DNA Master Hybridization Probe Mix with the Fast Start Taq DNA polymerase (Roche Molecular Biochemicals), 0.05 μM of fluorogenic probe labeled with FAM and TAMRA (33-E6-TM [5′-ACCACGAA-CATTGCATGATTTGTG-3′] or 45 E6-TM nucleotide position 491–514 [5′ AGCTGGACAGTACCGAGGGCAGTG-3′]), and 0.3 pmoles of each primer (33-E6-F [5′-TACTGCACGACTATGTTTCAAG-3′] and 33-E6-R [5′-TCTTGAGGACACAAAGGTCTT-3′], or 45-E6-F nucleotide position 463–486 [5′-TTAAGGACAAACGAAGATTTCACA-3′] and 45-E6-R nucleotide position 670–647 [5′-ACACAACAGGTCAACAGGATCTAA-3′]) [27]. Cycling parameters for quantitation of HPV45 included an activation step at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s, 60 °C for 5 s and 65 °C for 45 s. Cycling parameters for quantitation of HPV33 were as described previously [27]. For each of the HPV genotypes, cycle thresholds obtained for each sample were compared to those of a titration curve obtained by serial ten-fold dilutions of HPV16, 18, 31, 33 or 45 plasmids in a fixed amount of 75 ng of human genomic DNA (Roche Diagnostics) in 10 mM Tris-HCl [pH 8.2].

### 2.3. Statistical analysis

All analyses were restricted to cases and control women who were positive for HPV16, 18, 31, 33, or 45, and had valid viral load and β-globin results. The unit of analysis was the type-specific HPV infection. Analyses were performed using SAS 9.4 and R 3.5.3.

HPV viral loads were calculated as the number of HPV DNA copies per human cell (per two copies of β-globin DNA). We calculated the log-transformed viral loads and the geometric mean viral loads by HPV type, histological diagnosis, and by women's

**Table 1**
Characteristics of HPV16/18/31/33/45 positive women, their geometric mean viral load (copies per cell), and geometric mean viral load ratios, predicted by GEE linear regression models.

| Characteristic | Number HPV16/18/31/33/45 positive | | | | | | Geometric mean viral load (95% CI) | | Geometric mean viral load ratio (95% CI) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Controls | (%) | Cases | (%) | Total | (%) | | | | |
| All HPV positive | 73 | (100%) | 559 | (100%) | 632 | (100%) | 5.6 | (4.4–7.2) | | |
| Diagnosis | | | | | | | | | | |
| Normal | 73 | (100%) | – | – | 73 | (12%) | 0.7 | (0.3–1.5) | 1.0 | (ref) |
| CIN1 | – | – | 32 | (6%) | 32 | (5%) | 3.1 | (1.2–8.3) | 4.7 | (1.5–15.0) |
| CIN2 | – | – | 131 | (23%) | 131 | (21%) | 4.8 | (2.7–8.5) | 6.8 | (2.7–17.3) |
| CIN3/AIS | – | – | 232 | (42%) | 232 | (37%) | 7.2 | (5.1–10.1) | 10.1 | (4.5–22.7) |
| Cancer | – | – | 164 | (29%) | 164 | (26%) | 12.4 | (7.4–20.7) | 17.7 | (7.3–43.0) |
| HPV type | | | | | | | | | | |
| HPV16 | 38 | (52%)[a] | 385 | (69%)[a] | 423 | (67%)[a] | 10.4 | (7.6–14.2) | 13.7 | (5.7–32.8) |
| HPV18 | 13 | (18%)[a] | 79 | (14%)[a] | 92 | (15%)[a] | 2.4 | (1.2–4.5) | 3.2 | (1.1–9.2) |
| HPV31 | 14 | (19%)[a] | 67 | (12%)[a] | 81 | (13%)[a] | 1.5 | (0.8–2.8) | 2.0 | (0.7–5.7) |
| HPV33 | 4 | (5%)[a] | 42 | (8%)[a] | 46 | (7%)[a] | 7.5 | (2.9–19.5) | 9.8 | (2.9–33.4) |
| HPV45 | 7 | (10%)[a] | 37 | (7%)[a] | 44 | (7%)[a] | 0.7 | (0.3–1.7) | 1.0 | (ref) |
| Number of infecting types | | | | | | | | | | |
| Single infection | 28 | (38%) | 320 | (57%) | 348 | (55%) | 8.0 | (5.7–11.2) | 2.0 | (1.3–3.3) |
| Coinfection[b] | 45 | (62%) | 239 | (43%) | 284 | (45%) | 3.9 | (2.7–5.5) | 1.0 | (ref) |
| Age | | | | | | | | | | |
| <30 y | 51 | (70%) | 188 | (34%) | 239 | (38%) | 4.0 | (2.7–5.9) | 1.0 | (ref) |
| ≥30 y | 22 | (30%) | 366 | (65%) | 388 | (61%) | 6.9 | (5.0–9.5) | 1.7 | (1.1–2.9) |
| Missing | – | – | 5 | (1%) | 5 | (1%) | 18.0 | (0.2–1879.7) | | |
| Current smoker | | | | | | | | | | |
| Yes | 27 | (37%) | 213 | (38%) | 240 | (38%) | 6.5 | (4.4–9.6) | 1.2 | (0.8–2.1) |
| No | 45 | (62%) | 331 | (59%) | 376 | (59%) | 5.1 | (3.7–7.1) | 1.0 | (ref) |
| Missing | 1 | (1%) | 15 | (3%) | 16 | (3%) | 4.4 | (0.9–22.2) | | |
| Number of live births | | | | | | | | | | |
| 0 | 54 | (74%) | 253 | (45%) | 307 | (49%) | 4.8 | (3.4–6.8) | 1.0 | (ref) |
| 1 | 5 | (7%) | 106 | (19%) | 111 | (18%) | 8.7 | (4.9–15.3) | 1.9 | (0.9–3.7) |
| 2+ | 12 | (16%) | 183 | (33%) | 195 | (31%) | 5.4 | (3.4–8.6) | 1.2 | (0.7–2.1) |
| Missing | 2 | (3%) | 17 | (3%) | 19 | (3%) | 7.9 | (1.4–44.1) | | |
| Current oral contraceptive use | | | | | | | | | | |
| Regularly | 41 | (56%) | 174 | (31%) | 215 | (34%) | 3.3 | (2.2–4.9) | 0.5 | (0.3–0.8) |
| Sometimes | 5 | (7%) | 20 | (4%) | 25 | (4%) | 9.4 | (2.2–40.1) | 1.3 | (0.3–4.7) |
| Never | 25 | (34%) | 346 | (62%) | 371 | (59%) | 7.2 | (5.2–9.9) | 1.0 | (ref) |
| Missing | 2 | (3%) | 19 | (3%) | 21 | (3%) | 11.6 | (2.7–49.4) | | |
| Current condom use | | | | | | | | | | |
| Regularly | 15 | (21%) | 92 | (16%) | 107 | (17%) | 3.5 | (1.9–6.4) | 0.5 | (0.3–1.0) |
| Sometimes | 18 | (25%) | 99 | (18%) | 117 | (19%) | 5.0 | (2.9–8.5) | 0.8 | (0.4–1.5) |
| Never | 35 | (48%) | 342 | (61%) | 377 | (60%) | 6.8 | (4.9–9.3) | 1.0 | (ref) |
| Missing | 5 | (7%) | 26 | (5%) | 31 | (5%) | 4.9 | (1.5–15.8) | | |
| Lifetime number of Pap tests | | | | | | | | | | |
| 1 | 5 | (7%) | 34 | (6%) | 39 | (6%) | 24.5 | (9.6–63.0) | 4.8 | (1.7–13.6) |
| 2–3 | 13 | (18%) | 83 | (15%) | 96 | (15%) | 7.6 | (3.9–15.0) | 1.7 | (0.8–3.6) |
| 4–5 | 18 | (25%) | 80 | (14%) | 98 | (16%) | 5.4 | (2.8–10.5) | 1.2 | (0.6–2.5) |
| 6–10 | 13 | (18%) | 122 | (22%) | 135 | (21%) | 3.9 | (2.4–6.4) | 0.9 | (0.5–1.7) |
| >10 | 21 | (29%) | 222 | (40%) | 243 | (38%) | 4.3 | (2.9–6.4) | 1.0 | (ref) |
| Missing | 3 | (4%) | 18 | (3%) | 21 | (3%) | 17.8 | (4.7–67.6) | | |
| Ever diagnosed with chlamydia[c] | | | | | | | | | | |
| Yes | 8 | (11%) | 73 | (13%) | 81 | (13%) | 4.2 | (2.2–7.9) | 0.7 | (0.4–1.5) |
| No | 59 | (81%) | 438 | (78%) | 497 | (79%) | 5.6 | (4.2–7.4) | 1.0 | (ref) |
| Don't know/missing | 6 | (8%) | 48 | (9%) | 54 | (9%) | 9.2 | (4.3–19.6) | 1.6 | (0.7–3.4) |
| Ever diagnosed with a yeast infection[c] | | | | | | | | | | |
| Yes | 34 | (47%) | 227 | (41%) | 261 | (41%) | 3.7 | (2.5–5.4) | 0.6 | (0.4–1.0) |
| No | 30 | (41%) | 260 | (47%) | 290 | (46%) | 6.1 | (4.2–8.8) | 1.0 | (ref) |
| Don't know/missing | 9 | (12%) | 72 | (13%) | 81 | (13%) | 17.2 | (9.5–31.3) | 2.8 | (1.4–5.5) |
| Number of male sex partners in last year | | | | | | | | | | |
| 0 | 2 | (3%) | 67 | (12%) | 69 | (11%) | 11.7 | (4.8–28.9) | 1.0 | (ref) |
| 1 | 36 | (49%) | 362 | (65%) | 398 | (63%) | 5.6 | (4.2–7.5) | 0.5 | (0.2–1.1) |
| 2+ | 31 | (42%) | 106 | (19%) | 137 | (22%) | 4.3 | (2.5–7.1) | 0.4 | (0.1–0.9) |
| Missing | 4 | (5%) | 24 | (4%) | 28 | (4%) | 4.1 | (0.8–20.5) | | |

CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; OR = odds ratio, STI = sexually transmitted infection.
[a] Sum is >100% due to coinfections.
[b] Women with >1 infection with any of the 36 HPV types tested for in the Linear array assay.
[c] Self-reported.

self-reported characteristics to identify potential variables associated with HPV viral load. We selected the following variables to examine a priori as potential confounders: selection factors (diagnosis, age), risk factors for cervical cancer (Pap test frequency, HPV type, number of recent sex partners, number of live births, smoking), and hormonal, sexual, and microbial factors which we hypothesized might influence immune response and viral load (current oral contraceptive and condom use, ever had a yeast infection, and ever had a STI diagnosis). Ratios of geometric mean titers were calculated using linear regression models, with the log-transformed viral load as the outcome. The ratios of geometric mean viral loads between predictor categories were calculated

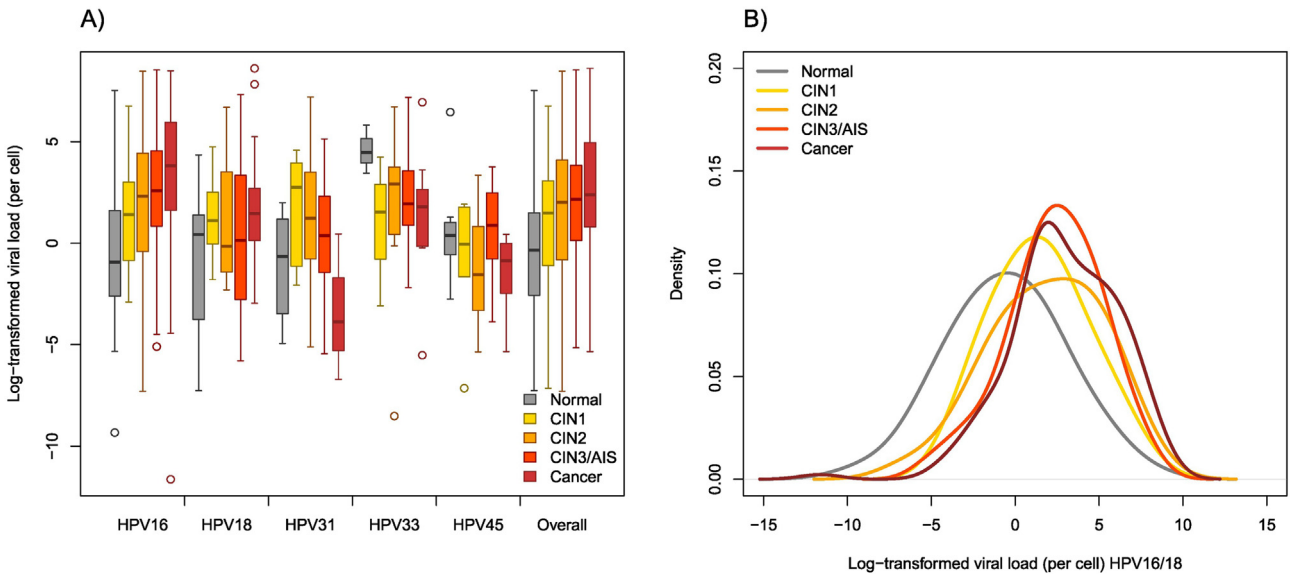


**Fig. 1.** Distribution of log-transformed viral load stratified by HPV type and diagnosis for A) all HPV types and B) HPV16/18. A Gaussian kernel function was used to smooth viral load density in B).

using the exponents of the coefficients from the regression model. We used a generalized estimating equations (GEE) approach to account for correlated data from multiple observations per woman due to coinfections with more than one HPV type. We used an exchangeable working correlation matrix between observations from the same woman, based on the assumption that outcomes with different HPV types would share a similar correlation.

We analyzed whether cervical disease diagnosis was associated with log-transformed type-specific viral loads using logistic and ordinal multinomial regression models using a GEE approach. Logistic regressions modeled the odds of being CIN1+ vs. normal (case vs. control) and of being CIN2+ vs. <CIN2. Multinomial regressions modeled the cumulative odds of being in a more severe vs. a less severe diagnosis category, the decreasing order of severity being Cancer > CIN3&AIS > CIN2 > CIN1 > Normal. Viral load was modeled as a log-transformed continuous exposure. All models were adjusted for age (continuous variable) and HPV type, in order to assess the predictive power of viral load independently of the carcinogenicity of the infecting type. We allowed the viral load association to vary by HPV type using a model interaction term. We assessed whether the association between diagnosis and viral load was due to confounding in multivariable models, including variables above which were either associated with viral load or with cervical disease diagnosis. We also fitted separate models by age (<30 y and ≥30 y) to assess whether the association is modified by age. The diagnostic accuracy of the log-transformed viral load in HPV-positive women was assessed using the area under the receiver operating characteristic (ROC) curve (AUC).

### 2.4. Multiple imputation of missing data

Many potential confounder variables had missing data due to partially filled-out questionnaires. A complete case analysis entailed a loss of 10% of observations due to missing data in multivariable analyses. The analysis of missing data profiles did not reveal any particular pattern, suggesting the missing data is due to arbitrary non-response to some questions. There were sufficient answers for related questionnaire items to assume the data were missing at random conditional on observed data. To keep these women in the analysis, we used multiple imputation to impute missing values for age, current smoking status, number of live births, number of partners in the past year, current contraceptive use, condom use, pap test frequency, and hormone use. We performed 10 imputations using a fully conditional specification (FCS) method [28]. The imputation model for missing values included all imputed variables as well as histological diagnosis, marital status, socioeconomic status, smoking history, pregnancy history, vaginal sex frequency, lifetime contraceptive use, and the number of lifetime male partners. Because results were nearly identical between the complete case analysis and the multiple imputation analysis, we only present regression model results using multiple imputed datasets.

## 3. Results

Of the 1611 women (766 cases and 845 controls) recruited into the BCCR study, there were 635 who were positive for HPV16/18/31/33/45 by Linear Array, of which 632 had valid viral load results. These 632 women had 684 type-specific infections with HPV16, 18, 31, 33, or 45. Most of these women were cases (88%), with only 73 (12%) normal control women due to the much higher HPV prevalence in cases than in controls. The sociodemographic and behavioral characteristics of these women are presented in Table 1. The average age of HPV16/18/31/33/45 positive women was 37 years old (SD 12) for cases and 31 years old (SD 9) for controls. The restriction to HPV16/18/31/33/45 positive women led to a younger age distribution in controls than in cases.

Viral load varied by HPV type and by diagnosis (Table 1, Fig. 1). The geometric mean viral load was highest for HPV16 at 10.4 (95%CI 7.6–14.2) copies per cell, and lowest for HPV45 at 0.7 (95%CI 0.3–1.7) copies per cell. HPV16 infections had a 13.7 times higher (95%CI 5.7–32.8) geometric mean viral load than HPV45 infections. The geometric mean viral load was highest in cancer cases at 12.4 (95%CI 7.4–20.7) copies per cell, and lowest in normal women at 0.7 (95%CI 0.3–1.5) copies per cell. Cancer cases had a 17.7 times higher (95%CI 7.3–43.0) geometric mean viral load than normal women. Viral load was also associated with coinfection status, current contraceptive use, lifetime Pap test frequency, knowledge of ever having a yeast infection diagnosis, and the number of male partners in the last year.

**Table 2**
Odds of having a more severe diagnosis per log-transformed viral load unit increase in HPV16/18/31/33/45 positive women, predicted by logistic and multinomial regression models.

| HPV type | Diagnosis | | | | | | | | | | Logistic regression (CIN1+ vs Normal) | | | Logistic regression (CIN2+ vs < CIN2) | | | Multinomial regression (Cancer > CIN3& AIS > CIN2 > CIN1 > Normal) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | | CIN1 | | CIN2 | | CIN3/AIS | | Cancer | | | | | | | | | | | |
| | N | (%) | N | (%) | N | (%) | N | (%) | N | (%) | OR | 95% CI | Per log viral load increase (adjusted)[b] | OR | 95% CI | Per log viral load increase (adjusted)[b] | OR | 95% CI | Per log viral load increase[a] | Per log viral load increase (adjusted)[b] |
| HPV16 | 38 | (9%) | 15 | (4%) | 86 | (20%) | 176 | (42%) | 108 | (26%) | 1.3 | (1.2–1.5) | 1.4 | (1.2–1.5) | 1.3 | (1.1–1.4) | 1.3 | (1.2–1.4) | 1.2 | (1.1–1.2) | 1.2 | (1.1–1.2) |
| HPV18 | 13 | (14%) | 6 | (7%) | 12 | (13%) | 21 | (23%) | 40 | (43%) | 1.2 | (1.0–1.5) | 1.2 | (1.0–1.4) | 1.1 | (1.0–1.3) | 1.1 | (0.9–1.3) | 1.2 | (1.0–1.4) | 1.2 | (1.1–1.4) |
| HPV31 | 14 | (17%) | 8 | (10%) | 22 | (27%) | 34 | (42%) | 3 | (4%) | 1.4 | (1.1–1.8) | 1.4 | (1.1–1.9) | 1.1 | (0.9–1.4) | 1.1 | (0.9–1.4) | 1.0 | (0.9–1.1) | 1.0 | (0.8–1.1) |
| HPV33 | 4 | (9%) | 3 | (7%) | 11 | (25%) | 17 | (39%) | 9 | (20%) | 0.6 | (0.3–1.2) | 0.6 | (0.3–1.3) | 0.9 | (0.7–1.2) | 0.9 | (0.7–1.2) | 0.9 | (0.7–1.1) | 0.9 | (0.7–1.1) |
| HPV45 | 7 | (16%) | 5 | (11%) | 9 | (20%) | 16 | (36%) | 7 | (16%) | 0.9 | (0.7–1.3) | 0.9 | (0.7–1.3) | 1.0 | (0.8–1.3) | 1.0 | (0.8–1.4) | 1.1 | (0.9–1.3) | 1.1 | (0.9–1.3) |
| HPV16/18/31/33/45 | 76 | (11%) | 37 | (5%) | 140 | (20%) | 264 | (39%) | 167 | (24%) | 1.2 | (1.1–1.4) | 1.3 | (1.1–1.4) | 1.2 | (1.1–1.3) | 1.2 | (1.1–1.3) | 1.1 | (1.1–1.2) | 1.2 | (1.1–1.3) |

CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; OR = odds ratio, STI = sexually transmitted infection.
[a] Model adjusting only for HPV type and age.
[b] Model adjusting for HPV type and potential confounders (age, coinfection with other HPV types, number of live births, number of lifetime sex partners, number of partners in the last year, current contraceptive use, current condom use, previous STI diagnosis, previous yeast infection diagnosis, and lifetime pap test frequency).

Each log-transformed viral copy per cell increase in an HPV16/18/31/33/45 positive woman was associated with a 1.2 (95%CI 1.1–1.3) times higher odds of a CIN1+ histology diagnosis after adjustment for age and HPV type (Table 2). This association was mainly driven by HPV16, 18, and 31, for which a higher viral load was associated with higher odds of a CIN1+ diagnosis, while HPV33 and 45 viral loads were not associated with higher odds of a CIN1+ diagnosis (Fig. 1A, Table 2). Similar associations with viral load were observed for the odds of a CIN2+ diagnosis. Each log-transformed viral copy per cell increase in an HPV-positive woman was associated with a 1.1 (95%CI: 1.1–1.2) times higher odds of having a more severe diagnosis vs. a less severe diagnosis in multinomial regression. This association was again mainly due to HPV16 and 18, for which a higher viral load was associated with a higher odds of a more severe diagnosis, while HPV31, 33, and 45 viral loads were not associated with higher odds of a more severe diagnosis across all diagnosis categories (Fig. 1A, Table 2).

While the above odds ratio may appear small, this was due to modeling the association on the log scale. The same associations for specific viral load threshold values are shown in Table 3 for all HPV types combined and in Table 4 for HPV16. For example, a woman with a viral load of 100 copies per cell had 6.9 (95%CI: 3.3–14.8) times higher odds of being CIN1+ than a woman with a viral load of 0.1 copies per cell after adjusting for age and HPV type. This association with viral load was modified by age (Table 3). Women under 30 years with a viral load of 100 copies per cell had 4.7 (95%CI 1.9–11.5) times higher odds of being CIN1+ than a woman with a viral load of 0.1 copies per cell, while women 30 years and over had 14.0 (95%CI 3.5–55.5) times higher odds of being CIN1+, after adjusting for age and HPV type. Adjustment for potential confounders slightly increased the magnitude of the association between viral load and diagnosis in most cases. The logistic regression models in general predicted larger odds ratios than the ordinal multinomial regression model. This is because the largest difference in viral load occurred between normal and CIN1+ women, while differences in viral load between CIN grades were more modest (Fig. 1).

The AUC for discriminating between normal and CIN1+ women was 0.70 (95%CI 0.64–0.76) for all HPV types combined, and was 0.74 (95%CI 0.67–0.82) for HPV16/18. The AUC for discriminating between <CIN2 and CIN2+ women was 0.66 (95%CI 0.61–0.72) for all HPV types combined, and was 0.70 (95%CI 0.64–0.77) for HPV16/18. For all HPV types combined, the AUC was higher for women ≥30 y at 0.76 (95%CI 0.66–0.86) than for women <30 y at 0.66 (95%CI 0.58–0.74) to discriminate CIN1+, and also higher for women ≥30 y at 0.70 (95%CI 0.62–0.78) than for women <30 y at 0.63 (95%CI 0.55–0.70) to discriminate CIN2+ (Fig. 2). The differences in AUC between age groups were however not statistically significant (Fig. 2).

## 4. Discussion

In this study, we assessed whether the HPV viral load was associated with concurrent cervical lesion diagnoses, conditional on a woman being type-specific HPV-positive. We found a dose-response relationship, where higher HPV16/18/31 viral load was associated with a higher likelihood of being diagnosed with CIN and cancer. While HPV33 and 45 viral loads were not associated with diagnosis, this might be due to the low number of observations with these HPV types since confidence intervals overlapped with estimates for HPV16/18/31. The association between viral load and higher cervical lesion grade was independent of lifestyle factors potentially affecting immunity, infection, and cancer risk. Despite a strong association, the diagnostic accuracy of viral load for distinguishing normal from abnormal HPV-positive women was

**Table 3**
Odds of having a more severe diagnosis by viral load threshold in HPV16/18/31/33/45 positive women, predicted by logistic and multinomial regression models.

| Age | Viral load threshold (copies/cell) | Above threshold (%)[a] | | | | Logistic regression (CIN1+ vs Normal) | | | Logistic regression (CIN2+ vs <CIN2) | | | Multinomial regression (Cancer > CIN3&AIS > CIN2 > CIN1 > Normal) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Normal[b] | CIN1+[c] | CIN2+[c] | CIN3+[c] | OR[d] | 95% CI | OR[e] | OR[d] | 95% CI | OR[e] | OR[d] | 95% CI | OR[e] |
| All | 0.01 | 88% | 97% | 97% | 98% | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 |
| | 0.1 | 72% | 91% | 91% | 92% | 1.6 | (1.3–2.0) | 1.7 | 1.4 | (1.2–1.7) | 1.5 | 1.3 | (1.2–1.5) | 1.5 |
| | 1.0 | 46% | 74% | 75% | 78% | 2.6 | (1.8–3.9) | 2.8 | 2.1 | (1.5–2.9) | 2.2 | 1.7 | (1.4–2.1) | 2.1 |
| | 10.0 | 16% | 47% | 47% | 48% | 4.3 | (2.4–7.6) | 4.8 | 3.0 | (1.9–4.8) | 3.2 | 2.2 | (1.6–3.1) | 3.1 |
| | 100.0 | 8% | 22% | 22% | 23% | 6.9 | (3.3–14.8) | 8.1 | 4.3 | (2.3–8.1) | 4.6 | 2.9 | (1.9–4.5) | 4.5 |
| | 1000.0 | 1% | 5% | 5% | 5% | 11.3 | (4.4–29.1) | 13.6 | 6.2 | (2.8–13.7) | 6.8 | 3.8 | (2.2–6.5) | 6.5 |
| <30 y | 0.01 | 91% | 96% | 97% | 97% | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 |
| | 0.1 | 74% | 90% | 90% | 93% | 1.5 | (1.2–1.8) | 1.6 | 1.3 | (1.1–1.6) | 1.4 | 1.4 | (1.2–1.7) | 1.2 |
| | 1.0 | 46% | 70% | 70% | 77% | 2.2 | (1.4–3.4) | 2.5 | 1.8 | (1.2–2.7) | 2.0 | 2.1 | (1.5–2.9) | 1.4 |
| | 10.0 | 19% | 45% | 45% | 48% | 3.2 | (1.6–6.3) | 3.9 | 2.4 | (1.3–4.4) | 2.7 | 3.0 | (1.8–4.9) | 1.7 |
| | 100.0 | 9% | 20% | 20% | 23% | 4.7 | (1.9–11.5) | 6.1 | 3.3 | (1.5–7.3) | 3.8 | 4.2 | (2.2–8.2) | 2.0 |
| | 1000.0 | 2% | 5% | 5% | 7% | 6.9 | (2.2–21.3) | 9.5 | 4.4 | (1.6–12.0) | 5.4 | 6.1 | (2.7–13.9) | 2.4 |
| ≥30 y | 0.01 | 82% | 98% | 98% | 98% | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 | 1.0 | (ref) | 1.0 |
| | 0.1 | 68% | 91% | 91% | 92% | 1.9 | (1.4–2.7) | 2.1 | 1.6 | (1.2–2.1) | 1.6 | 1.2 | (1.1–1.4) | 1.3 |
| | 1.0 | 45% | 76% | 77% | 78% | 3.7 | (1.9–7.4) | 4.3 | 2.5 | (1.5–4.3) | 2.5 | 1.5 | (1.1–2.0) | 1.7 |
| | 10.0 | 9% | 48% | 49% | 48% | 7.2 | (2.6–20.3) | 8.9 | 4.0 | (1.8–8.8) | 3.9 | 1.8 | (1.2–2.8) | 2.3 |
| | 100.0 | 5% | 22% | 23% | 23% | 14.0 | (3.5–55.5) | 18.5 | 6.4 | (2.2–18.2) | 6.2 | 2.2 | (1.2–4.0) | 3.0 |
| | 1000.0 | 0% | 5% | 5% | 4% | 27.0 | (4.8–151.4) | 38.4 | 10.1 | (2.7–37.7) | 9.7 | 2.7 | (1.3–5.6) | 4.0 |

CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; OR = odds ratio, STI = sexually transmitted infection.

[a] Proportion of women with viral load higher than value.

[b] 1-Specificity of threshold value.

[c] Sensitivity of threshold value.

[d] Model adjusting only for HPV type and age.

[e] Model adjusting for HPV type and potential confounders (age, coinfection with other HPV types, number of live births, number of lifetime sex partners, number of partners in the last year, current contraceptive use, current condom use, previous STI diagnosis, previous yeast infection diagnosis, and lifetime pap test frequency).

only moderate. This was due to the substantial overlap in viral load distributions between different cervical lesion diagnosis categories.

Most studies have observed that higher HPV viral loads are associated with increased odds of an abnormal cytological or histological diagnosis [7–12,16,29,30], though some studies did not observe an association [13–15]. This association has been most consistently found for HPV16 viral load [29,31]. While many studies have examined the role of viral load for HPV16, fewer studies have looked at the type-specific role of viral load for HPV18, 31, 33, and 45, possibly due to small numbers of observations for each type [11,32]. Viral loads of other oncogenic HPV types have also been associated with abnormal diagnoses, but associations are less consistent across studies. Similarly to us, some studies have found that the largest difference in viral load is between normal and abnormal women, while differences between CIN grades are less important [10,13,29]. Results are generally difficult to quantitatively compare between studies due to different methods used to measure and analyze viral loads. The studies with the most comparable reporting to ours were Xi et al. and Liu et al., who found that 10-fold increases in HPV16 and HPV31 viral load were respectively associated with a 1.6 (95%CI: 1.3–2.0) and a 1.5 (95%CI: 1.2–2.4) higher odds of a high-grade lesion diagnosis [31,32]. This was similar to our estimated 1.5 (95%CI: 1.2–1.7) higher odds of a more severe diagnosis per 10-fold increase in HPV viral load (Table 3). Stronger associations than these have however been observed [29]. Cricca et al. found an AUC of 0.76 for discriminating between HSIL and LSIL with HPV16 viral load, which is slightly higher than our AUC of 0.70 (95%CI 0.64–0.77) for HPV16/18 [33].

We observed a stronger association between viral load and lesion cervical grade was stronger in women aged 30 years and over than in women aged <30 years, though this difference was not significant. While HPV testing is only recommended for women 30 years and over in some countries [2,3], others also recommend HPV testing for women under 30 [18]. Screening with HPV testing is more controversial in younger women as many at this age are HPV infected but have no underlying lesions, leading to high colposcopy referral rates. More effective triage methods in HPV positive women to identify those at higher risk may therefore help reduce the colposcopy referral rate in younger women. However, our results do not suggest that viral load is a more effective triage method in women under 30 years old.

It is unclear whether the viral load has a causal effect in increasing the risk of cervical lesions and cancer in HPV infected women. While our study was cross-sectional, other studies have found that high viral load may precede lesion and cancer diagnoses by many years [8,9,30,31], so reverse causality is unlikely. Our multivariate model analyses suggest that the observed association is also unlikely to be due to confounding from age or lifestyle factors. We adjusted and stratified analyses by HPV type, so the association is not due to confounding from more carcinogenic HPV types having a higher viral load. Viral integration into the host genome has been associated with a decreased viral load [27,33], which suggests viral integration does not explain the correlation between viral load and lesion grade. High viral load is however associated with persistence of HPV infection [12,30,34–37], so viral load may be a marker of HPV persistence. The association between viral load and risk of higher grade lesions diminishes after controlling for HPV persistence [12], which supports this hypothesis. It is also possible that higher viral load may be correlated with immunological factors affecting cancer progression risk, such as genetic susceptibility or immunosuppressive conditions like HIV, but we did not have the data to control for these factors.

In our study, we restricted analyses to type-specific HPV-positive women to assess the additional value of viral load as a potential triage test when it is known that a woman is HPV-positive. While viral load was predictive of underlying disease in HPV-positive

**Table 4**
Odds of having a more severe diagnosis by viral load threshold in HPV16 positive women, predicted by logistic and multinomial regression models.

| Age | Viral load threshold (copies/cell) | Above threshold (%)[a] | | | | Logistic regression (CIN1+ vs normal) | | | Logistic regression (CIN2+ vs <CIN2) | | | Multinomial regression (Cancer > CIN3&AIS > CIN2 > CIN1 > Normal) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Normal[b] | CIN1+[c] | CIN2+[c] | CIN3+[c] | OR[d] | 95% CI | OR[e] | 95% CI | OR[d] | 95% CI | OR[e] | 95% CI | OR[d] | 95% CI | OR[e] | 95% CI |
| All | 0.01 | 89% | 99% | 99% | 99% | 1.0 | (ref) | 1.0 | (ref) | 1.0 | (ref) | 1.0 | (ref) | 1.0 | (ref) | 1.0 | (ref) |
| | 0.1 | 71% | 93% | 93% | 95% | 1.9 | (1.5–2.5) | 1.9 | (1.5–2.6) | 1.7 | (1.4–2.2) | 1.7 | (1.4–2.2) | 1.4 | (1.3–1.7) | 1.5 | (1.3–1.7) |
| | 1.0 | 42% | 82% | 83% | 86% | 3.8 | (2.2–6.4) | 3.8 | (2.2–6.6) | 3.0 | (1.9–4.7) | 3.0 | (1.8–4.7) | 2.1 | (1.6–2.8) | 2.1 | (1.6–2.8) |
| | 10.0 | 16% | 55% | 55% | 57% | 7.3 | (3.3–16.2) | 7.4 | (3.2–17.0) | 5.1 | (2.6–10.0) | 5.1 | (2.5–10.3) | 3.0 | (2.0–4.6) | 3.1 | (2.0–4.8) |
| | 100.0 | 11% | 28% | 29% | 30% | 14.3 | (5.0–40.9) | 14.4 | (4.8–43.6) | 8.8 | (3.6–21.7) | 8.7 | (3.4–22.4) | 4.4 | (2.5–7.7) | 4.5 | (2.5–8.0) |
| | 1000.0 | 3% | 6% | 6% | 6% | 27.7 | (7.4–103.4) | 28.1 | (7.1–112.0) | 15.2 | (5.0–46.8) | 15.0 | (4.6–48.7) | 6.4 | (3.2–12.8) | 6.5 | (3.2–13.4) |

CIN = cervical intraepithelial neoplasia; HPV = human papillomavirus; OR = odds ratio, STI = sexually transmitted infection.
[a] Proportion of women with viral load higher than value.
[b] 1-Specificity of threshold value.
[c] Sensitivity of threshold value.
[d] Model adjusting only for HPV type and age.
[e] Model adjusting for HPV type and potential confounders (age, coinfection with other HPV types, number of live births, number of lifetime sex partners, number of partners in the last year, current contraceptive use, current condom use, previous STI diagnosis, previous yeast infection diagnosis, and lifetime pap test frequency).

women, there is a large overlap between normal women and CIN grades in ours and other studies [6,9,11,13]. Because of this, viral load is likely to perform less well for triage of HPV-positive women than cytology or HPV genotyping, which have higher diagnostic accuracies [4,6,38]. In comparison, conventional Pap smears and liquid-based cytology had an estimated AUC of 0.91–0.94 in a meta-analysis [39]. However, viral load triage presents the advantages that it does not require cytology and can be performed using the same sample as for HPV testing. Finding triage tests that can be performed using the same sample would be particularly useful in the context of self-sampled HPV tests. There is increasing interest in using self-sampling to screen populations who are difficult to see in clinic, such as women who have not responded to screening invitations [40] or women in geographically remote communities [41].

The positive predictive value of a triage test is also important to determine whether a woman is sent to colposcopy under risk based-management [5]. Due to our case-control design, we could assess the sensitivity and specificity, but not the positive predictive value of the viral load, which requires population-based studies. Because the positive predictive value depends on underlying population prevalence, it is possible that viral load may be clinically useful for distinguishing HPV-positive women at higher risk of high-grade lesions in some settings [42], but not in others [6,14,38]. It is also possible that other viral load assays or type-specific measures of viral load could have sufficiently high positive predictive values to be clinically useful.

In conclusion, the HPV viral load tends to be higher in women with CIN and cancer diagnoses, especially for HPV16/18/31. This association does not appear to be due to confounding from lifestyle factors, but may potentially be due to persistent infections having higher viral loads, which has been observed in other studies [12,34–36]. The association between viral load and cervical lesion grade was stronger in women aged 30 years and over than in women aged <30 years. While HPV viral load has moderate diagnostic accuracy for CIN grade diagnosis in HPV-positive women, it is less accurate than other triage tests such as cytology or HPV genotyping. However, the viral load might be useful for triage in settings where cytology testing is unfeasible or lacks quality assurance [8,42].

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## Author contributions

AK and ELF conceived and designed BCCR and obtained funding. ELF was the principal investigator for the study. FC supervised laboratory analyses and PCR assays. AK and AR maintained and curated the research data. TM, KL, and ELF designed the analysis. TM and KL performed statistical analyses and wrote the first draft of the manuscript. All authors reviewed the manuscript for intellectual content and assisted in the interpretation of results.

## Declaration of competing interest

ELF reports grants from Canadian Institutes of Health Research during the conduct of the study; grants and non-financial support

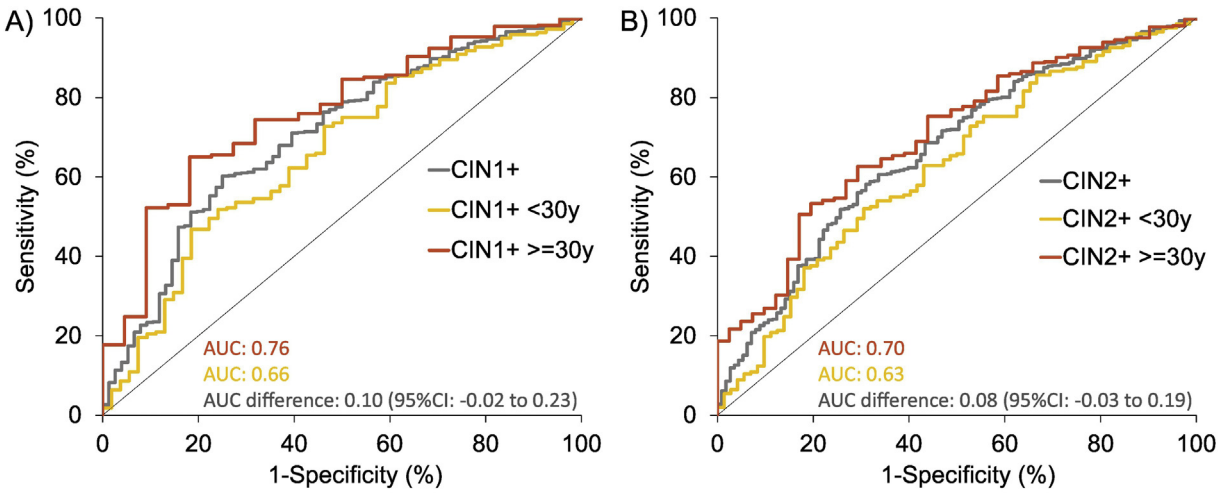


**Fig. 2.** Receiver operating characteristic (ROC) curves for the diagnostic ability of viral load to discriminate CIN1+ from normal A) and CIN2+ from <CIN2 B). CIN = cervical intraepithelial neoplasia; AUC = area under the curve; CI= confidence interval.

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## CRediT authorship contribution statement

**Talía Malagón:** Methodology, Formal analysis, Writing - original draft. **Karolina Louvanto:** Methodology, Formal analysis, Writing - original draft. **Agnihotram V. Ramanakumar:** Data curation, Writing - review & editing. **Anita Koushik:** Conceptualization, Methodology, Project administration, Funding acquisition, Writing - review & editing. **François Coutlée:** Validation, Investigation, Writing - review & editing. **Eduardo L. Franco:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

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*Data statement*

The study data is not publicly available to preserve participant confidentiality. To access the data, communicate with the corresponding author.

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