Received: 14 January 2025 | Revised: 25 February 2025 | Accepted: 2 March 2025 | Published online: 19 March 2025

DOI: 10.1002/ijgo.70074

CLINICAL ARTICLE

Gynecology

Improved cervical screening using HPV type restriction and cycle threshold limit setting with the AmpFire assay: A prospective screening cohort of women with and without HIV in Botswana

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Abstract

Objective: The aim of this study was to evaluate the performance of HPV type restriction and cycle threshold (Ct)-limit setting to optimize detection of cervical intraepithelial neoplasia (CIN) with primary HPV testing.

Methods: Baseline cervical screening at time of entry into a prospective longitudinal cohort of women with and without HIV was conducted from February 2021 to July 2022 in Botswana. All women underwent HPV testing of 15 individual types using the AmpFire assay; all HPV-positive and a random subset of HPV negative had histopathology collected. Performance parameters of HPV type restriction groupings were calculated, and sensitivity by individual HPV type Ct-value limits were plotted.

Results: Among 2964 women who underwent primary HPV screening, 1293 (43.6%) tested HPV-positive. Among women with HIV (WWH), HPV types 16/18/33 were associated with the greatest burden of CIN2+/CIN3+ (53%/56%). In WWH, grouping by HPV types separately reported in commercial assays (16/18/45) had low sensitivity (44% [CI: 36%–52%]) but high specificity (86% [CI: 84%–88%]) for CIN2+; 8-type HPV restriction (16/18/31/33/35/45/52/58) improved sensitivity (79% [CI: 72%–86%]) and maintained reasonable specificity (67% [CI: 65%–70%]) for CIN2+. Similar results were seen in women without HIV. Ct-limit setting for medium oncogenic HPV types (31,33,35,52,58) maintained a sensitivity of 72% in WWH while reducing overdetection of non-pathogenic HPV.

Conclusion: Eight-type HPV restriction and Ct-limit setting are promising strategies for improving the performance of primary HPV screening. A potential strategy to

Clinical trials registration: This study is registered on Clinicaltrials.gov, number NCT04242823, https://clinicaltrials.gov/ct2/show/NCT04242823.

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Funding information
National Cancer Institute, Grant/Award Number: 1K08CA271949; Fogarty International Center, Grant/Award Number: K43 TW012350-01

improve 8-type HPV restriction would be to treat all with HPV 16/18/45; treat HPV 31/33/35/52/58 if below the type-specific Ct limit and repeat HPV testing in 1-year for other positive HPV results.

KEYWORDS
8-type HPV genotype restriction, cervical dysplasia, cervical screening, Ct-value, HPV viral load, human immunodeficiency virus (HIV), human papillomavirus (HPV), low- and middle-income countries (LMICs)

1 | INTRODUCTION

The burden of cervical cancer continues to grow in low- and middle-income countries (LMICs) despite global trends demonstrating reduced incidence and mortality worldwide.[1–4] In response, the WHO's strategy to eliminate cervical cancer advocates to increase the effectiveness and accessibility of screening with primary HPV testing.[5–8] The strategy's screening target is 70% population coverage with two lifetime HPV-based screening tests. At the time of the strategy launch in 2020, only 37% of countries had reached 70% coverage with any modality of cervical screening and very few had implemented primary HPV screening.[9,10]

While primary HPV screening is highly sensitive and reduces the time and resource intensity of traditional screening modalities, HPV-based screening strategies require optimization, particularly in settings without efficient cytopathology.[11–13] HPV testing is complicated by low specificity and positive predictive value (PPV), necessitating further evaluation of women with positive HPV results or refinement of the test itself to avoid overtreatment. Data suggest that using HPV type restriction as a primary screening tool, with restriction to a limited set of types most associated with cervical cancer, may be an effective method of maintaining sensitivity while improving specificity of primary HPV screening.[14] Further optimization of HPV type restriction by setting HPV viral load cutoffs, as approximated by cycle threshold (Ct)-value, also has demonstrated promise in improving the precision of HPV-based cervical screening.[15] Viral load is approximated in PCR-based assays by Ct-value, where a low Ct-value corresponds to a high viral load and is more likely to correspond to pathogenic disease.[16]

The aim of the present study was to evaluate the performance of HPV type restriction and Ct-value limit setting with the AmpFire assay. These data are stratified by HIV-status, which is important in guiding the practical implementation of high-performance HPV screening in LMICs.

2 | MATERIALS AND METHODS

We conducted a prospective cohort study among women residing in a rural and peri-urban district in Botswana. Non-pregnant women aged 25 or older with an intact cervix and without a prior diagnosis of cervical cancer were approached at health facilities from February 22, 2021 to July 29, 2022. Eligible and interested women provided written informed consent. Participants completed a demographic and health questionnaire. HIV treatment and cervical screening history was obtained from the electronic medical record.

Participants obtained a vaginal self-swab for HPV testing after receiving instructions to squat, insert the swab into their vagina, and swirl it around the vagina five times. Specimens were transported to the Botswana Harvard HIV Reference Laboratory for processing, which has been accredited by the South African National Accreditation System, and by the Southern African Development Community Accreditation Service, both of which are members of the International Laboratory Accreditation Cooperation. For each vaginal dry swab, 1 mL of 1× lysis buffer was added to the collection tube, vortexed briefly (~15 s), and then transferred to a 1.5 mL Eppendorf tube. The sample was incubated for 20 min and then centrifuged for 30 min at a maximum speed (17000 *g* or greater), followed by a complete supernatant removal. Subsequently, 30 μL of 1× lysis buffer was added to the cell pellet and then thoroughly vortexed to ensure complete resuspension. The suspension from each sample was transferred to a 0.2 mL PCR tube and incubated at 95°C for 10 min in a thermocycler. HPV DNA genotyping was then conducted using the Atila AmpFire HPV detection kit (Atila BioSystems, Mountain View, California), which individually reports 15 high-risk HPV types (16/18/31/33/35/39/45/51/52/53/56/58/59/66/68). The assay was run using an isothermal qPCR of 60°C, taking fluorescence reading at the FAM/HEX/CY5/ROX channels once every minute for a total of 60 min. The results of the Ct values for each amplification curve in all fluorescence channels were automatically reported by the Bioer Line-gene 9600 plus real time PCR system. For each sample, genotyping results were coded and identified based on exponential amplification curves in the CY5, ROX, FAM, and HEX channels in the above layout of four reaction tubes. All samples with detectable fluorescence were considered positive. The amplification curves were visually inspected to ensure sigmoid typical amplification curves. The lack of exponential amplification curve in the HEX channel from primer mix 3 was interpreted as an invalid result. Positive and negative control for each run, as well as human β-globin gene amplification, were included to check for the adequacy of extraction for each sample.

All participants with positive HPV testing and a random sample of 10% of participants with negative testing were asked to return for evaluation at the district hospital. During this evaluation, a gynecologist performed colposcopic-directed biopsy. If there was a visible lesion, a punch biopsy was collected if the lesion appeared low-grade or a loop electrosurgical excision procedure (LEEP) was performed if the lesion appeared high-grade, according to Botswana's see-and-treat policy. If no lesion was visible, a small endocervical sample was collected. Pathology specimens underwent standard processing and pathology interpretation at the Botswana National Health Laboratory. Histopathology data were reported by the National Health Laboratory according to the cervical intraepithelial neoplasia (CIN) classification system[17] and were categorized by severity. CIN3 or worse (CIN3+) included CIN3, CIN3 with invasive features, adenocarcinoma in situ (AIS), squamous cell cancer (SCC), and adenocarcinoma. CIN2 or worse (CIN2+) included CIN2 and CIN3+.

2.1 | Data analysis

The primary study outcome was the performance of HPV type restriction to detect CIN2+ according to three HPV groupings: 3-type HPV restriction using the HPV types separately reported in commercial assays (HPV 16/18/45), 8-type HPV restriction (HPV 16/18/31/33/35/45/52/58), and the International Agency for Research on Cancer (IARC) list of the 12 most oncogenic HPV types (HPV 16/18/31/33/35/39/45/51/52/56/58/59).[18] Among women with multiple detected HPV types, the type with highest expected oncogenic potential as determined by IARC was utilized (highest to lowest oncogenic potential: 16/18/45/33/58/31/52/35/59/39/68/51/56/66/53).[19] Histopathology collected at the triage visit served as the primary endpoint reference.

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of HPV type restriction to detect CIN2+ were calculated for each HPV grouping. Given that only a sample of the HPV-negative women underwent histopathologic evaluation, test characteristics could not be calculated with the existing data. Therefore, the existing data was extrapolated to represent the entire cohort had resources been available to test all HPV-negative women. The extrapolated data was used to calculate the final test characteristics presented in the results. Though that estimates are imprecise given the small sample of biopsies collected from women with HPV-negative results, the sensitivity would not be affected.

The secondary outcome was evaluating the role of Ct-limit setting for individual HPV types to improve the performance of 8-type HPV restriction. We used the observed data from the cohort to calculate the sensitivity for detection of CIN2+ by Ct-value and plotted the corresponding coordinates. We then selected the Ct-limits corresponding to the 75% percentile of individuals with CIN2+ for each of the individual eight most oncogenic HPV types, stratified by HIV status.

Data were analyzed with SAS 9.4 (SAS Institute, Cary, North Carolina) and Stata 17 (StataCorp, College Station, Texas). Descriptive statistics are presented as mean with standard deviation, median with interquartile range, or proportion. Continuous variables were compared with a *t* or Wilcoxon rank sum test and categorical variables with a chi-square or Fisher exact test.

This was a secondary analysis of the larger cohort study, and thus for this analysis, the sample size was one of convenience and included all study participants who had histopathology results available.

The institutional review boards of the Botswana Ministry of Health and Wellness (01060), the University of Botswana, and the Beth Israel Deaconess Medical Center (2019P001130) approved this study. The South East District Health Management Team also approved this study. This study is registered on Clinicaltrials.gov (NCT04242823).

3 | RESULTS

Study enrollment of 3000 participants and follow-up visits took place from February 2021 to February 2023. A total of 2957 participants completed HPV testing. Of the 1479 WWH with HPV results for analysis, 823 (56%) tested HPV-positive and 710 (86%) of these had histopathology available for analysis; of 1478 women without HIV, 655 (44%) tested HPV-positive and 583 (89%) had histopathology available for analysis. Of the random subset of 10% of women testing HPV-negative who were recalled for biopsy, 136 (9%) had a histopathology specimen collected for verification bias, including 51 WWH and 85 women without HIV (Figure 1). Sensitivity analysis showed the only notable difference between HPV negative participants who were recalled for biopsy to those who were not, was longer duration of HIV (Table S1). The mean age was 42 years (±SD 11) (Table 1). Notably, WWH were generally well-controlled on antiretroviral therapy (ART) with a long duration since both HIV diagnosis and initiation of ART as detailed in Table 1.

3.1 | Cervical dysplasia prevalence by HPV type

The prevalence of CIN2+ and CIN3+ was assessed among 1293 (87%) women with detected HPV who had available histopathology results (Table 2). Prevalence of CIN2+ and CIN3+ was higher among the 710 WWH testing HPV positive who were assessed—135 (PPV 19.0%) and 100 (PPV 14.1%), respectively—than

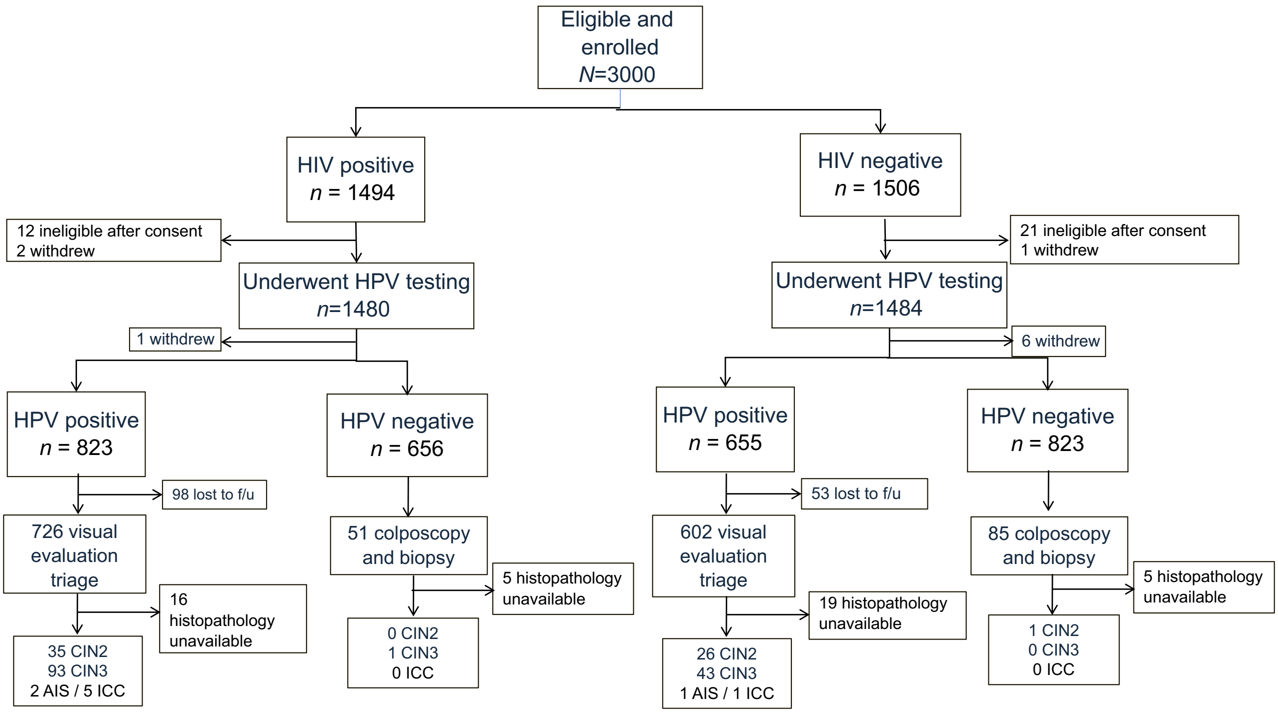

FIGURE 1 Study recruitment flow chart. CIN, cervical intraepithelial neoplasia; f/u, follow-up; HIV, human immunodeficiency virus; HPV, human papillomavirus; ICC, invasive cervical cancer.

583 women without HIV assessed—71 (PPV 12.1%) and 45 (PPV 7.7%), respectively.

In WWH, HPV 16 was associated with the highest number of cases of CIN2+ (34) corresponding to a PPV of 32%. HPV 18 carried the next highest burden with 23 CIN2+ cases and a PPV of 28%, followed by HPV 33 with 14 cases and a PPV of 30%. These same three HPV types—16, 18, and 33—were also associated with the greatest burden of CIN3+ in WWH. Trends in HPV types associated with CIN2+/CIN3+ were broadly similar in women without HIV.

Using the hierarchical HPV type assignment, only eight of the 100 cases (8.0%) of CIN3+ in WWH and two of the 45 cases (4.4%) of CIN3+ in women without HIV were associated with HPV types not included in the eight highest-risk HPV types (16,18,31,33,35,45,52,58). There were only three cases of AIS and five cases of cervical cancer, only one of which was associated with an HPV type not included in the 8-type HPV grouping (HPV51).

3.2 | Performance of HPV type restriction to detect cervical dysplasia

We evaluated the ability of HPV type restriction to detect CIN2+ and CIN3+, stratified by HIV status. Among the WWH who tested negative for HPV and who attended a follow-up visit for biopsy, there were two cases of CIN2+ (1 CIN2 and 1 CIN3) detected, leading to an overall simulated sensitivity of primary HPV testing alone (with the 15 HPV types in the AmpFire HPV assay) of 90% (confidence interval [CI]: 84%–94%) for detection of CIN2+ and 87% (CI: 79%–93%) for detection of CIN3+ (Table 3). Specificity for any positive HPV type by AmpFire assay was 53% (CI: 50%–56%) for CIN2+ and 51% (CI: 48%–54%) for CIN3.

In WWH, a strategy of restricting to the three HPV types individually reported in commercial assays (16/18/45) resulted in low sensitivity of 44% (CI: 36%–52%) for CIN2+ and 47% (CI: 38%–56%) for CIN3+ but had high specificity of 86% (CI: 84%–88%) for CIN2+ and 85% (CI: 83%–87%) for CIN3+. A strategy of restricting to the eight most oncogenic types (HPV16/18/31/33/35/45/52/58) had a sensitivity of 79% (CI: 72%–86%) for detection of CIN2+ and 80% (CI: 72%–87%) for CIN3+. Specificity was higher with this strategy than primary HPV testing alone at 67% (CI: 65%–70%) for CIN2+ and 66% (CI: 63%–69%) for CIN3+. We found performance parameters when restricting to the 12 HPV types classified as oncogenic by IARC were similar to primary HPV testing with the 15 HPV types in the AmpFire assay. Similar trends were seen in women without HIV but with overall lower sensitivity of HPV testing alone and HPV type restriction for detection of CIN2+/CIN3+.

3.3 | HPV Ct-value limit setting and correlation to cervical dysplasia

Ct-values of 543 participants who had HPV-positive results and complete histopathology data were included in the evaluation of Ct-values and correlation to cervical dysplasia. Data were combined for women with and without HIV due to scarcity of cases in lesser oncogenic HPV types when stratified by HIV status.

TABLE 1 Demographic characteristics of 2957 participants who underwent HPV testing in South East District, Botswana stratified by HIV status.

Characteristic	All n = 2957	Women with HIV n = 1479	Women without HIV n = 1478
Age, years—mean ± standard deviation	42 ± 11	43 ± 9	41 ± 11
Education			
≤Primary	562 (19)	315 (21)	247 (17)
≥Secondary	2395 (81)	1164 (79)	1231 (83)
Employed	1672 (57)	838 (57)	834 (56)
Marital status			
Single	2118 (72)	1106 (75)	1012 (68)
Married	674 (23)	285 (19)	389 (26)
Divorced/separated	38 (1)	20 (1)	18 (1)
Widowed	127 (4)	68 (5)	59 (4)
Gravidity			
0	190 (6)	85 (6)	108 (7)
1–3	1897 (64)	927 (63)	970 (66)
≥4	870 (29)	467 (32)	403 (27)
Parity			
0	222 (8)	102 (7)	120 (8)
1–3	2056 (70)	1010 (68)	1046 (71)
≥4	679 (23)	367 (25)	312 (21)
Age of sexual debut, years—mean ± standard deviation	19 ± 3	19 ± 3	19 ± 3
Lifetime sexual partners[a]			
0	4 (0.1)	0 (0)	4 (0.3)
1-5	1940 (66)	893 (61)	1047 (71)
≥6	995 (34)	577 (39)	418 (28)
Smoking	172 (6)	114 (8)	58 (4)
Self-reported history of cervical cancer screening	1989 (67)	1180 (80)	809 (55)
Self-reported history of cervical excisional procedure	25 (1)	17 (1)	8 (0.5)
Duration of HIV diagnosis, years ± standard deviation	-	10 ± 6	-
Currently taking ART	-	1478 (99.9)	-
Length of time on ART, years—mean ± standard deviation	-	8 ± 5	-
CD4 count (per μL)-			
<200	-	26 (2)	-
200-500	-	291 (20)	-
>500	-	1162 (79)	-
Detectable viral load[a]	-	14 (1)	-

Abbreviation: ART, antiretroviral therapy.

[a]Missing data: 18 participants missing number of sexual partners, 1 missing viral load.

For each individual HPV type in the 8-type HPV restriction group, the sensitivity for detection of CIN2+ by Ct-values is presented in Figure 2. Each individual HPV type reached a sensitivity of 75% at different Ct-values, with HPV 35 achieving a sensitivity of 75% detection for CIN2+ at the lowest Ct-value (27) relative to other HPV types. This is in sharp contrast to other types, such as HPV 45, which only reached 75% sensitivity for CIN2+ at a Ct-value of 44, indicating pathogenic disease (CIN2+) was present at a lower HPV viral load.

3.4 | Implications for policy

In Table 4, we present the outcomes of a simulated strategy utilizing individual Ct-value limits that correspond to a sensitivity of at least 75% for CIN2+ for an 8-type HPV restriction strategy, stratified by HIV status. We calculated the number of cases detected below the Ct-limit, as well as the number of cases of CIN2+ that would have been missed because they were above the Ct-limit. The proposed strategy includes assuming any positive HPV 16/18/45 is considered positive whereas results of HPV 33/58/31/52/35 are only reported as positive if the Ct-value is below the pre-specified limit (indicating high viral load). Overall, this strategy identifies 90% of the CIN2+ cases in the HPV 8-type restricted group, while potentially avoiding 20% of colposcopy examinations or treatments (depending on the context of implementation). Given the sensitivity of an 8-type restriction strategy in our entire screened cohort of WWH of 79% (Table 3), the overall sensitivity of a combined approach with 8-type HPV restriction and Ct-limit setting would be 72% in WWH. The lower sensitivity of 8-type HPV restriction in women without HIV of 75% combined with the Ct-value adjustment sensitivity of 88% would result in a sensitivity of the combined approach of only 66%.

TABLE 2 Prevalence of high-grade cervical dysplasia by individual HPV type using the IARC hierarchical HPV type assignment,[a] stratified by HIV status.

		Women with HIV			Women without HIV		
HPV status	Number undergoing triage and with biopsy results (*n*)	Number undergoing triage and with biopsy results (*n*)	CIN2+[b], *n* (%)	CIN3+[c], *n* (%)	Number undergoing triage and with biopsy results (*n*)	CIN2+[b], *n* (%)	CIN3+[c], *n* (%)
HPV negative	126	46	1 (2.2%)	1 (2.2%)	80	1 (1.3%)	0 (0%)
HPV positive	1293	710	135 (19.0%)	100 (14.1%)[d]	583	71 (12.1%)	45 (7.7%)[e]
Hierarchical HPV assignment							
16	191	106	34 (32)	29 (27)	85	12 (14)	7 (8)
18	126	82	23 (28)	18 (22)	44	6 (14)	5 (11)
45	101	52	9 (17)	7 (13)	49	3 (6)	3 (6)
33	100	46	14 (30)	9 (20)	54	10 (19)	9 (17)
58	86	58	10 (17)	9 (16)	28	7 (25)	6 (21)
31	70	41	9 (22)	8 (20)	29	5 (17)	4 (14)
52	112	67	9 (13)	4 (6)	45	11 (24)	6 (13)
35	113	63	11 (17)	8 (13)	50	7 (14)	3 (6)
59	63	33	3 (9)	2 (6)	30	1 (3)	0 (0)
39	70	33	2 (6)	1 (3)	37	5 (14)	2 (5)
68	89	43	1 (2)	0 (0)	46	1 (2)	0 (0)
51	58	25	2 (8)	2 (8)	33	2 (6)	0 (0)
56	42	24	4 (17)	1 (4)	18	1 (6)	0 (0)
66	21	12	2 (17)	2 (17)	9	0 (0)	0 (0)
53	50	24	1 (4)	0 (0)	26	0 (0)	0 (0)

Abbreviations: CIN, cervical intraepithelial neoplasia; IARC, International Agency for Research on Cancer.

[a]Hierarchy delineated by International Agency for Research on Cancer, *Cervical cancer screening handbook*, Volume 14, page 67. IARC hierarchy of HPV oncogenicity from highest to lowest oncogenic potential: 16/18/45/33/58/31/52/35/59/39/68/51/56/66/53.

[b]CIN2+ includes cervical intraepithelial neoplasia (CIN) 2, CIN3, CIN3 with microinvasion, adenocarcinoma in-situ, squamous cell carcinoma, adenocarcinoma.

[c]CIN3+ includes CIN3, CIN3 with microinvasion, adenocarcinoma in-situ, squamous cell carcinoma, adenocarcinoma.

[d]This includes 2 cases of CIN3 with invasive features, 1 case of squamous cell carcinoma, 2 cases of adenocarcinoma and 2 cases of adenocarcinoma in-situ. HPV 18 was associated with 2 cases of adenocarcinoma, HPV 16 and 31 were each associated with 1 case of CIN3 with invasive features; HPV 51 was associated with 1 case of squamous cell carcinoma; HPV 52 and 58 were each associated with 1 case of adenocarcinoma in-situ.

[e]This includes 1 case of CIN3 with invasive features and 1 case of adenocarcinoma in-situ; HPV 58 was associated with the case of CIN3 with invasive features, HPV 16 was associated with the case of adenocarcinoma in-situ.

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TABLE 3 Ability of HPV type restriction to detect CIN2+ and CIN3+, stratified by HIV status.

Performance parameters	Biopsy result[a]		Biopsy result[a]		Sensitivity		Specificity		PPV		NPV	
Histology endpoints	<CIN2	CIN2+	<CIN3	CIN3+	CIN2+	CIN3+	CIN2+	CIN3+	CIN2+	CIN3+	CIN2+	CIN3+
Women with HIV												
HPV positive	575	135	610	100	90 (84–94)	87 (79–93)	53 (50–56)	51 (48–54)	19 (16–22)	14 (12–17)	98 (96–99)	98 (96–99)
HPV 16/18/45 pos	174	66	186	54	44 (36–52)	47 (38–56)	86 (84–88)	85 (83–87)	28 (22–34)	23 (17–28)	93 (91–94)	95 (93–96)
HPV 8-type[b] pos	396	119	423	92	79 (72–86)	80 (72–87)	67 (65–70)	66 (63–69)	23 (20–27)	18 (15–21)	96 (95–98)	97 (96–98)
HPV IARC 12[c] pos	500	130	532	98	87 (80–92)	85 (77–91)	59 (56–62)	57 (55–60)	21 (18–24)	16 (13–19)	97 (96–98)	98 (96–99)
Women without HIV												
HPV positive	508	70	534	44	86 (77–93)	100 (92–100)	62 (59–64)	61 (58–63)	12 (10–15)	8 (6–10)	99 (98–99)	100 (100–100)
HPV 16/18/45 pos	156	21	162	15	26 (17–37)	34 (20–50)	88 (86–90)	88 (86–90)	12 (8–18)	8 (5–14)	95 (94–96)	98 (97–98)
HPV 8-type[b] pos	321	60	339	42	74 (63–83)	95 (85–99)	76 (73–78)	75 (73–77)	16 (12–20)	11 (8–15)	98 (97–99)	100 (99–100)
HPV IARC 12[c] pos	429	69	454	44	85 (76–92)	100 (92–100)	68 (65–70)	67 (64–69)	14 (11–17)	9 (6–12)	99 (98–99)	100 (100–100)

Abbreviations: CIN, cervical intraepithelial neoplasia; IARC, International Agency for Research on Cancer.

[a]Extrapolated figures based on 1 CIN3 case among 46 WWH who were HPV negative and underwent biopsy and 1 CIN2 case among 79 women without HIV who were HPV negative and underwent biopsy.

[b]HPV 8-type restriction includes HPV types 16/18/31/33/35/45/52/58.

[c]HPV IARC 12 includes HPV types 16/18/31/33/35/39/45/51/52/56/58/59.

4 | DISCUSSION

In this large cervical screening cohort in Botswana, we observed a high prevalence of HPV and cervical dysplasia in both women with and without HIV. The great majority of CIN3+ lesions occurred among women with the eight highest risk HPV types. A strategy of targeting clinical resources for follow-up examination and biopsy for women with 8-type HPV restriction could limit procedural burden and possible overtreatment of non-pathogenic HPV infection, while maximizing detection of cancer and precancerous lesions. Ct-value limit setting may further optimize this strategy, though requires careful consider of the burden of disease and resources available in individual contexts.

The Enduring Guidelines from the American Society for Colposcopy and Cervical Pathology recently updated recommendations for incorporation of extended genotyping into cervical screening based on one FDA approved assay in the United States.[20] Our findings similarly support differential screening, triage, and treatment strategies based on the individual eight highest risk HPV types. Although our findings show that HPV 16, 18, and 33 were most associated with CIN2+, no currently available commercial assay individually reports HPV 33. Pragmatically, we evaluated the performance of restriction to the three HPV types most often individually reported in commercial assays, which are HPV 16, 18, and 45. Given the high specificity of HPV 16/18/45 combined in this cohort and recent data still showing that these are the three types most strongly associated with invasive cervical cancer,[21] it would be a reasonable strategy to treat all women who test positive for these types without further optimization. While our observed data show that HPV33 carries a greater burden of CIN2+ than HPV45 in our setting, HPV33 was not associated with any cases of ICC in our cohort and may have lesser likelihood of progressing from preinvasive to invasive disease. For the next five HPV types most associated with cervical cancer (HPV 31/33/35/52/58), optimized Ct-limits can be applied, and women with these HPV types with Ct-values below the prespecified threshold (higher viral loads) could be triaged to treatment. More or less stringent Ct-limits could be considered, balancing resources for triage and treatment services, as well as the tradeoff between overtreatment and missed CIN2+ lesions.

Given current constraints in HPV triage strategies, optimizing laboratory-based HPV screening is likely to be the most effective strategy to identify those most at risk for developing cervical cancer, whether through HPV type restriction, Ct-value limit setting as a proxy for HPV viral load, or other molecular biomarker triage strategies. Kuhn et al. reported that reducing Ct-value cutoffs for positive results from three channels on the Xpert platform (HPV 16, HPV 18/45, and HPV 31/33/35/52/58) improved the specificity of primary HPV testing.[15] Using the Ct-value thresholds identified in Kuhn's analysis, Denny et al. found that same-day ablative treatment of 8-type HPV restriction at high viral loads reduced overtreatment.[22] Two studies using Roche Cobas4800 for primary HPV screening found that lower Ct-values were associated with an

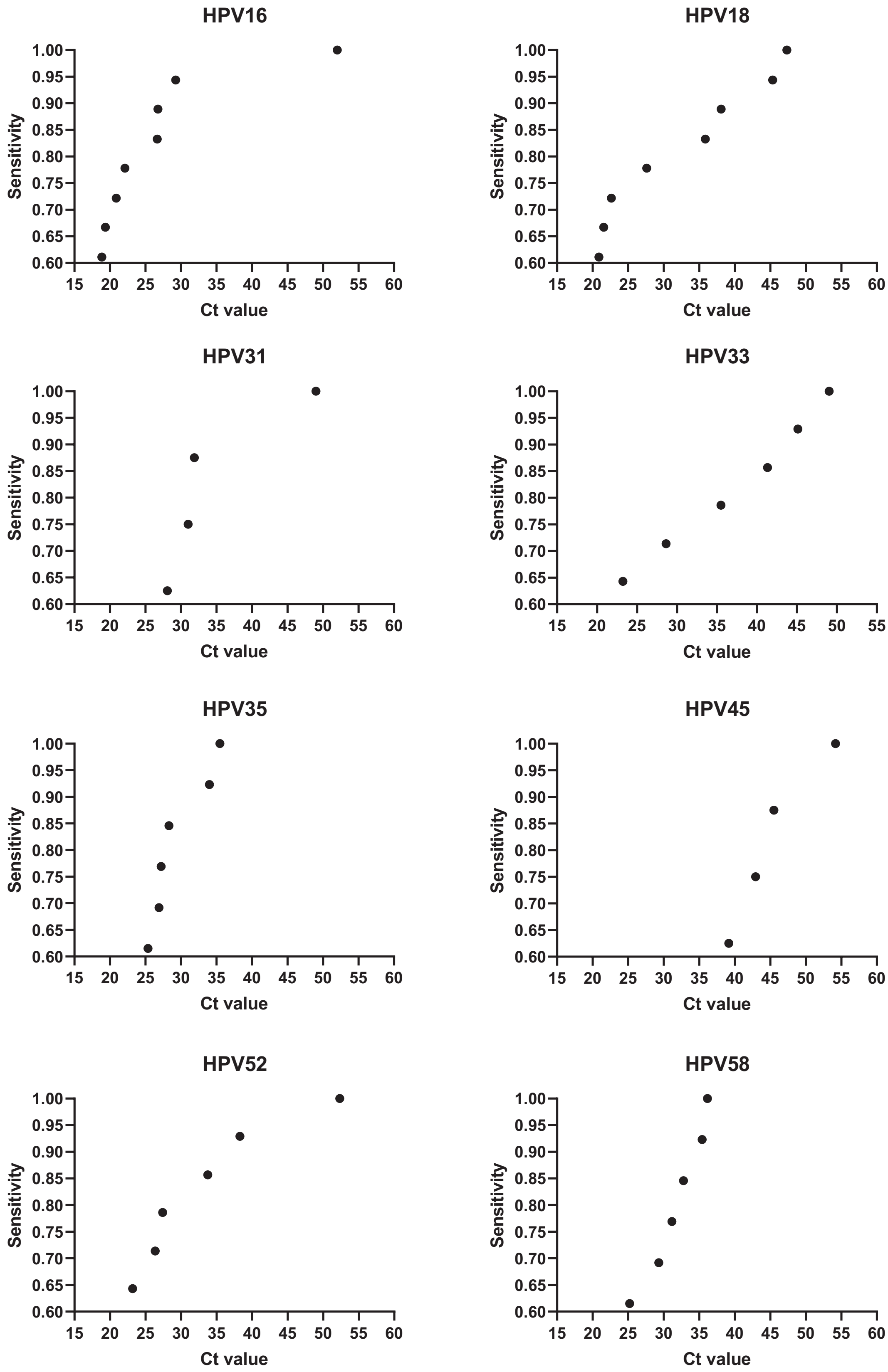

FIGURE 2 Ct-values and associated sensitivities for the eight most oncogenic HPV types among all women. HPV, human papillomavirus.

TABLE 4 Performance of a strategy using HPV 8-type restriction with immediate treatment of 16/18/45 and individual Ct-value limit setting for HPV 31/33/35/52/58, stratified by HIV status.

	Optimized Ct-value	Number with positive result	CIN2+ cases identified	Number with negative result[a]	CIN2+ cases missed	Sensitivity (%)	Specificity (%)
Women with HIV							
HPV 16	n/a	48	9	n/a	0	100	21
HPV 18	n/a	36	13	n/a	0	100	9
HPV 45	n/a	29	5	n/a	0	100	8
HPV 31	<28	11	3	5	1	75	33
HPV 33	<46	19	6	1	1	86	0
HPV 35	<29	21	6	15	2	75	46
HPV 52[b]	<19	8	4	26	0	100	87
HPV 58	<32	22	5	7	1	83	26
Total		194	51	54	5	91	26
Women without HIV							
HPV 16	n/a	68	9	n/a	0	100	31
HPV 18	n/a	34	5	n/a	0	100	45
HPV 45	n/a	44	3	n/a	0	100	22
HPV 31	<32	16	3	8	1	75	35
HPV 33	<36	24	6	6	1	86	22
HPV 35	<25	12	4	22	1	80	72
HPV 52	<34	29	8	9	2	80	25
HPV 58	<36	16	6	7	1	86	38
Total		243	44	52	6	88	18

Abbreviations: CIN, cervical intraepithelial neoplasia; Ct-value, cycle threshold value.

[a]This column represents those cases that would not be pursued for further work-up (i.e. colposcopy) based on HPV result with optimized Ct-value cut-off.

[b]HPV 52 had 4 cases, case 3 and 4 had CT values between 18 and 19, so any whole number Ct-value threshold would not discriminate between these 2 cases.

increased risk for CIN2+.[23,24] Similarly, Martinelli et al. reported an association between a high HPV viral load (calculated from Ct-value and cell count) and CIN2+ using the OncoPredict HPV assay.[25] Our data add to this growing body of literature and we believe is the first to demonstrate the wide variation in HPV type specific Ct-values associated with pathogenic disease, which has been a limitation of these prior studies.

Strengths of this study include being a large screening cohort of both women with and without HIV, and the evaluation of the correlation between individual HPV Ct-value limits and CIN2+.

Our study has limitations. The AmpFire assay has not undergone the same clinical validation process as other commercial assays and thus appears more sensitive in detection of HPV relative to other HPV assays.[26,27] We focused our Ct-value analysis on the HPV types in the 8-type HPV restriction approach, because data are sparse for the other seven types reported by the AmpFire assay when a hierarchical HPV assignment is made. The hierarchical assignment of HPV types does not account for a potential synergy in risk for cervical dysplasia imparted by multiple HPV infections. Evaluation of CIN3+ as an endpoint for the Ct-value analysis was not possible due to scarcity of data by individual HPV type. Due to a lack of detected cases of CIN3+ in women without HIV who tested negative for HPV, assessment of performance parameters may have limited precision. Finally, we only had a random sample of women who tested HPV negative and extrapolation was necessary in order to calculate the test characteristics and therefore we did not include receiver-operating characteristic (ROC) curves. These results should be interpreted with caution, particularly because HPV-negative participants sampled had longer duration of HIV which may have overestimated the observed number of cases of CIN2+ and consequently underestimated specificity.

5 | CONCLUSION

AmpFire assay 8-type HPV restriction and individual HPV type Ct-limit setting is a promising strategy for improving the performance of HPV primary screening. Recognizing that there will always be a trade-off between detection of CIN2+ lesions and the risk of overtreatment, our findings suggest that the following specific triage strategy may be useful: (1) Treat all with HPV 16/18/45 given its high specificity. (2) Treat those with HPV 31/33/35/52/58 with established Ct-values below type-specific limit to avoid over-treatment. (3) Repeat evaluation at 1-year for individuals with HPV39/51/56/59 and those with HPV31/33/35/52/58 with established Ct-values above the type-specific limit.

AUTHOR CONTRIBUTIONS

RL: conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, validation, visualisation, writing– original draft, and writing – review & editing. DRM: conceptualisation, data curation, funding acquisition, investigation, methodology, project administration, resources, validation, visualisation, writing – review & editing. SM: methodology, supervision, writing – review & editing. AG: software, data curation, formal analysis, writing – review & editing. AM: formal analysis, writing-review & editing. NM: methodology, writing – review & editing. TK: project administration, writing – review & editing. LT: methodology, writing – review & editing. FN: methodology, writing – review & editing. LK: methodology, writing – review & editing. SG: writing – review & editing. SDP: writing – review & editing. GD: supervision, writing – review & editing. JM: supervision, writing – review & editing. MHB: supervision, formal analysis, writing – review & editing. MRH: methodology, supervision, formal analysis, writing – review & editing. RLS: supervision, formal analysis, resources, writing – review & editing.

ACKNOWLEDGMENTS

We would like to thank all study participants. We also appreciate our ongoing support from the Ministry of Health and Wellness of Botswana, the South East District Health Management Team, and the Administrative leadership at Bamalete Lutheran Hospital. We are very grateful for the support of Chief Kgosi Mosadi, for her support of this study in her district and her commitment to improving the health and wellness of her constituency.

FUNDING INFORMATION

Funding for this study was provided by the Young Investigator Award from the Department of Obstetrics and Gynecology at Beth Israel Deaconess Medical Center, the National Cancer Institute, National Institutes of Health Award 1K08CA271949, and National Institutes of Health Fogarty International Center K43 TW012350-01. The funders required external peer review for scientific quality. The funders had no role in the conduct of the study, data analysis or manuscript preparation.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets used for the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Luckett R, Ramogola-Masire D, Moyo S, et al. Improved cervical screening using HPV type restriction and cycle threshold limit setting with the AmpFire assay: A prospective screening cohort of women with and without HIV in Botswana. *Int J Gynecol Obstet*. 2025;170:882-892. doi:10.1002/ijgo.70074