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Accuracy of Liferiver HarmoniaHPV and VenusHPV Assays on Urine and Vaginal Self-Samples

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Keywords: cervical cancer screening | diagnostic test accuracy | HPV | self-sampling | VALHUDES

ABSTRACT

In this report, the clinical performance of Liferiver HarmoniaHPV and Liferiver VenusHPV was evaluated under the VALHUDES framework. Five hundred and twenty-three women collected first-void urine (FVU) with Colli-Pee and vaginal samples with Evalyn Brush or Qvintip. Cervical samples were taken with the Cervex Brush by a clinician. Both vaginal and cervical samples were resuspended in 20 mL ThinPrep. Triplet samples from 499 women were tested with HarmoniaHPV and VenusHPV tests. The clinical accuracy of HarmoniaHPV did not differ in FVU and vaginal self-samples versus cervical samples. The relative sensitivity for CIN2+ on FVU and vaginal samples was 0.95 [95% CI 0.89–1.02] and 0.95 [95% CI 0.88–1.02], respectively. Relative specificity for < CIN2 was 0.95 [0.86–1.04] on FVU and 0.93 [0.86–1.01] on vaginal samples. VenusHPV demonstrated lower sensitivity on both self-sample types, whereas the specificity was similar to cervical samples. Post-hoc adjustment of the VenusHPV C_t-values improved sensitivity (ratio FVU/cervical = 0.94 [95% CI 0.88–1.00]; ratio vaginal/cervical = 0.96 [95% CI 0.92–1.01]) without compromising specificity (ratio FVU/cervical = 1.00 [0.92–1.09]; ratio vaginal/cervical = 0.95 [95% CI 0.88–1.02]) on both self-samples. In conclusion, HarmoniaHPV and VenusHPV tests demonstrated similar clinical accuracy on FVU and vaginal self- versus cervical samples, although VenusHPV test required cut-off optimization.

1 | Introduction

Despite the availability of effective cervical cancer prevention programmes, cervical cancer remains global public health challenge [1]. Human Papillomavirus (HPV) testing has proven to be more effective for cervical cancer screening compared to a cytology [2], leading many countries to transition from the conventional Pap smear to primary HPV testing [3]. However, cervical cancer screening participation rates remain suboptimal in many regions [4]. The integration of self-sampling into cervical cancer screening programs could increase screening coverage, particularly among women who are under-screened or never screened [5, 6].

In recent years, HPV testing on self-samples has developed as a promising alternative to traditional screening [7]. Self-sampling allows women to collect vaginal or urine samples at home not requiring clinical visits. Several studies have demonstrated that self-collected samples are highly acceptable to women and can achieve similar diagnostic accuracy using polymerase-chain reaction based (PCR) high-risk (hr) HPV testing when compared to clinician-collected samples [5, 8]. However, the implementation of HPV testing on self-samples in routine screening settings requires strong evidence of its clinical accuracy which is currently limited. To address this gap, the VALHUDES (VALidation of HUman papillomavirus assays and collection DEvices for Self-samples and urine samples) project was initiated to assess the performance of various HPV assays and self-sampling devices, providing essential data to support the use of self-sampling in cervical cancer screening programs [9]. The results of the VALHUDES project provide crucial missing evidence to support the inclusion of self-sampling in cervical cancer screening guidelines, both at national and international levels.

Here we evaluated clinical accuracy of Liferiver HarmoniaHPV and VenusHPV tests on first-void urine (FVU) self-samples collected with Colli-Pee and vaginal self-samples collected with the Evalyn Brush (EB) or Qvintip (QT) using the VALHUDES diagnostic accuracy protocol. HarmoniaHPV is a partially genotyping test identifying HPV16 and 18 individually, and other 12 HPV genotypes in bulk, whereas VenusHPV is a full genotyping test targeting 15 HPV genotypes. HarmoniaHPV has been part of VALGENT4 framework and was considered partially validated for cervical cancer screening as it required post-hoc cut-off optimisation [10].

2 | Materials and Methods

2.1 | Study Design

A total of 523 women were recruited in five colposcopy clinics in Belgium for the VALHUDES diagnostic test accuracy study (NCT03064087) according to the STARD guidelines [9, 11]. Colposcopy referral was indicated due to previous HPV infection or cytological abnormality. Two self-samples were collected: FVU and vaginal self-samples (EB or QT). FVU was collected at home a day before colposcopy with the Colli-Pee device (Novosanis, Wijnegem, Belgium), whereas the vaginal sample was taken at the colposcopy clinic with the EB or QT device. The Colli-Pee device collects ~13 mL of FVU in a tube containing 7 mL of Urine Conservation Medium (UCM). Thereafter, a cervical sample was taken by a clinician using the Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) and placed into 20 mL ThinPrep PreservCyt solution (Hologic Inc, Marlborough, MA, USA) followed by colposcopy with a biopsy if clinically required. FVU was then stored at the Centre for the Evaluation of Vaccination ([CEV], University of Antwerp; [Biobank Antwerp, Antwerp, Belgium; ID: BE 71030031000]). Cervical vials and dry vaginal devices were sent to Algemeen Medisch Laboratorium (AML [Antwerp, Belgium]) where EB and QT were resuspended in 20 mL ThinPrep. All specimens were stored at −80°C before HPV testing.

2.2 | HPV Testing

HPV testing with the HarmoniaHPV and VenusHPV assays (Shanghai ZJ Bio-Tech Co. Ltd., China) was performed in batches of 48 tests and 12 tests per run excluding controls using lyophilized reagents, respectively. Aliquots of 250 μL were taken from specimens and placed into an AutraMic mini4800 Plus (also named as ChinKing mini) for HPV testing with fully automated workflow. ChinKing mini system performs DNA extraction, amplification, and interpretation of the test results. The system yielded 75 μL of DNA extract, of which 4 μL was used for amplification by each test. Both tests are based on multiplex real-time PCR. The HarmoniaHPV assay can identify and distinguish HPV16 and HPV18, while the other 12 HPV genotypes are pooled together (HPV58, 33, 45, 31, 52, 35, 39, 59, 51, 56, 66, and 68). The HarmoniaHPV PCR primers target the *E6* gene of HPV18, *L1/L2* genes of HPV52 and HPV58, and the *E1* gene of the remaining HPV types, including HPV16. VenusHPV assay identifies 15 HPV genotypes individually targeting *E1* gene of HPV16, HPV82, *E6* of HPV18, HPV68, *E7* of HPV33, *E2* of HPV35, HPV66, *L1* of HPV31, HPV39, HPV45, HPV52, HPV56, HPV59 and *L2* of HPV51, HPV58 genotypes. Both tests include single-copy human minibrain gene (*MNBH)* as an internal control to assess sample validity, negative control, and positive HPV controls to assess contamination and sample integrity/validity. Samples are considered valid if the C_t-value of the internal control *MNBH* is ≤ 30. HPV positivity was defined at C_t-values ≤ 30 and ≤ 35 for HarmoniaHPV and VenusHPV, respectively.

2.3 | Statistical Analysis

To evaluate clinical accuracy, we used biopsy outcomes as a gold standard and colposcopy if no biopsy was performed. Biopsy outcomes were categorized as cervical intraepithelial neoplasia grade 0 (CIN0), CIN1, CIN2, and CIN3. If no biopsy outcome was available, normal or minor colposcopy impressions were categorized as < CIN2. Differences in clinical accuracy between HPV testing on self-samples (the index) and HPV testing on cervical samples (comparator) were evaluated using the McNemar test. Accuracy was estimated for following outcomes: < CIN2 for specificity and ≥ CIN2+ and CIN3 for sensitivity. As VenusHPV is a full genotyping test individually detecting HPV82 in addition to 14 hrHPV, HPV82 was excluded

from accuracy estimation. A post hoc cut-off optimisation was performed applying an iterative statistical procedure for VenusHPV assay to reach optimal balance between specificity and sensitivity. HPV test concordance between specimens was evaluated with Cohen's kappa: 0.00–0.19 poor, 0.20–0.39 fair, 0.40–0.59 moderate, 0.60–0.79 good and 0.80–1.00 excellent concordance. The difference in median C_t-values between samples was evaluated using the Mann–Whitney test for matched comparison and Wilcoxon signed-rank test for non-matched comparison. Statistical analyses were performed using Stata 16.1 (College Station, Texas, USA).

2.4 | Ethical Approval

The VALHUDES (NCT03064087) study was designed in accordance with the Helsinki declaration of 1964 and was approved by the central Ethics Committee of the University Hospital of Antwerp/University of Antwerp (B300201733869) and the local Ethics Committees of all other centers involved in the study.

3 | Results

3.1 | Study Population

Out of 523 samples, 24 were excluded due to major protocol violations as previously described [12, 13] and 499 triplets were tested with Liferiver HarmoniaHPV and VenusHPV assays (Figure 1). Of this cohort 17.6% (88/411) participants were categorised as CIN2+ and 9.0% (45/454) as CIN3. Median age of CIN2 negative cases (median age = 41 years) was significantly higher than median age of women with CIN2+ (median age = 38 years, $p < 0.05$). HarmoniaHPV data set contained 495 matched valid cervical and vaginal samples, and 496 matched valid cervical and FVU samples. VenusHPV data set contained 497 matched valid cervical and vaginal, and 496 valid matched cervical and FVU samples. Figure 1 shows number of CIN2+ and < CIN2 cases for each data set. Study characteristics of the population are reported in Table 1.

3.2 | HarmoniaHPV

HarmoniaHPV assay on FVU versus cervical samples was as accurate to identify CIN2+ (sensitivity ratio = 0.95; 95% CI 0.89–1.02) and < CIN2 (specificity ratio = 0.95; 95% CI 0.86–1.04) compared to cervical samples, whereas sensitivity for CIN3 was 10% lower than on cervical samples. Sensitivity for CIN2+ and CIN3 for each device separately resembled combined sensitivity. HarmoniaHPV was 16% less specific with QT compared to EB (non-matched comparison ratio = 0.84 95% CI 0.67–1.06) (Table 2). Absolute accuracy and relative accuracy for women of age 30 and older are presented in Supporting Information S1: Tables S1 and S2.

Higher concordance was observed between vaginal and cervical samples ($k = 0.73$–0.86), than between FVU and cervical samples ($k = 0.60$–0.69) for overall hrHPV, HPV16, HPV18, and other hrHPV (Table 3). Concordance by disease status is reported in Supporting Information S1: Tables S3–S4.

HrHPV, HPV16, and other hrHPV median C_t-values were significantly higher in FVU compared to cervical samples ($p < 0.05$) (Supporting Information S1: Table S5). Similarly, significantly higher median C_t-values were detected in vaginal self-samples for overall hrHPV and HPV16 ($p < 0.05$), but not for other hrHPV. *MNBH* gene median C_t-values were lower in cervical samples compared to vaginal ($p < 0.05$), but not compared to FVU (Supporting Information S1: Table S6). Median C_t-value comparison by disease status demonstrated significantly higher C_t-values in < CIN2 vs. CIN2+ cases in all sample types for overall hrHPV, in cervical and vaginal samples for

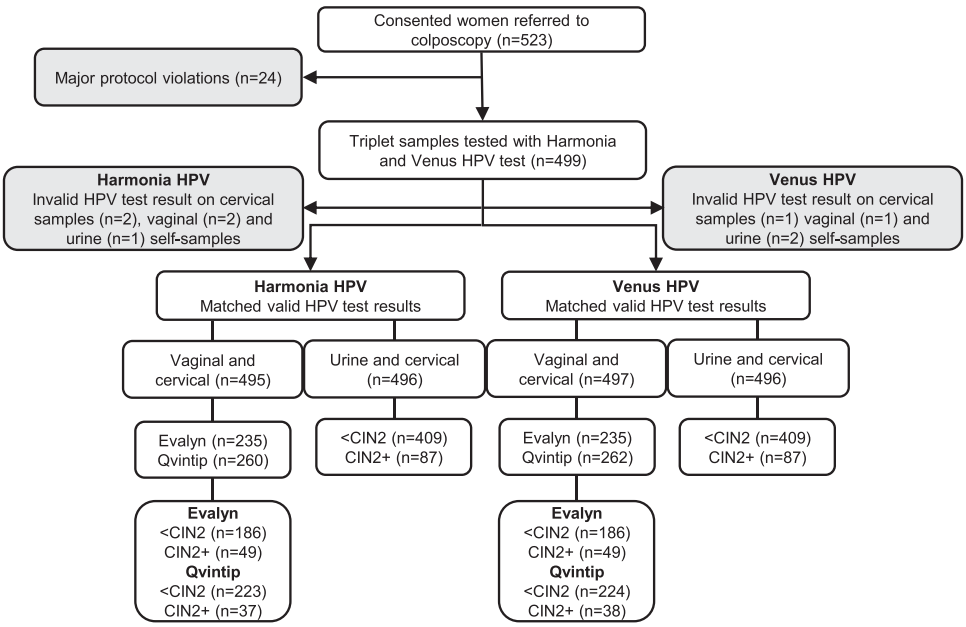

FIGURE 1 | Flow chart of samples included in the VALHUDES trial tested with the Liferiver HarmoniaHPV and VenusHPV assays. Grey boxes represent excluded samples. Detailed exclusions are reported elsewhere [12, 13].

TABLE 1 | Study population characteristics.

Age category (years)	Participants *N* (%)	Cervical hrHPV Pos *N* (%)	Vaginal hrHPV Pos *N* (%)	Urine hrHPV Pos *N* (%)
HarmoniaHPV				
< 30	97 (19.5)	62 (23.0)	63 (24.7)	52 (20.2)
30–39	141 (28.4)	79 (29.3)	72 (28.2)	73 (28.4)
40–49	136 (27.4)	64 (23.7)	58 (22.8)	69 (26.9)
50–59	96 (19.3)	52 (19.3)	50 (19.6)	49 (19.1)
60+	27 (5.4)	13 (4.8)	12 (4.7)	14 (5.5)
Total	497 (100.0)	270 (100.0)	255 (100.0)	257 (100.0)
VenusHPV				
< 30	97 (19.5)	65 (22.5)	69 (24.3)	61 (23.0)
30–39	142 (28.4)	82 (28.4)	80 (28.2)	74 (27.9)
40–49	136 (23.7)	68 (23.5)	62 (21.8)	61 (23.0)
50–59	96 (19.3)	60 (20.7)	59 (20.8)	57 (21.5)
60+	27 (5.4)	14 (4.8)	14 (4.9)	12 (4.5)
Total	498 (100.0)	289 (100.0)	284 (100.0)	265 (100.0)

Abbreviations: CIN, cervical intraepithelial neoplasia; hrHPV, high-risk HPV; Pos, HPV positivity.

TABLE 2 | Relative accuracy of the Liferiver HarmoniaHPV assay on self-samples compared to clinician-collected cervical samples.

	Relative sensitivity [95% CI] CIN2 +	Relative sensitivity [95% CI] CIN3	Relative specificity [95% CI] < CIN2
HarmoniaHPV			
Manufacturer's cut-off all types C_t-value ≤ 30			
FVU	0.95 [0.89–1.02]	0.90 [0.82–0.998]	0.95 [0.86–1.04]
Evalyn/Qvintip[a]	0.95 [0.88–1.02]	0.91 [0.82–0.998]	0.93 [0.86–1.01]
Evalyn	0.96 [0.88–1.04]	0.91 [0.81–1.04]	1.01 [0.91–1.14]
Qvintip	0.94 [0.83–1.06]	0.90 [0.77–1.04]	0.86 [0.77–0.96]
VenusHPV			
Manufacturer's cut-off all types C_t-value ≤ 35			
FVU	0.84 [0.76–0.93]	0.78 [0.66–0.92]	1.05 [0.96–1.14]
Evalyn/Qvintip[a]	0.91 [0.85–0.98]	0.91 [0.82–0.998]	0.99 [0.92–1.06]
Evalyn	0.91 [0.83–0.998]	0.91 [0.80–1.04]	0.98 [0.88–1.09]
Qvintip	0.91 [0.83–1.01]	0.90 [0.78–1.04]	0.99 [0.90–1.09]
New cut-off: HPV16, 18, 45 C_t ≤ 38.4; HPV31, 33, 35, 39, 52, 58 C_t ≤ 37; all others C_t ≤ 32			
FVU	0.94 [0.88–1.002]	0.90 [0.82–0.998]	1.00 [0.92–1.09]
Evalyn/Qvintip[a]	0.96 [0.92–1.01]	0.95 [0.89–1.02]	0.95 [0.88–1.02]
Evalyn	0.98 [0.94–1.02]	0.95 [0.87–1.05]	0.97 [0.87–1.08]
Qvintip	0.94 [0.87–1.02]	0.95 [0.86–1.05]	0.93 [0.83–1.03]

Note: Relative sensitivity and specificity for women ≥ 30 years old are shown in Supporting Information S1: Table S3. Matched numbers of cases used to estimate relative accuracy are present in Supporting Information S1: Table S4.
Abbreviations: CI, confidence interval; CIN, cervical intraepithelial neoplasia; FVU, first-void urine; *N*, number.
[a]Samples were collected with Evalyn Brush or Qvintip combined.

HPV16 and only in cervical samples for other hrHPV ($p < 0.05$) (Supporting Information S1: Tables S7–S9). Interestingly, in both self-sample types, but not in cervical samples *MNBH* gene median C_t-values were lower in CIN2+ cases ($p < 0.05$) compared to < CIN2 (Supporting Information S1: Tables S8 and S9).

3.3 | VenusHPV

Sensitivity of the VenusHPV assay on both self-sample types was significantly lower than on cervical samples for CIN2+ and CIN3 ($p < 0.05$), whereas specificity was similar. Cut-off

TABLE 3 | Type-specific agreement and test concordance between cervical and self-samples with HarmoniaHPV assay.

HPV type	+/+[a]	+/−	−/+	−/−	Concordance [%]	Kappa[b] [95% CI]
FVU vs. cervical						
Total population ($n = 496$)						
hrHPV[c]	263	42	48	143	81.9	0.615 (0.543–0.686)
HPV16	69	11	36	380	90.5	0.689 (0.607–0.771)
HPV18	19	5	13	459	96.4	0.660 (0.513–0.806)
Other	205	46	53	192	80.0	0.601 (0.530–0.671)
Evalyn Brush/Qvintip vs. cervical						
Total population ($n = 495$)						
hrHPV	279	25	34	157	88.1	0.746 (0.686–0.807)
HPV16	73	8	13	401	95.8	0.849 (0.786–0.912)
HPV18	20	4	2	469	98.8	0.863 (0.755–0.971)
Other	220	29	38	208	86.5	0.729 (0.669–0.789)
Evalyn Brush vs cervical						
Total population ($n = 235$)						
hrHPV	132	16	13	74	87.7	0.737 (0.648–0.827)
HPV16	41	4	4	186	96.6	0.890 (0.815–0.965)
HPV18	13	3	1	218	98.3	0.858 (0.720–0.995)
Other	96	18	16	105	85.5	0.710 (0.620–0.800)
Qvintip vs. cervical						
Total population ($n = 260$)						
hrHPV	147	9	21	83	88.5	0.755 (0.673–0.837)
HPV16	32	4	9	215	95.0	0.802 (0.698–0.906)
HPV18	7	1	1	251	99.2	0.871 (0.694–1.000)
Other	124	11	22	103	87.3	0.745 (0.664–0.826)

Note: Concordance by disease status is shown in Supporting Information S1: Table S3.
Abbreviations: CI, confidence interval;?hr, high risk; HPV, human papillomavirus; FVU, first-void urine; *N*, number.
[a] + /+ positive on self- and cervical samples, +/− positive only on cervical samples, −/+ positive only on self-samples, −/− negative on both sample types.
[b] Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: 0.00–0.20 Poor; 0.21–0.40 Fair; 0.41–0.60 Moderate; 0.61–0.80 Good; 0.81–1.00 Excellent.
[c] Fourteen carcinogenic HPV genotypes.

optimization resulted in sensitivity improvement for CIN2+ and CIN3 on FVU and vaginal self-samples with 95% CI including unity. When stratified by the vaginal devices EB and QT, both sensitivity and specificity were slightly higher for EB than QT (Table 2).

The concordance between vaginal and cervical samples ($k = 0.49$–0.91) was higher than the concordance between FVU and cervical samples ($k = 0.00$–0.84) for overall hrHPV and individual genotypes (Table 4). Concordance by disease status is reported in Supporting Information S1: Tables S10–S13.

Absolute accuracy and relative accuracy for women of 30 years and older are presented in Supporting Information S1: Tables S2 and S14.

Higher median C_t-values were identified in FVU samples for overall hrHPV, individual HPV genotypes 16, 31, 33, 35, 39, 52, 56, 58 and in vaginal samples for hrHPV, HPV16, 31, and 58 compared to cervical ($p < 0.05$) (Supporting Information S1: Tables S15 and S16).

MNBH gene median C_t-values were significantly higher in both self-sample types compared to cervical ($p < 0.05$) (Supporting Information S1: Tables S15 and S16).

When stratified by disease status, significantly lower median C_t-values of hrHPV and α-9 (HPV16, 31, 33, 35, 52, 58) genotypes were detected in all specimen types in CIN2+ cases versus < CIN2 ($p < 0.05$). C_t-values of α-5 (HPV51, 82) and α-6 (HPV56, 66) genotypes in FVU samples and C_t-values of α-6 (HPV56, 66) and α-7 (HPV18, 39, 45, 59, 68) genotypes in vaginal samples were lower in CIN2+ cases compared to < CIN2 ($p < 0.05$). In CIN2+ cases median *MNBH* gene C_t-values were always lower compared to < CIN2 in both self-sample types, but not in cervical samples (Supporting Information S1: Tables S17–S19).

4 | Discussion

In this report, the clinical accuracy of Liferiver HarmoniaHPV and VenusHPV tests on FVU and vaginal self-samples was evaluated. HarmoniaHPV demonstrated similar clinical accuracy

TABLE 4 | Type-specific agreement and test concordance between cervical and self-samples with VenusHPV assay.

HPV type	+/+[a]	+/−	−/+	−/−	Concordance [%]	Kappa[b] [95% CI]
FVU vs. cervical						
Total population ($n = 496$)						
hrHPV	229	58	36	173	81.1	0.617 (0.547–0.686)
HPV16	57	11	8	420	96.2	0.835 (0.763–0.907)
HPV18	17	7	6	466	97.4	0.710 (0.560–0.860)
HPV31	18	27	7	444	93.2	0.481 (0.333–0.628)
HPV33	13	5	6	472	97.8	0.691 (0.518–0.864)
HPV35	10	2	7	477	98.2	0.681 (0.484–0.877)
HPV39	14	9	8	465	96.6	0.604 (0.432–0.776)
HPV45	10	9	4	473	97.4	0.593 (0.392–0.794)
HPV51	34	12	16	434	94.4	0.677 (0.565–0.789)
HPV52	36	16	8	436	95.2	0.723 (0.618–0.828)
HPV56	33	7	11	445	96.4	0.766 (0.662–0.870)
HPV58	23	7	14	452	95.8	0.664 (0.530–0.798)
HPV59	2	4	6	484	98.0	0.276 (−0.037–0.588)
HPV66	27	5	7	457	97.6	0.805 (0.698–0.912)
HPV68	0	11	2	483	97.4	−0.007 (−0.016–0.003)
HPV82	5	2	5	484	98.6	0.581 (0.300–0.862)
Evalyn Brush/Qvintip vs. cervical vs. cervical						
Total population ($n = 497$)						
hrHPV	256	32	28	181	87.9	0.753 (0.694–0.812)
HPV16	64	5	6	422	97.8	0.908 (0.854–0.962)
HPV18	20	4	2	471	98.8	0.863 (0.755–0.971)
HPV31	34	10	9	444	96.2	0.761 (0.657–0.864)
HPV33	16	2	4	475	98.8	0.836 (0.707–0.965)
HPV35	10	2	4	481	98.8	0.763 (0.580–0.947)
HPV39	17	6	6	468	97.6	0.726 (0.578–0.874)
HPV45	12	7	4	474	97.8	0.674 (0.493–0.855)
HPV51	40	6	9	442	97.0	0.825 (0.739–0.912)
HPV52	40	12	6	439	96.4	0.796 (0.705–0.887)
HPV56	36	5	9	447	97.2	0.822 (0.731–0.913)
HPV58	28	2	7	460	98.2	0.852 (0.757–0.947)
HPV59	4	2	6	485	98.4	0.492 (0.190–0.795)
HPV66	28	4	5	460	98.2	0.852 (0.757–0.947)
HPV68	8	3	5	481	98.4	0.658 (0.437–0.880)
HPV82	6	1	5	485	98.8	0.661 (0.407–0.915)
Evalyn Brush vs. cervical						
Total population ($n = 235$)						
hrHPV	122	16	14	83	87.2	0.737 (0.650–0.825)
HPV16	38	3	3	191	97.5	0.911 (0.841–0.981)
HPV18	14	4	0	217	98.3	0.866 (0.737–0.995)
HPV31	11	4	3	217	97.0	0.743 (0.560–0.925)
HPV33	5	2	1	227	98.7	0.763 (0.503–1.000)

(Continues)

TABLE 4 | (Continued)

	HPV type	+/+[a]	+/−	−/+	−/−	Concordance [%]	Kappa[b] [95% CI]
	HPV35	4	1	2	228	98.7	0.721 (0.418–1.000)
	HPV39	5	2	4	224	97.5	0.612 (0.327–0.897)
	HPV45	2	2	3	228	97.9	0.434 (0.024–0.843)
	HPV51	19	4	5	207	96.2	0.787 (0.653–0.921)
	HPV52	19	4	5	207	96.2	0.787 (0.653–0.921)
	HPV56	16	2	3	214	97.9	0.853 (0.727–0.980)
	HPV58	16	2	3	214	97.9	0.853 (0.727–0.980)
	HPV59	0	0	4	231	98.3	0.000 (0.000–1.000)
	HPV66	13	3	3	216	97.5	0.799 (0.642–0.955)
	HPV68	4	1	1	229	99.2	0.796 (0.519–1.000)
	HPV82	1	1	2	231	98.7	0.394 (−0.152–0.940)
Qvintip vs. cervical							
Total population ($n = 262$)							
	hrHPV	134	16	14	98	88.6	0.767 (0.688–0.845)
	HPV16	26	2	3	231	98.1	0.902 (0.816–0.987)
	HPV18	6	0	2	254	99.2	0.853 (0.653–1.000)
	HPV31	23	6	6	227	95.4	0.767 (0.641–0.894)
	HPV33	11	0	3	248	98.9	0.874 (0.734–1.000)
	HPV35	6	1	2	253	98.9	0.794 (0.567–1.000)
	HPV39	12	4	2	244	97.7	0.788 (0.623–0.953)
	HPV45	10	5	1	246	97.7	0.757 (0.571–0.944)
	HPV51	21	2	4	235	97.7	0.862 (0.754–0.970)
	HPV52	21	8	1	232	96.6	0.805 (0.682–0.928)
	HPV56	20	3	6	233	96.6	0.797 (0.669–0.926)
	HPV58	12	0	4	246	98.5	0.849 (0.704–0.994)
	HPV59	4	2	2	254	98.5	0.659 (0.345–0.972)
	HPV66	15	1	2	244	98.9	0.903 (0.794–1.000)
	HPV68	4	2	4	252	97.7	0.560 (0.245–0.875)
	HPV82	5	0	3	254	98.9	0.764 (0.505–1.000)
	hrHPV	134	16	14	98	88.6	0.767 (0.688–0.845)

Note: Concordance by disease status is shown in Supporting Information S1: Table S5.
Abbreviations: CI, confidence interval; HPV, human papillomavirus; hr, high risk; FVU, first-void urine; *N*, number.
[a] + /+ positive on self- and cervical samples, +/− positive only on cervical samples, −/+ positive only on self-samples, −/− negative on both sample types.
[b]Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: 0.00–0.20 Poor; 0.21–0.40 Fair; 0.41–0.60 Moderate; 0.61–0.80 Good; 0.81–1.00 Excellent.

on both self-samples compared to cervical samples. VenusHPV on the other hand was significantly less sensitive but after cut-off optimisation sensitivity improved with 95% CI including unity. HarmoniaHPV has been previously evaluated within The VALidation of HPV GENotyping Tests framework (VALGENT4) [10], which was designed to evaluate the accuracy of HPV tests using a standardized set of cervical cell samples collected from cervical cancer screening programs enriched with CIN2 cases from colposcopy settings [14]. To date four VALGENT installments have been completed with more than dozen HPV tests assessed [15]. HarmoniaHPV assay was part of the fourth installment and was evaluated on cervical samples stored in 10 mL SurePath [10]. The assay was not specific enough and required post-hoc cut-off optimisation and, therefore, was considered partially validated. In the current VALHUDES settings, HPV testing was performed on cervical and vaginal samples resuspended in 20 mL ThinPrep, whereas FVU was collected with the 20 mL Colli-Pee device containing approximately 7 mL of UCM. Moreover, lyophilized reagents were used for HarmoniaHPV and VenusHPV in the current VALHUDES study. These variations in preanalytical parameters led to the application of different cut-off values for HPV positivity in both studies. Nevertheless, in current report HarmoniaHPV test did not require post hoc optimization.

On the other hand, this is the first clinical accuracy study for the VenusHPV test. The test was less sensitive on both FVU and

vaginal samples when HPV positivity was defined at ≤ 35 C_t-value for all types, while specificity was similar to cervical samples. Following cut-off optimisation, sensitivity improved without compromising specificity. We applied new type specific cut-offs according to carcinogenic risk as described by Wei et al. [16].

Yet two VALHUDES installments were set up. In the Belgian VALHUDES, women collected FVU with the Colli-Pee device, while vaginal self-samples were taken with either EB or QT and placed in 20 mL ThinPrep [9]. Whereas in the European VALHUDES, including specimens from Italy and Scotland, FVU was collected with Colli-Pee, but vaginal sample was collected with FloqSwab and then placed in 5 mL eNAT medium (Copan Italia Spa, Brescia, Italy) [17, 18]. To date, eight assays were evaluated in the Belgian (Alinity m HR HPV, BD Onclarity HPV, Cobas 4800, Cobas HPV, Liferiver HarmoniaHPV and VenusHPV, RealTime High-Risk HPV, and Xpert HPV) [12, 13, 19–22] (Van Keer et al., submitted) and two in the European VALHUDES (OncoPredict QT and OncoPredict SCR) projects [17, 18]. All assays were evaluated on both FVU and vaginal self-samples, except for the Cobas 4800 and Cobas HPV assays, which were tested solely on urine samples, and the Xpert HPV assay, which was assessed only on vaginal samples. Five out of ten assays required post-hoc cut-off optimization to ensure similar accuracy on self- compared to clinician samples. Cut-off optimization is often required to achieve satisfactory clinical performance of the test.

Previous discussions have highlighted that variations in pre- and post-analytical laboratory workflows can influence clinical accuracy [23–26]. Sensitivity for detecting cervical intraepithelial neoplasia tends to be robust and less impacted by different workflows, whereas specificity can vary more substantially. For instance, in our previous VALHUDES reports, we often observed that when a smaller volume of resuspension medium is used, specificity tends to be lower [20, 26, 27]. Lower resuspension volume yields more concentrated sample and, therefore, more viral DNA particles are detected leading to lower specificity. These differences can be addressed through cut-off optimization to balance sensitivity and specificity. Another variable that could potentially impact the accuracy of such a diagnostic study is the sampling order. In our study, sampling order was defined as from least to most invasive minimising the impact of prior sampling on subsequent samples. Urine samples were collected at home 1 day before colposcopy as often women urinate before doctors visit to address concerns that women might urinate before their colposcopy appointment, potentially washing away exfoliated cells and compromising sample quality. The clinician-collected cervical sample was performed last, as it involves physical manipulation of the cervix, which could influence the quality of subsequent samples by causing slight bleeding or other complications.

We also investigated whether C_t-values varied by type of collection (first-void urine, vaginal self, cervical clinician-collected) or disease status (< CIN2, CIN2+). Previous studies have shown that the amount of viral DNA decreases toward the lower genital tract as viral infection and replication occurs in transformation zone, from where cervical cells and viral particles are then shed towards lower genital tract [28]. Similar trend was observed in current and previous VALHUDES data with lower viral median C_t-values detected in cervical compared to self-samples indicating a higher amount of viral DNA in cervical samples [17, 22]. Additionally, C_t-values were correlated with clinical outcome [29, 30], which was also reported in our current and previous VALHUDES reports with median viral C_t-values lower in women with cervical neoplasia compared to healthy women [17–19]. C_t values reflect viral load and viral load may correlate to some degree with disease development. While our study did not directly measure viral load, but C_t-value as an indication, it is widely used today. However, more accurate method for viral load estimation such as viral copies per cell or per μL may be clinically relevant to appreciate changes over time and predict cervical disease progression.

In conclusion, Liferiver HarmoniaHPV assay was similarly sensitive and specific for outcome CIN2+ in both FVU and vaginal samples compared to clinician collected samples. For Liferiver VenusHPV test, cut-off optimisation was necessary to improve sensitivity without compromising specificity with 95% CI of relative values including unity for both self-sample types versus cervical samples.

Author Contributions

Principal investigator and conceptualization of VALHUDES: Marc Arbyn. Protocol development: Marc Arbyn, Ardashel Latsuzbaia, Severien Van Keer, Davy Vanden Broeck, Alex Vorsters, and Gilbert Donders. Funding acquisition: Marc Arbyn and Alex Vorsters. Project administration: Ardashel Latsuzbaia and Marc Arbyn. Enrolment of patients: Steven Weyers, Gilbert Donders, Philippe De Sutter, Wiebren Tjalma, and Jean Doyen. Data Curation and formal analysis: Ardashel Latsuzbaia, and Marc Arbyn. Sample handling: Davy Vanden Broeck and Severien Van Keer. Drafting original manuscript: Ardashel Latsuzbaia. Critical review and editing of manuscript: all authors.

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Ethics Statement

VALHUDES trial (NCT03064087) was approved by the central Ethics Committee of the University Hospital of Antwerp/University of Antwerp (B300201733869) and the local Ethics Committees of all the other involved centres. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants before enrolment.

Conflicts of Interest

The VALHUDES project is a researcher-induced study, designed by Sciensano (Principal Investigator; Brussels, Belgium), CEV (University of Antwerp, Antwerp, Belgium), and AML (Antwerp, Belgium). Manufacturers of HPV assays and devices can participate in the VALHUDES

framework contributing equipment for laboratory testing and financial support for statistical analysis under the condition of accepting independent publication of results. This research was supported by Liferiver Bio-Tech (San Diego, Ca), Novosanis NV (Wijnegem, Belgium), and University of Antwerp (Antwerp, Belgium). The study group received sample collection devices from Rovers Medical Devices B.V. (Oss, The Netherlands) and Aprovix AB (Uppsala, Sweden).

The funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. A. Vorsters is cofounder and former board member of Novosanis (Belgium), a spin-off company of the University of Antwerp, and was minority shareholder until January 2019. The University of Antwerp received payment for participation of S. Van Keer in an Advisory Board of Novosanis (subsidiary of OraSure Technologies Inc, Pennsylvania, USA). All funds are handled and managed by the University of Antwerp. D. Vanden Broeck is employed by AML (Antwerp, Belgium), part of the National Reference Centre HPV, a private lab performing routine cervical cytology and HPV testing.

Data Availability Statement

Final study datasets generated by VALHUDES will be stored locally and securely at Sciensano. Anonymized data will be available by request to the corresponding author on a case-by-case basis pending approval from the information security coordinator at Sciensano.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.