Analytical and clinical performance of OncoPredict HPV Screening (SCR) assay on self-collected vaginal and urine specimens within the VALHUDES Framework

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Abstract

The introduction of self-sampling in cervical cancer screening has raised the importance of HPV testvalidation on self-collected samples. This study aimed to evaluate the clinical performance of the OncoPredict HPV Screening (SCR) assay on self-collected vaginal and first-void urine (FVU) as part of the VALHUDES framework. Vaginal (FLOQSwabs) and FVU (Colli-Pee) samples were self-collected by 500 women referred to colposcopy, followed by a clinician-collected cervical sample prior to colposcopy, which were all tested using OncoPredict HPV SCR. OncoPredict HPV SCR demonstrated similar relative clinical sensitivity to detect cervical intraepithelial neoplasia grade 2 or worse ([?] CIN2) in urine (ratio: 0.95 [95%CI 0.88-1.02]) and vaginal self-samples (ratio: 0.96 [95%CI 0.90-1.02]) compared to cervical samples. The clinical specificity was lower in vaginal but not in urine samples compared to cervical which improved following cut-off optimization. A higher cellularity was found in vaginal as compared to cervical and FVU samples. Moderate to excellent agreement in HPV detection in self-collected samples and cervical scrapes was demonstrated (Kappa values: 0.53 to 1.00). OncoPredict HPV SCR assay demonstrated similar accuracy on self-collected vaginal and FVU samples compared to cervical samples, although cut-off adjustment improved clinical specificity when applied to vaginal samples.

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Shortened title: OncoPredict SCR accuracy on self-samples

Abstract

The introduction of self-sampling in cervical cancer screening has raised the importance of HPV testvalidation on self-collected samples. This study aimed to evaluate the clinical performance of the OncoPredict HPV Screening (SCR) assay on self-collected vaginal and first-void urine (FVU) as part of the VALHUDES framework.

Vaginal (FLOQSwabs) and FVU (Colli-Pee) samples were self-collected by 500 women referred to colposcopy, followed by a clinician-collected cervical sample prior to colposcopy, which were all tested using OncoPredict HPV SCR.

OncoPredict HPV SCR demonstrated similar relative clinical sensitivity to detect cervical intraepithelial neoplasia grade 2 or worse ([?] CIN2) in urine (ratio: 0.95 [95%CI 0.88-1.02]) and vaginal self-samples (ratio: 0.96 [95%CI 0.90-1.02]) compared to cervical samples. The clinical specificity was lower in vaginal but not in urine samples compared to cervical which improved following cut-off optimization. A higher cellularity was found in vaginal as compared to cervical and FVU samples. Moderate to excellent agreement in HPV detection in self-collected samples and cervical scrapes was demonstrated (Kappa values: 0.53 to 1.00).

OncoPredict HPV SCR assay demonstrated similar accuracy on self-collected vaginal and FVU samples compared to cervical samples, although cut-off adjustment improved clinical specificity when applied to vaginal samples.

Key words (3 to 6): cervical cancer, self-sampling, diagnostic accuracy study, Human Papillomavirus (HPV), OncoPredict HPV SCR, European VALHUDES

Introduction

As proposed in the World Health Organization (WHO) call to action, screening of 70% of women a key target to support the elimination of cervical cancer by 2030 ([1]).

Human Papillomavirus (HPV) testing has been demonstrated to be more effective than cytology for secondary prevention of cervical cancer ([2,3]) and is therefore recommended as a primary screening tool in current

screening algorithms ([4]). Additionally, meta-analyses have shown that the clinical accuracy of PCR-based HPV tests on self-samples is similar to that of cervical samples ([5,6]).

In 2021, 48 countries recommended primary HPV-based screening and 17 introduced self-sampling in their national programs or guidelines ([7]) as response to WHO Call to Action ([1]).

While several HPV tests are currently validated for use in cervical cancer screening ([8]), only a few are formally validated for use on self-collected specimens. The VALidation of HUman papillomavirus assays and collection Devices for Self-samples and urine samples (VALHUDES) Framework has defined a standardized protocol to assess the clinical performance of HPV tests in combination with self-collection devices ([9]). Results of a first installment of VALHUDES demonstrated similar accuracy of first void urine (FVU) collected with the Colli-Pee device and vaginal specimens compared to clinician-collected cervical samples using different HPV assays ([10–15]). We now report on a second iteration of VALHUDES undertaken in a different geographic setting and utilizing a different approach to vaginal sampling.

This present study aimed to evaluate the clinical performance of the OncoPredict HPV Screening (SCR) assay on vaginal self-samples collected with FLOQSwab resuspended in 5 ml eNat and FVU with Colli-Pee as compared to clinician-collected cervical scrapes to detect high-grade cervical lesions. Secondarily, we investigated the analytical performance of the assay and evaluated the adequacy of self-collected samples.

Material and Methods

2.1 Study design

Within the European VALHUDES Framework, 600 women, referred to colposcopy following a previous cervical abnormality or HPV positivity, were enrolled between July 2020 and February 2022 in four colposcopy centers (NHS Lothian, Edinburgh; ASST degli Spedali Civili di Brescia, Brescia, Italy; European Institute of Oncology IRCCS, Milan, Italy; U.O. Coordinamento Consultori Familiari, ASSL Sassari - ATS Sardegna, Sassari, Italy). Exclusion criteria have already been described (Latsuzbaia et al., submitted).

All women were asked to collect a urine sample followed by a vaginal specimen. FVU was collected using Colli-Pee FV5000 (Novosanis, Wijnegem, Belgium). The device captures approximately 13 ml of FVU that are mixed with 7 ml nucleic acid preservative included within the collection device. Vaginal self-collection was performed using a FLOQSwab (Copan Italia Spa, Brescia, Italy). During gynecological examination, a cervical specimen was collected by a clinician with Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) and immediately transferred in 20 ml PreservCyt (Hologic Inc., Bedford, Massachusetts, USA).

All women underwent colposcopy and biopsy was performed if clinically required. The histological result of the biopsy was used to determine the disease outcome.

Self-collected vaginal samples were transported dry to the laboratory together with the 20 ml PreservCyt vial containing cervical samples and the Colli-Pee tube containing FVU. All specimens were transported to the laboratories affiliated with the enrolling colposcopy centers. After arrival in the laboratories, cervical and FVU specimens were shaken for 30 seconds and divided into 1.5 ml aliquots. The dry vaginal swabs were resuspended in 5 ml of PreservCyt (Hologic Inc., Bedford, Massachusetts, USA) or 5 ml eNat (Copan Italia Spa, Brescia, Italy). Vaginal samples were further aliquoted into 0.4 ml volumes. All aliquots were stored at -20 °C until transferred to MIRRI-IT Biobank of the University of Milano-Bicocca where they were stored at -80 °C. Results reported in this manuscript are from the 500 women whose vaginal swabs were resuspended in eNat.

2.2 HPV testing

Testing of all specimens was performed at the Laboratory Clinical Microbiology and Virology, School of Medicine and Surgery, University of Milano-Bicocca (Monza, Italy). Nucleic acid extraction was performed using a Fluent 480 (Tecan, Männedorf, Switzerland) automated platform with *Quick* -DNA/RNA MagBead (Zymo, USA) starting from 400 µl of sample. Fluent 480 workstation was also used to set-up the real-time PCR plate of OncoPredict HPV SCR assay (Hiantis, Milan, Italy) according to manufacturer instructions

with 10 µl of mastermix and 5 µl of sample's DNA extract. The OncoPredict HPV SCR assay, previously validated for testing on cervical scrapes in a screening setting ([16]), is a partial genotyping assay targeting E6 and E7 DNA sequences of 13 high-risk Human Papillomavirus (hrHPV) types (HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68). The test is composed of two separate real-time PCR reactions. A quality control tube (QC) well allows the assessment of nucleic acid extraction recovery with an exogenous control gene target added to the sample before preanalytical processing, additionally the QC tube determines sample adequacy in terms of human cellularity by the quantification of*C-C Motif Chemokine Receptor 5* (CCR5) gene. A second reaction well is used to assess the presence of HPV-16, HPV-18 individually and the 11 other hrHPV types as a pool. Both wells contain an amplification control to evaluate the potential PCR inhibition. The PCR was carried out using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA). All results were considered valid if HPV positive. In the case of HPV negative result(s) samples were defined as inadequate if i) the extraction efficiency was below 10%; ii) less than 400 cells/reaction in cervical samples ([16]) and 150 cells/reaction in urine and vaginal samples were detected and iii) there was PCR inhibition in any of the two reaction wells.

2.3 Statistical analysis

Clinical sensitivity was estimated for cervical intraepithelial neoplasia grade 2 or worse ([?]CIN2) and for cervical intraepithelial neoplasia grade 3 or worse ([?]CIN3). Specificity was estimated for <CIN2 outcome or accepting negative colposcopy as clinical endpoint when the gynecologist did not take a biopsy. We used McNemar tests to evaluate the accuracy differences between index and comparator tests with statistical significance accepted if p-values <0.05 or when the 95% confidence intervals excluded 1. Cohen's kappa was employed to assess HPV test concordance between self- and clinician taken samples for the entire study population and according to disease status among specimens and categorized as: poor (0.00–0.19), fair (0.20–0.39), moderate (0.40–0.59), good (0.60–0.79), and excellent (0.80–1.00). Mann-Whitney test was used to evaluate differences in median Ct-values and median number of cells/reaction. All statistical analyses were conducted using Stata 16.1 (Statacorp, College Station, TX, USA).

2.4 Ethical Approval

The European VALHUDES study (ClincalTrail.gov: NCT04312737) was conducted in accordance with the Declaration of Helsinki and approved by the central Ethics Committee of the Coordinating Centre, ASST degli Spedali Civili di Brescia, Brescia, Italy (Ethics approval number: NP 3879- Studio WP6-HPVONC) on the 16th of July 2020, subsequently by the local Ethics Committees of the other participating centres. All women signed a written informed consent form before to enrolment.

Consorzio Italiano per la Ricerca in Medicina (C.I.R.M.), Milano, Italy, performed on site and remote monitoring of the study conduction, as previously described (Latsuzbaia et al., submitted).

Results

3.1 Study population

490 out of the 500 women were included in the study as reported in Figure 1. The median age of the women included in the study was 37 years (IQR: 31-47 years, range: 25-64 years), with median age of women with [?]CIN2 lesions being significantly lower of those with <CIN2 result, as previously described (Latsuzbaia et al., submitted). 489 women had colposcopy with the following outcomes: 134 (27.4%) negative, 245 (50.1%) minor colposcopy findings, 104 (21.3%) major colposcopy and 6 (1.2%) suspicion of cancer. 55% (271/490) of women underwent biopsy and diagnosis of [?]CIN2 was confirmed in 41.3% (112/271) of cases. Table 1 reports the characteristics of the study population by age group and colposcopy center. 28 cervical specimens, 13 vaginal swabs and 19 FVU samples were excluded from the analysis because they were inadequate and HPV-negative (Figure 1).

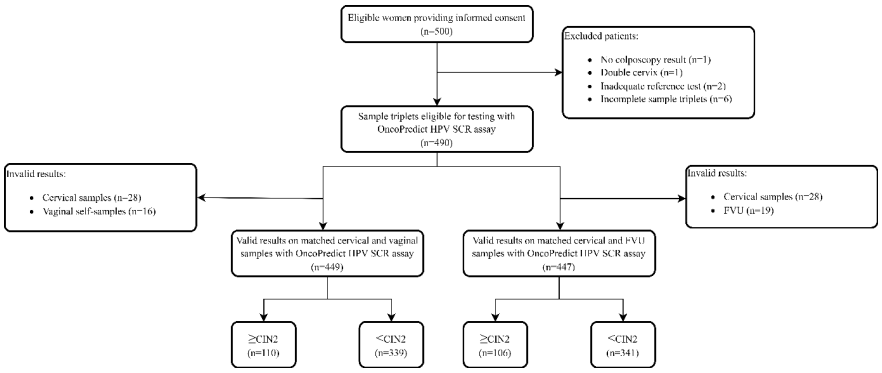

Figure 1: Flowchart of samples included in the analysis for the accuracy of OncoPredict HPV SCR assay within the VALHUDES Framework.

Table 1: Histologically confirmed outcomes by age group and colposcopy center.

Age group (years)	**Participants (n (%))**	**Biologically confirmed disease outcome**		
		[?]CIN2 (n (%))	**[?]CIN3 (n (%))**	**<CI**
<30	93 (19.0)	23 (20.5)	14 (20.0)	70 (1
30	397 (81.0)	89 (79.5)	56 (80.0)	308 (
Total	490 (100.0)	112 (100.0)	70 (100.0)	378 (
Colposcopy center	**Participants (n (%))**	**Biologically confirmed disease outcome**		
		[?]CIN2 (n (%))	**[?]CIN3 (n (%))**	**<CI**
Edinburgh	191 (39.0)	37 (33.0)	27 (38.6)	154 (
Brescia	49 (10.0)	5 (4.5)	0 (0.0)	44 (1
Milan	150 (30.6)	63 (56.3)	41 (58.6)	87 (2
Sassari	100 (20.4)	7 (6.3)	2 (2.9)	93 (2
Total	490 (100.0)	112 (100.0)	70 (100.0)	378 (

CIN: cervical intraepithelial neoplasia

*217 cases were categorized as <CIN2 based on clinical/colposcopic criteria without biopsy.

3.2 Sample's adequacy

All hrHPV-positive samples were considered valid. 5.7% (28/490) cervical, 3.3% (16/490) vaginal and 3.9% (19/490) FVU hrHPV-negative specimens were inadequate. Most cervical (23/28) and FVU (12/19) samples were invalid because of a low cellularity, while only 3 vaginal samples showed cellularity below the cut-off. Invalidity in this group of samples was mainly related to extraction efficiency (13/16). As shown in Table 2, the cellularity of vaginal self-collected specimens (transferred into 5 ml of eNat was demonstrated to be more than 10-fold higher than that of cervical (transferred into 20 ml PreservCyt) and FVU samples.

Table 2: Median values of cellularity (cells/reaction) across different types of samples.

	Matched cervical and vaginal specimens (n=449)	**Matched cervical and FVU s**
	Clinician- collected cervical samples	Vaginal self-collected samples
Median cellularity (IQR)	3875 (1469-8956)	42049 (258523-59300)

3.3 Clinical accuracy of OncoPredict HPV SCR assay

Clinical sensitivity for the detection of [?]CIN2 and [?]CIN3 and specificity for the detection of <CIN2 of OncoPredict HPV SCR assay on self-collected samples relative to cervical scrapes are reported in Table 3. Using manufacturer's cut-offs, clinical sensitivity of OncoPredict HPV SCR assay for [?]CIN2 on FVU (ratio=0.95 [95%CI 0.88-1.02]) and vaginal (ratio=0.96 [95%CI 0.90-1.02]) samples was not different to that of cervical specimens. Specificity for <CIN2 on FVU was similar to cervical (ratio=1.03 [95%CI 0.96-1.12]), whereas specificity on vaginal samples was slightly lower (ratio=0.90 [95%CI 0.84-0.96]).

After cut-off adjustment, the specificity on vaginal samples improved (ratio=0.94 [0.88-1.01]). Supplementary Table 1 provides data in terms of clinical accuracy in women older or equal 30.

Table 3**:** Relative accuracy of OncoPredict HPV SCR assay on vaginal and FVU self-samples versus cervical specimens.

	Relative sensitivity [95%CI] for [?]CIN2 detection	**Relative sensitivity [95%CI] fo**
Manufacturer cut-offs[1]		
Vaginal self-sample	0.96 [0.90-1.02]	0.95 [0.87-1.04]
FVU	0.95 [0.88-1.02]	0.93 [0.85-1.03]
New cut-offs[2]		
Vaginal self-sample	0.95 [0.90-1.00]	0.93 [0.86-1.01]

CI, confidence interval; CIN, cervical intraepithelial neoplasia;

[1] Manufacturer's positivity threshold for all hrHPV types in cervical samples and vaginal self-sample: Ct [?] 40;

[2] New a posteriori cut-offs vaginal self-sample: HPV16 Ct [?] 39, HPV18 Ct [?] 37, other hrHPV16 Ct [?] 38.

3.4 hrHPV positivity and concordance

Out of 449 women with matched cervical and vaginal specimens, 256 (57.0%) cervical and 270 (60.1%) vaginal specimens were hrHPV-positive. Out of 447 matched cervical and FVU samples, 250 (55.9%) cervical swabs and 256 (57.3%) FVU tested hrHPV-positive.

Moderate to excellent agreement with Kappa values ranging from 0.53 to 1.00 between both vaginal and FVU self-collected samples and cervical scrapes was demonstrated (Tables 4 and 5). In general, vaginal samples showed higher test agreement with cervical specimens than FVU.

In matched cervical and vaginal hrHPV-positve samples, median Ct values were significantly lower in vaginal samples compared to cervical for 11 other hrHPV, but not for HPV16 or HPV18 (Supplementary Figure 1). In matched cervical and FVU hrHPV-positive samples, median Ct values were always higher in FVU as compared to cervical samples. However, the difference was not significant for HPV18 (Supplementary Figure 2).

Table 4: Concordance between vaginal self-collected and clinician-collected cervical samples using manufacturer's cut-offs.

Total population (n=450)	**HPV type**	+/+	+/-	-/+	-/-	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	241	15	29	164	90.2	0.798 (0.742 - 0.855)
	HPV16	60	7	10	372	96.2	0.854 (0.786 - 0.921)
	HPV18	13	2	4	430	98.7	0.806 (0.654 - 0.957)
	Other hrHPV	185	13	38	213	88.6	0.773 (0.714 - 0.831)
[?]CIN2 (n=110)	**HPV type**	+/+	+/-	-/+	-/-	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	90	6	2	12	92.7	0.708 (0.519 - 0.897)
	HPV16	34	4	5	67	91.8	0.820 (0.708 - 0.933)

	HPV18	3	1	0	106	99.1	0.853 (0.568 - 1.000)
	Other hrHPV	64	4	5	37	91.8	0.826 (0.717 - 0.935)
<CIN2 (n=339)	**HPV type**	**+/+**	**+/-**	**-/+**	**-/-**	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	151	9	27	152	89.4	0.788 (0.723 - 0.853)
	HPV16	26	3	5	305	97.7	0.854 (0.754 - 0.953)
	HPV18	10	1	4	324	98.5	0.792 (0.616 - 0.969)
	Other hrHPV	121	9	33	176	87.6	0.747 (0.677 - 0.818)

N: number; CI: 95% confidence interval; CIN: cervical intraepithelial neoplasia

+/+ positive on urine and cervical samples, +/- positive only on cervical samples, -/+ positive only on urine samples, -/- negative on both sample types.

Note: Color legend: for the concordance: dark green (1.00 [?] K > 0.80): excellent; light green (0.80 [?] K > 0.60): good; yellow (0.60 [?] K > 0.40): moderate; orange (0.40 [?] K > 0.20): fair; red (0.20 [?] K > 0.00): poor.

Table 5: Concordance between FVU and clinician-collected cervical samples using manufacturer's cut-offs.

Total population (n=447)	**HPV type**	**+/+**	**+/-**	**-/+**	**-/-**	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	212	38	27	170	85.5	0.707 (0.641 - 0.773)
	HPV16	56	10	6	375	96.4	0.854 (0.784 - 0.924)
	HPV18	11	3	3	430	98.7	0.779 (0.607 - 0.951)
	Other hrHPV	156	38	35	218	83.6	0.667 (0.597 - 0.737)
[?]CIN2 (n=106)	**HPV type**	**+/+**	**+/-**	**-/+**	**-/-**	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	84	8	3	11	89.6	0.607 (0.398 - 0.816)
	HPV16	31	6	5	64	89.6	0.770 (0.642 - 0.898)
	HPV18	2	1	1	102	98.1	0.657 (0.212 - 1.000)
	Other hrHPV	56	10	5	35	85.9	0.706 (0.569 - 0.843)
<CIN2 (n=342)	**HPV type**	**+/+**	**+/-**	**-/+**	**-/-**	**Agreement [%]**	**Kappa [95% CI]**
	hrHPV	128	30	24	159	84.2	0.681 (0.603 - 0.759)
	HPV16	25	4	1	311	98.5	0.901 (0.816 - 0.987)
	HPV18	9	2	2	328	98.8	0.812 (0.632 - 0.992)
	Other hrHPV	100	28	30	183	83.0	0.638 (0.554 - 0.723)

N: number; CI: 95% confidence interval; CIN: cervical intraepithelial neoplasia

+/+ positive on FVU and cervical samples, +/- positive only on cervical samples, -/+ positive only on FVU samples, -/- negative on both sample types.

Note: Color legend: for the concordance: dark green (1.00 [?] K > 0.80): excellent; light green (0.80 [?] K > 0.60): good; yellow (0.60 [?] K > 0.40): moderate; orange (0.40 [?] K > 0.20): fair; red (0.20 [?] K > 0.00): poor.

Discussion

The introduction of self-sampling in cervical cancer screening programs, further enhanced by the COVID19 pandemics ([17]), is an important instrument to reach 70% screening coverage as proposed in the WHO call to action ([1]). The similar clinical accuracy of PCR-based HPV tests on self-samples and clinician-collected cervical scrapes has been demonstrated in previous validation studies for other assays ([10–15]).

The present study demonstrated that the use of OncoPredict HPV SCR assay on self-collected vaginal specimens FLOQSwabs and resuspended in 5 ml of eNat and FVU collected using Colli-Pee FV5000 has

a similar clinical accuracy to detect [?]CIN2 and CIN3 lesions as compared to clinician-collected cervical samples. Clinical sensitivity of OncoPredict HPV SCR assay on FVU and vaginal samples was similar to cervical specimens, however specificity on vaginal samples was lower when applying manufacturer cut-off values. Cut-off optimization on vaginal self-collected samples resulted in an improvement in specificity without compromising sensitivity. A lower specificity for the detection of [?]CIN2 as compared to cervical specimen was also reported for the validation of BD Onclarity HPV test on FLOQSwabs resuspended in 3 ml of BD HPV self-collection diluent ([18]). On the other hand, in the Belgian VALHUDES, where vaginal samples were resuspended in 20 ml of PreservCyt, a posteriori cut-off determination was necessary to improve the clinical sensitivity, but not for the specificity ([11,12]).

Different preanalytical workflows of self-collected vaginal samples may affect on clinical accuracy of the test. Therefore, optimization and standardization of the procedures for handling and testing of self-samples are fundamental to ensure an optimal performance of the assay ([19]). Presently, the VALHUDES protocol has been developed to assess the performance of HPV tests in combination with self-collection devices ([9]). In the European VALHUDES, vaginal swabs have been collected using FLOQSwabs resuspended in 5 ml of eNat, while FVU was collected using a 20 ml Colli-Pee device. eNat is a transport medium that allows the preservation of nucleic acids, denaturation of proteins and inactivation of microbial agents. It has been previously used in combination with HPV molecular assays ([20,21]);two studies demonstrated a good analytical performance of FLOQSwabs resuspended in 5 ml of eNat as compared to cervical samples ([22,23]). Moderate to excellent agreement between vaginal and cervical specimens was also demonstrated in the present study.

Both urine and vaginal self-collected samples are well accepted by women ([24]), in particular this study confirmed that FVU is a non-invasive collection method with clinical accuracy for [?]CIN2 lesions comparable to cervical specimens, as previously reported ([14,15]).

Ensuring sample adequacy is crucial, particularly for self-collection, in order to prevent false-negative results ([25–27]). One of the main advantages of OncoPredict HPV SCR assay is the inclusion of a thorough quality assessment, both for the preanalytical and analytical phases. The assay allows to determine the efficiency of nucleic acid extraction and potential PCR inhibition through the use of external calibrators, as well as assessing adequacy of sample collection through a quantitative cellularity assessment. In most molecular assays an internal housekeeping gene is used for both sample adequacy and amplification assessment. Recent studies have underlined the importance of identifying the cause of invalidity ([25]) of performing quality controls for the assay in a separate reaction well ([26]). In the present study, no invalid result related to PCR inhibition was detected, underlying the good performance of the analytical process. The invalidity due to a low extraction efficiency could be attributed to errors in specific nucleic acid extraction runs that may be resolved by retesting samples after a new extraction. On the contrary, in case of low cellularity samples, in absence of other invalidity reasons, sample collection should be repeated ([25]). In general, the invalidity rate was higher in cervical samples than in self-collected samples. This could be related to different limits of acceptable cellularity for cervical and self-collected samples. Moreover, as previously discussed, in the present study vaginal samples were resuspended in 5 ml of eNat while cervical swabs in 20 ml of PreservCyt which may have resulted in lower sample cellularity. Finally, the inclusion of the QC module in OncoPredict HPV SCR assay allows to accurately verify the sample adequacy, avoiding false negative results in cervical cancer screening.

OncoPredict HPV SCR assay is a limited genotyping assay, identifying HPV16, HPV18 and/or "other" hrHPV genotypes, whereas OncoPredict HPV Quantitative Typing (QT) is a full genotyping assay that can distinguish all the 12 hrHPV types separately. Both assays have been independently validated on cervical and self-samples withing VALGENT and VALHUDES frameworks (Latsuzbaia et al., submitted, [16,28]), respectively. hrHPV-positive specimens identified with OncoPredict HPV SCR assay may benefit from complete genotyping using QT assay as a reflex test.

In conclusion, following a posteriori cut-offs adjustment the OncoPredict HPV SCR assay demonstrated similar clinical accuracy for [?]CIN2 lesions on self-collected vaginal and FVU samples compared to testing on clinician-collected cervical samples.

Authors Contributions

Principal investigator and conceptualization: CEC, MA. Protocol development: MA, CEC. Funding

acquisition: CEC, MA. Project administration: CEC, MA. Enrolment of patients: ADI, RP. Data curation and formal analysis: AL, MA, MM. Sample collection, handling and Methodology: ADI, RP MM, CG, FB, AFP. Drafting original manuscript: CG. Critical review and editing of manuscript: AL, ADI, AFP, CEC, CG, FB, FO, GT, HE, KC, MA, MM, RP.

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Copan Italia Spa (Brescia, Italy) has provided free FLOQSwab devices and eNat solution. Novosanis (Wijnegem, Belgium) has provided free Colli-Pee devices. Hiantis (Milan, Italy) has provided free kits and reagents for HPV testing.

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Conflict of Interest

The European VALHUDES is a researcher-induced study, coordinated by University of Milano-Bicocca (Monza, Italy), Sciensano (Bruxelles, Belgium), Istituto Europeo di Oncologia (Milan, Italy), University of Sassari (Sassari, Italy), U.O. Coordinamento Consultori Familiari, ASSL Sassari – ATS Sardegna (Sassari, Italy), NHS Lothian, University of Edinburgh (Edinburgh, Scotland), Trinity College Dublin (Dublin, Ireland).

Manufacturers of HPV assays (GeneFirst, Oxford, UK and Hiantis, Milan, Italy) and devices (Copan Italia Spa, Brescia, Italy and Novosanis, Belgium) participated in the European VALHUDES framework contributing equipment for laboratory testing under the condition of accepting independent publication of results. The study group received free self-sample collection devices from Copan Italia Spa (Brescia, Italy) and Novosanis (Belgium) and free OncoPredict HPV assay from (Hiantis, Milan, Italy). CEC declares to have received research support from BD Diagnostics, Seegene, Arrows Diagnostics, Copan, GeneFirst, Hiantis and VITRO. CEC is a minority shareholder of Hiantis. ADI, AFP, FB, FO, GT, HE, KC, MM, CG, RP declare no conflict of interest.

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Ethics Approval Statement

The European VALHUDES study (ClincalTrail.gov: NCT04312737) was conducted under the Declaration of Helsinki. The study was approved by the central Ethics Committee of the Coordinating Centre, ASST degli Spedali Civili di Brescia, Brescia, Italy (Ethics approval number: NP 3879- Studio WP6-HPVONC) and subsequently by the local Ethics Committees of the other participating centres. All women signed a written informed consent form before to enrolment.

Data availability Statement

Final study data sets generated by the study 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.

References

1. World Health Organization. Global strategy to accelerate the elimination of cervical cancer as a public health problem. Published online January 3, 2020.2. Arbyn M, Ronco G, Anttila A, et al. Evidence Regarding Human Papillomavirus Testing in Secondary Prevention of Cervical Cancer. *Vaccine* . 2012;30:F88-F99. doi:10.1016/j.vaccine.2012.06.0953. Arbyn M, Gultekin M, Morice P, et al. The European response to the WHO call to eliminate cervical cancer as a public health problem. *Intl Journal of Cancer* . 2021;148(2):277-284. doi:10.1002/ijc.331894. Kyrgiou M, Arbyn M, Bergeron C, et al. Cervical screening: ESGO-EFC position paper of the European Society of Gynaecologic Oncology (ESGO) and the European Federation of Colposcopy (EFC). *Br J Cancer* . 2020;123(4):510-517. doi:10.1038/s41416-020-0920-95. Arbyn M, Verdoodt F, Snijders PJF, et al. Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. *The Lancet Oncology* . 2014;15(2):172-183. doi:10.1016/S1470-2045(13)70570-96. Arbyn M, Smith SB, Temin S, Sultana F, Castle P. Detecting cervical precancer and reaching underscreened women by using HPV testing on self samples: updated meta-analyses. *BMJ* . Published online December 5, 2018:k4823. doi:10.1136/bmj.k48237. Serrano B, Ibáñez R, Robles C, Peremiquel-Trillas P, de Sanjosé S, Bruni L. Worldwide use of HPV self-sampling for cervical cancer screening. *Preventive Medicine* . 2022;154:106900. doi:10.1016/j.ypmed.2021.1069008. Arbyn M, Simon M, Peeters E, et al. 2020 list of human papillomavirus assays suitable for primary cervical cancer screening. *Clinical Microbiology and Infection* . 2021;27(8):1083-1095. doi:10.1016/j.cmi.2021.04.0319. Arbyn M, Peeters E, Benoy I, et al. VALHUDES: A protocol for validation of human papillomavirus assays and collection devices for HPV testing on self-samples and urine samples. *Journal of Clinical Virology* . 2018;107:52-56. doi:10.1016/j.jcv.2018.08.00610. Latsuzbaia A, Vanden Broeck D, Van Keer S, et al. Comparison of the Clinical Accuracy of Xpert HPV Assay on Vaginal Self-Samples and Cervical Clinician-Taken Samples within the VALHUDES Framework. *The Journal of Molecular Diagnostics* . 2023;25(9):702-708. doi:10.1016/j.jmoldx.2023.06.00411. Latsuzbaia A, Van Keer S, Vanden Broeck D, et al. Clinical Accuracy of Alinity m HR HPV Assay on Self- versus Clinician-Taken Samples Using the VALHUDES Protocol. *The Journal of Molecular Diagnostics* . 2023;25(12):957-966. doi:10.1016/j.jmoldx.2023.09.00812. Latsuzbaia A, Vanden Broeck D, Van Keer S, et al. Clinical Performance of the RealTi*m* e High Risk HPV Assay on Self-Collected Vaginal Samples within the VALHUDES Framework. Lainhart W, ed. *Microbiol Spectr* . 2022;10(5):e01631-22. doi:10.1128/spectrum.01631-2213. Latsuzbaia A, Vanden Broeck D, Van Keer S, et al. Validation of BD Onclarity HPV Assay on Vaginal Self-Samples versus Cervical Samples Using the VALHUDES Protocol. *Cancer Epidemiology, Biomarkers & Prevention* . 2022;31(12):2177-2184. doi:10.1158/1055-9965.EPI-22-075714. Van Keer S, Latsuzbaia A, Vanden Broeck D, et al. Analytical and clinical performance of extended HPV genotyping with BD Onclarity HPV Assay in home-collected first-void urine: A diagnostic test accuracy study.*J Clin Virol* . 2022;155:105271. doi:10.1016/j.jcv.2022.10527115. Van Keer S, Peeters E, Vanden Broeck D, et al. Clinical and analytical evaluation of the RealTime High Risk HPV assay in Colli-Pee collected first-void urine using the VALHUDES protocol. *Gynecol Oncol* . 2021;162(3):575-583. doi:10.1016/j.ygyno.2021.06.01016. Dhillon SK, Cocuzza CE, Chung PYJ, et al. Evaluation of the clinical performance of OncoPredict HPV® SCR assay within the VAL-

GENT-2 framework. *Journal of Medical Virology* . 2023;95(1):e28417. doi:10.1002/jmv.2841717. Arbyn M, Bruni L, Kelly D, et al. Tackling cervical cancer in Europe amidst the COVID-19 pandemic. *The Lancet Public Health* . 2020;5(8):e425. doi:10.1016/S2468-2667(20)30122-518. Martinelli M, Latsuzbaia A, Bonde J, et al. Performance of BD Onclarity HPV assay on FLOQSwabs vaginal self-samples. Starolis M, ed. *Microbiol Spectr* . 2024;12(3):e02872-23. doi:10.1128/spectrum.02872-2319. Arbyn M, Costa S, Latsuzbaia A, et al. HPV-based Cervical Cancer Screening on Self-samples in the Netherlands: Challenges to Reach Women and Test Performance Questions. *Cancer Epidemiology, Biomarkers & Prevention* . 2023;32(2):159-163. doi:10.1158/1055-9965.EPI-22-104120. Juliana NCA, Juma MH, Heijmans R, et al. Detection of high-risk human papillomavirus (HPV) by the novel AmpFire isothermal HPV assay among pregnant women in Pemba Island, Tanzania. *Pan Afr Med J* . 2020;37. doi:10.11604/pamj.2020.37.183.2336721. Seyoum A, Seyoum B, Gure T, et al. Genotype heterogeneity of high-risk human papillomavirus infection in Ethiopia. *Front Microbiol* . 2023;14:1116685. doi:10.3389/fmicb.2023.111668522. Giubbi C, Martinelli M, Vallini I, et al. Human papillomavirus (HPV) detection in vaginal self-samples: evaluation of eNat(r) as an alternative suspension medium to ThinPrep(r)PreservCyt(r) for vaginal swabs. *Open Res Europe* . 2022;2:35. doi:10.12688/openreseurope.14344.223. Giubbi C, Martinelli M, Di Meo ML, et al. Evaluation of two alternative non-alcohol-based media for the suspension of self-collected vaginal swabs for HPV testing in cervical cancer screening. *Heliyon* . 2024;10(10):e31032. doi:10.1016/j.heliyon.2024.e3103224. De Pauw H, Donders G, Weyers S, et al. Cervical cancer screening using HPV tests on self-samples: attitudes and preferences of women participating in the VALHUDES study. *Arch Public Health* . 2021;79(1):155. doi:10.1186/s13690-021-00667-425. Pasquier C, Raymond S, Duchanois D, et al. Human papillomavirus testing using HPV APTIMA(r) assay and an external cellularity control in self-collected samples. *Journal of Medical Virology* . 2023;95(12):e29283. doi:10.1002/jmv.2928326. Brukner I, Eintracht S, Papadakis AI, et al. Maximizing confidence in a negative result: Quantitative sample adequacy control. *Journal of Infection and Public Health* . 2020;13(7):991-993. doi:10.1016/j.jiph.2020.01.30727. Brukner I, Resendes A, Eintracht S, Papadakis A, Oughton M. Sample Adequacy Control (SAC) Lowers False Negatives and Increases the Quality of Screening: Introduction of "Non-Competitive" SAC for qPCR Assays.*Diagnostics* . 2021;11(7):1133. doi:10.3390/diagnostics1107113328. Cocuzza CE, Dhillon SK, Martinelli M, et al. Clinical performance of the novel full-genotyping OncoPredict HPV Quantitative Typing assay using the VALGENT framework. *Intl Journal of Cancer* . 2024;154(3):538-547. doi:10.1002/ijc.34754