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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 a grant from Abbott Laboratories (Abbott Molecular Diagnostics, Des Plaines, IL, USA), 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 co-founder and former board member of Novosanis (Belgium), a spin-off company of the University of Antwerp, and was minority shareholder until January 2019. 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.

Abstract

The VALHUDES was established to evaluate clinical accuracy of HPV assays to detect cervical precancer on first-void urine (FVU) and vaginal self-samples (VSS) versus matched clinician-collected cervical samples (CCS). In this study, we evaluated clinical performance of Alinity m HR HPV assay (Alinity) in a colposcopy referral population. Home-collected FVU (Colli-Pee FV 5020) one day before colposcopy (n=492), at-clinic collected dry VSS (*m*ulti-Collect Swab [*m*C; n=493], followed by Evalyn Brush [EB; n=233] or Qvintip [QT; n=260]) and matched CCS were available for the study. At the laboratory, *m*C swabs were resuspended in 2.5 mL Cervi-Collect buffer, EB and QT were transferred in 20 mL PreservCyt.

Sensitivity to detect CIN2+ of Alinity testing on FVU (ratio=0.94 [95%CI 0.85-1.03]), *m*C (ratio=1.00 [95%CI 0.94-1.06]) and EB/QT (ratio=0.92 [95%CI 0.85-1.00]) was not different to CCS. Specificity on FVU was similar to CCS (ratio=1.02 [95%CI 0.95-1.10]), whereas specificity on *m*C was lower (ratio=0.83 [95%CI 0.76-0.90]), but on EB/QT was higher (ratio=1.08 [95%CI 1.01-1.15]) than on CCS. Accuracy on EB (sensitivity ratio=0.96 [95%CI 0.87-1.05]; specificity ratio=1.18 [95%CI 1.06-1.31]) was slightly better than on QT (sensitivity ratio=0.88 [95%CI 0.75-1.03]; specificity ratio=1.00 [95%CI 0.92-1.09]). In conclusion, clinical sensitivity of Alinity assay on all self-sample types was similar to cervical specimens in a colposcopy referral population. Adjustment of signal-thresholds improved assay´s accuracy to detect CIN2+ in all self-sample types.

Introduction

Cervical cancer (CC) remains a global public health issue, particularly, in countries with limited resources [1]. CC can be largely prevented with screening and HPV vaccination. In western countries, CC screening programs were introduced in late 1950s and resulted in substantial decrease in CC incidence [2]. Evidence that HPV-based cervical cancer screening has superior sensitivity to cytology, triggered several western countries to change their recommendations and switch from cytological to primary HPV-based screening programs [3]. Moreover, HPV testing can be performed on self-samples which offers opportunities to reach out to women who do not attend screening regularly. Few countries had introduced HPV-based CC screening with self-sampling policies by 2022 [4].

Meta-analyses have shown that polymerase chain reaction (PCR)-based HPV DNA tests are similarly sensitive to detect cervical intraepithelial neoplasia grade two or higher (CIN2+) on vaginal self-samples compared to clinician-collected cervical samples (CCS) [5, 6]. Another meta-analysis reported pooled sensitivity on urine-self samples lower than on cervical, although some PCR-based tests were similarly sensitive compared to CCS [7]. The meta-analyses were pivotal in triggering a high level of acceptance of self-sampling among women and policymakers, however optimisation of pre- and post-analytical workflows are still lacking [8-11]. In order to tackle these challenges, the validation of HPV assays and collection devices for HPV testing on vaginal self-samples and urine samples (VALHUDES) protocol was established [12]. Five VALHUDES studies were published showing similar sensitivity and specificity on first-void urine (FVU) [13, 14] and vaginal self-samples versus CCS [15-17].

The current report aims to evaluate the accuracy of Alinity m HR HPV assay (Alinity; [Abbott Molecular Diagnostics, Des Plaines, IL, USA]) to detect cervical precancer on FVU and vaginal self-samples collected either with *m*ulti-Collect swab (*m*C; Abbott Molecular Diagnostics. Des Plaines, IL, USA), Evalyn Brush (EB; Rovers Medical Devices, Oss, The Netherlands) or Qvintip (QT; Aprovix AB, Stockholm, Sweden). In addition, we compared signal strength, expressed by the fraction cycle number (FCN) across the all specimen types.

Materials and Methods

Study design

The general design of the VALHUDES study (NCT03064087) has been described previously [12]. In total, 523 women were invited to one of five Belgian colposcopy clinics because of previous HPV infection or cervical abnormality [13, 15]. The day before the colposcopy appointment, enrolled women used the Colli-Pee device (Novosanis, Wijnegem, Belgium), which collects ~13 mL of FVU in a collector tube prefilled with 7 mL of urine conservation medium (UCM). Women were instructed to store the FVU specimen at room temperature. At the colposcopy clinic, women presented the FVU sample to the study nurse and subsequently collected two vaginal self-samples. The first vaginal self-sample was taken with the *m*C swab, followed by the second self-sample with either EB or QT. Self-samples were collected according to manufacturer's instructions at the time of sample collection. The EB was first offered to women in the colposcopy clinics of Antwerp and Ghent, whereas QT was first offered in Brussels, Liège, and Tienen. When about half of the sample size was reached sampling devices were switched across the colposcopy clinics. Subsequently, a cervical sample was taken by a gynaecologist using a Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) after visualisation of cervix and prior to colposcopy,

according to the standards as recommended by European guidelines [18]. The cervical specimen was resuspended into a vial containing 20 mL ThinPrep PreservCyt (Hologic, Inc, Marlborough, MA, USA). After all specimens had been collected, colposcopy was performed followed by biopsy if indicated.

All self- and clinician-collected specimens were stored at room temperature (20-22 °C) in the colposcopy clinic for a maximum of six days (median =2 days). Subsequently, samples were transferred at room temperature to Algemeen Medisch Laboratorium (AML [Antwerp, Belgium]) for further pre-processing and storage.

The FVU samples were transferred at 4°C to the Centre for the Evaluation of Vaccination ([CEV], University of Antwerp) where they were vortexed for 15-20 seconds, divided into secondary aliquots and stored at -80°C (Biobank Antwerp, Antwerp, Belgium; ID: BE 71030031000) [19]. Aliquots were frozen for 889 days on average before HPV testing (range: 491-1217 days).

In the AML laboratory, immediately upon arrival, 2.5 mL of Abbott Cervi-Collect buffer was added to dry *m*C swabs, whereas EB and QT brush heads were transferred in 20 mL ThinPrep PreservCyt solution. Vaginal and CCS were stored at 4°C for a maximum of up to three months, then vortexed for 15-20 seconds, aliquoted and frozen at -80°C (Biobank, BB190002). Aliquots were frozen for 885 days on average before HPV testing (range: 559-1183 days).

Ethical approval

The VALHUDES (NCT03064087) study 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 centres involved in the study. The study was conducted in accordance with the Helsinki declaration of 1964. All participants signed informed consent before enrolment.

HPV testing

Of 523 study participants, HPV testing with the Alinity assay was performed on 499 quadruplets. Twenty-four sample quadruplets were excluded due to major protocol violations as described elsewhere [13, 15]. HPV testing was performed on all quadruplets side by side by transferring 550 µl aliquots (400 µl of input volume is processed by the system) into the Alinity m System for fully automated integrated DNA extraction, amplification and result interpretation according to the manufacturer´s instructions. The Alinity assay is a qualitative multiplex real-time PCR assay targeting a conserved sequence within the L1 gene of 14 hrHPV genotypes. The assay separately reports the presence of HPV16, 18, 45, and other genotypes in two groups using genotype specific probes in five channels (Group A: HPV31/33/52/58 and Group B: 35/39/51/56/59/66/68) at clinically relevant infection levels. The assay is clinically validated for use in cervical cancer screening on clinician-taken cervical samples [20]. DNA extraction was automatically performed by the Alinity m HR HPV system using the sample preparation kit, including Alinity m Lysis Solution, Alinity m Ethanol Solution and Alinity m Diluent Solution. The Alinity m System employs magnetic microparticle technology to facilitate nucleic acid capture, wash and elution. Purified DNA was mixed with liquid activation and lyophilized amplification/detection reagents and transferred into a reaction vessel. Subsequently, Alinity m Vapor Barrier Solution was added to the reaction vessel followed by a transfer to an amplification/detection unit for PCR amplification, and real-time fluorescence detection of HPV targets, The amplification/detection reagents of the Alinity assay include primers and probes that amplify and detect an endogenous

human β-globin sequence as sample validity control for cell adequacy, sample extraction and amplification efficiency [21, 22]. Amplification signal strength is reported with FCN (fraction cycle numbers) which is inversely correlated with of β-globin DNA concentration and viral load in the sample. The reagents also contain Uracil-DNA Glycosylase as a contamination control, negative and positive controls.

Amount

Statistical analysis

To estimate the relative clinical accuracy, HPV testing on self-samples was considered as the index test and HPV testing on CCS as the comparator. Colposcopy and histology outcomes were used as a reference test for disease verification. Histological outcomes were classified as normal, CIN1, CIN2 and CIN3 (no patients with cancer were diagnosed in the study). Participants without biopsy outcome were categorised as <CIN2 when colposcopy was satisfactory and yielded a normal impression. The disease thresholds were <CIN2 for specificity and ≥CIN2+ and CIN3 for sensitivity. HPV positivity was defined according to the confidential channel specific cut-offs established by the manufacturer for CCS. Alternative cut-off thresholds were applied for the self-samples a posteriori using an iterative statistical procedure to improve the relative clinical accuracy of Alinity testing on self- versus CCS. After increasing/decreasing cut-off thresholds relative accuracy was reviewed. Alternative cut-offs were defined when an optimal balance was reached between specificity and sensitivity. Common new cut-offs were applied on FVU and vaginal self-samples collected with EB/QT for HPV16 (FCN<=32), Group A (FCN<=31) and Group B (FCN<=31.6), no changes were applied to HPV18 and 45 positivity thresholds. On *m*C samples new cut-offs were established for HPV16 (FCN <=28), HPV18/45 (FCN<=27), Group A (FCN<=25.3) and Group B (FCN<=26).

Five quadruplets were excluded due to invalid test results on the CCS. Two urine and three vaginal self-samples were excluded due to invalid test results on respective specimen (Figure 1). Fourteen quadruplets were retested as follows: a) four quadruplets required retesting due to system error on all specimen types and deemed valid after retesting; b) four quadruplets were retested due to β-globin inadequacy and one due to max ratio abnormal response on CCS, all specimens remained invalid after retesting; c) a single quadruplet underwent retesting due to β-globin inadequacy on FVU and remained invalid upon retesting; d) three quadruplets were retested due to β-globin inadequacy on vaginal sample collected with EB or QT devices, retesting was successful for two samples, while one sample remained invalid; e) three quadruplets underwent retesting due to failure on *m*C swabs and were found to be valid after the retesting. Two *m*C and one FVU samples were invalid but were not retested and were excluded from the analysis. The exclusion was limited to a specific sample type. The dataset used for statistical analysis contained 492 matched CCS, FVU and vaginal samples collected with *m*C swab, and 493 matched CCS and vaginal samples taken with EB/QT. Clinical accuracy of the Alinity assay was evaluated for the whole study population and stratified for women of 30 years and older. Characteristics of the study population were reported previously [13, 16].

McNemar test and matched 95% confidence intervals (CI) were used to assess the difference in clinical accuracy between cervical and all other sample types. Statistical significance was accepted when p-values <0.05 or 95% confidence intervals around relative accuracy measures excluded unity. Overall and type-specific HPV test concordance between different specimen types was assessed using Cohen's kappa values, which were categorized as follows: 0.00–0.19 as poor, 0.20–0.39 as fair, 0.40–0.59 as moderate, 0.60–0.79 as good and 0.80–1.00 as excellent concordance. Concordance was estimated for the total study population and stratified by disease outcome. The

differences in median signal level between matched samples was evaluated using Mann-Whitney test. For non-matched comparison (EB versus QT devices), Wilcoxon signed-rank test was used. Statistical analyses were performed using Stata 16.1 (College Station, Texas, USA).

Results

Clinical accuracy

Relative sensitivity of the Alinity assay on FVU versus CCS was 0.94 (95% CI 0.85-1.03) and 0.90 (95% CI 0.783-1.04) for CIN2+ and CIN3, respectively. Specificity for <CIN2 on FVU and CCS was similar (ratio=1.02; 95%CI 0.95-1.10]). hrHPV testing with the Alinity assay on *m*C swab self-samples was similarly sensitive for the detection of CIN2+ (ratio=1.00; 95% CI 0.94-1.06) and CIN3 (ratio=0.98; 95% CI 0.88-1.09) compared to CCS. The specificity for <CIN2 was significantly lower than on CCS (ratio=0.83; 95% CI 0.76-0.90).

On vaginal self-samples collected with EB or QT, sensitivity relative to CCS was 0.92 (95% CI 0.85-1.00) and 0.93 (95% CI 0.83-1.04) for CIN2+ and CIN3, respectively. The relative specificity for <CIN2 was significantly higher in vaginal self-samples compared to CCS (ratio=1.08; 95% CI 1.01-1.15, p=0.02). Relative sensitivity on samples collected with the EB (ratio=0.96; 95% CI 0.87-1.05) was slightly higher than on QT samples (ratio=0.88; 95%CI 0.75-1.03) (Table 1). The relative sensitivity of independent non-matched comparison between EB versus QT was 1.11 (95% CI 0.93-1.33).

Adjusting the cut-off values on FVU (higher FCN cut-off than established by the manufacturer for CCS), resulted in sensitivity improvement for CIN2+ (ratio=1.00; 95% CI 0.93-1.07) and CIN3

(ratio=1.00; 95% CI 0.91-1.10) with slight decrease in specificity (ratio=0.95; 95% CI 0.88-1.03) (Table 1).

After adjusting cut-off values on *m*C swab samples (lower FCN cut-off than established by the manufacturer for CCS), relative specificity improved to 1.01 (95% CI 0.95-1.08] with a slight decrease in sensitivity (ratio=0.97; 95% CI 0.90-1.05) (Table 1).

The sensitivity on vaginal samples collected with EB or QT combined compared to CCS improved as a result of cut-off adjustment (higher FCN cut-off than established by the manufacturer for CCS): ratio=0.96 (95% CI 0.90-1.03) for CIN2+ and 0.95 (95% CI 0.86-1.05) for CIN3. Sensitivity for CIN2+ also improved on EB (ratio=1.00; 95% CI 0.90-1.06) and QT samples (0.91; 95% CI 0.79-1.05) separately. The relative specificity on vaginal EB/QT samples decreased to 1.01 (95% CI 0.95-1.08). On EB samples, the specificity was still higher than on CCS after cut-off optimisation (ratio=1.11; 95% CI 1.01-1.22), but not on QT samples (ratio=0.94; 95% CI 0.86-1.02) (Table 1). Absolute sensitivity for the total population is reported in Supplementary Table S1 and for women 30 years and older in Supplementary Table S2. Relative accuracy for women 30 years or older is shown in Supplementary Table S3.

An a posteriori cut-off optimisation yielded detection of five additional CIN2+ cases on FVU, and three on vaginal EB/QT self-samples, whereas 14 FVU and 13 vaginal EB/QT <CIN2 cases became HPV positive. On *m*C swab samples 37 additional <CIN2 subjects became HPV-negative, while two additional CIN2+ cases were missed (Table 1). Optimized cut-offs resulted in a sensitivity improvement of the Alinity assay on FVU and EB/QT samples and in a specificity improvement on *m*C swab samples, in the sense that the 95% CI around the relative sensitivity and

relative specificity of testing on the respective self-sample versus on CCS included unity or exceeded unity.

Test concordance

Eighty four percent of FVU and CCS were concordantly hrHPV positive or negative ([232+181]/492, kappa=0.68). The hrHPV test concordance between vaginal *m*C swab and vaginal EB/QT self-samples versus cervical samples was 86% ([258+163]/492, kappa=0.70) and 88% ([236+197]493, kappa=0.76), respectively (Table 2).

After cut-off adjustments, the number of concordantly positive and negative samples between FVU and CCS increased to 86% ([247+177]/492, kappa=0.72) (Table 3). Similarly, between vaginal *m*C swab and vaginal EB/QT versus CCS, number of concordant samples increased to 87% ([246+190]/492; kappa=0.77) and 90% ([248+193]/493, kappa=0.79), respectively (Table 3). Type-specific agreement between the specimens according to the disease status is shown in Supplementary Table S5 and Supplementary Table S6.

Signal strength

Overall hrHPV, HPV16, other hrHPV Group A, other hrHPV Group B, and *β-globin* median FCN values were significantly higher in FVU compared to CCS. Similarly, hrHPV, HPV16 and other hrHPV Group A median viral FCN values were significantly higher in the vaginal samples collected with EB or QT than in CCS. In *m*C specimens, hrHPV, other hrHPV Group A, other hrHPV Group B, and *β-globin* FCN values were significantly lower than in CCS (Supplementary Table S7). When stratified by disease status, viral hrHPV and *β-globin* FCN values were

significantly lower in CIN2+ cases compared to <CIN2 for all sample types except *m*C swab (Figure 2 and Supplementary Table S8).

Discussion

In this VALHUDES study, the accuracy of the Alinity assay to detect high-grade cervical disease was assessed in diverse self-collected sample types compared to CCS. Four self-sampling devices were evaluated: FVU collected with the Colli-Pee device, and a vaginal self-sample taken with *m*C swab, EB or QT. All enrolled women were asked to collect a self-sample using Colli-Pee and *m*C, whereas only half of the women used either EB or QT. No significant difference in clinical sensitivity for CIN2+ and CIN3 was observed for Alinity assay on FVU and vaginal self-samples collected with EB/QT compared to CCS. Clinical specificity (<CIN2) on FVU was similar to CCS, whereas on vaginal self-samples collected with EB/QT, the clinical specificity was significantly higher than on CCS. The clinical sensitivity for CIN2+ and CIN3 on vaginal self-samples collected with *m*C swab was similar to CCS, while its clinical specificity was significantly lower. Following post-hoc optimisation of signal cut-off values an optimal balance was achieved between relative specificity and sensitivity for all self-sample types. Cut-off optimisation of signal thresholds improved the relative accuracy estimates with 95% CIs including unity or tending to include unity for CIN2+ for all self-sample types. Common cut-offs which were higher than the initial values were applied on FVU and vaginal self-samples collected with EB/QT, whereas lower cut-offs were used for the *m*C device.

Prior to cut-off optimisation, sensitivity of the Alinity assay on vaginal self-samples collected with EB or QT was somewhat lower in comparison to the *m*C swab, whereas specificity was higher.

This contrast in accuracy may be explained by the difference in resuspension volumes, transport media and sampling order [9, 10]. EB and QT devices were placed in 20 mL of ThinPrep PreservCyt solution, whereas 2.5 mL Abbott Cervi-Collect buffer was added to *m*C swab transport tube with *m*C swab in it. As Abbott Cervi-Collect Buffer was added to the *m*C swab transport tube, *m*C sample could have a higher concentration of cell material than EB or QT. These pre-analytical differences resulted in ~3FCN stronger signal in *m*C compared to EB/QT vaginal samples, which translated into lower specificity for <CIN2 but only a slightly higher sensitivity. On the other hand, Colli-Pee allows standardised volumetric collection of ~13 mL FVU prefilled with 7 mL UCM. However, identical alternative cut-offs could be applied to FVU and vaginal self-samples collected with EV or QT. Three HPV tests have already been evaluated within VALHUDES [13-17]. RealTi*m*e HPV and BD Onclarity showed similar accuracy on self-collected vaginal and FVU samples compared to CCS. To improve accuracy on self-samples and CCS with RealTi*m*e HPV, signal cut-off adjustment on FVU and vaginal samples was performed [13-16]. Clinical accuracy of Xpert HPV was likewise not different on self-collected vaginal versus CCS [17]. No urine samples were run on Xpert HPV. In agreement with the present report, clinical performance of hrHPV testing on EB was similarly sensitive and specific compared to the CCS, whereas sensitivity and specificity on vaginal QT samples tended to be lower.

Two previously published studies exploring the performance of self- versus CCS in colposcopy referral populations observed a lower accuracy when vaginal self-samples were taken with QT, which is in agreement with our findings [23, 24]. Cadman *et al.* assessed accuracy of four vaginal (dry flocked swab, Dacron swab, HerSwab, and QT) and one FVU device (Colli-Pee) in 600 women with the BD Onclarity HPV assay. Dry flocked and Dacron swabs performed better in detection of CIN2+ than QT and HerSwab [23]. FVU collected with 20 mL version of Colli-Pee device also

326 demonstrated high sensitivity, however at lower specificity. Jentschke *et al.* performed direct
327 comparison of QT and EB in a small study including 136 women [24]. Although, the sample size
328 was limited Jentschke's findings suggested somewhat better performance of EB compared to QT.
329 Both studies resuspended vaginal brushes in 20 mL PreservCyt medium.

330 Another study by Ørnskov and colleagues evaluated the cobas 4800 HPV assay on EB vaginal
331 samples and FVU recruiting 359 participants in colposcopy settings. Dry vaginal sample was
332 resuspended in 20 mL PreservCyt, while FVU (mndinimum of 8 mL collected in a urine cup) was
333 poured into 8 mL EDTA solution. The study showed that that the clinical sensitivity on both self-
334 collected vaginal samples with EB, and urine samples was not different compared to CCS [25]**.** In
335 the Dutch IMPROVE trial accuracy of hrHPV testing using GP5+/6+ PCR enzyme immunoassay
336 on EB vaginal self-samples was compared to CCS in women attending cervical cancer screening.
337 In total, 7,643 women were included in a self-sampling arm, and another 6,282 in a clinician-
338 collected arm. Here, EB resuspended in 1.5 mL of PreservCyt medium, was similarly accurate to
339 detect CIN2+ and CIN3+ versus CCS resuspended in 10 mL PreservCyt medium [26]. Moreover,
340 several meta-analyses have demonstrated that cross-sectional accuracy of HPV testing on vaginal
341 self- is similar to CCS under the condition of using validated PCR-based HPV DNA assays [5, 7]. A
342 meta-analysis on urine samples showed that only some PCR-based hrHPV tests are similarly
343 sensitive compared to CCS. From 21 included studies, four did not report which urine fraction was
344 used for hrHPV testing and six did not report information on the collection device or use of
345 preservative buffer. It has been demonstrated that when no preservation or first-void fraction is
346 used accuracy is worse in urine compared to CCS [8, 11, 27]. A meta-analysis on vaginal samples
347 found that out of 56 studies, only one did not report collection device and six did not document
348 storage medium, while volume of resuspension was not well documented [5, 10]. Recent work

underlined the challenge to optimize pre-analytical and post-analytical laboratory workflows which could play a determinant role in identification of cervical precancer using self-samples. For instance, sample collection device, transport medium and volume, DNA extraction methods, PCR input volume or HPV positivity criteria could influence accuracy and require thorough understanding and optimisation on both urine and vaginal self-samples [9-11, 28].

The Alinity assay has been clinically validated for cervical cancer screening using CCS [20, 22]. The assay has a broader extended genotyping ability than the Abbott RealTi*m*e assay, which might be useful for triage and risk management of HPV positive women [29]. Since HPV genotypes have different oncogenic potential, risk stratification might be an important clinical application for cervical cancer screening [30, 31]. Additionally, the Alinity assay's extended genotyping profile to separately identify all hrHPV types targeted by the nonavalent HPV vaccine (HPV16, HPV18, HPV45 and the combination of HPV31/33/52/58) could be useful in future screening and triage algorithms [32].

Our study had several notable strengths, including its large sample size which, given the colposcopy setting, enabled finding sufficient number of CIN2+ cases and power to address sensitivity hypotheses. The study was conducted in accordance with STARD guidelines for good diagnostic test accuracy research [33] with disease outcome for all enrolled women avoiding partial verification bias. It was not expected at the time of designing VALHUDES, that HPV testing on EB and QT devices would impact clinical accuracy. Pooling of the VALHUDES results from multiple tests suggested somewhat lower relative sensitivity and specificity on QT self-samples, but not with EB. To address this limitation, we presented results both jointly for both devices and separately for each device. Recruitment from colposcopy clinics may be object of criticism since not representative for a screening population where HPV testing on self-samples is typically used.

As a result, the absolute specificity was lower than what would be observed in a screening population. However, our conclusions are based on relative accuracy, which is a robust parameter over diverse settings as demonstrated empirically in meta-analyses (6). Another limitation was the fact that women aged 19 to 70 years were enrolled in our study, while the age recommended in most (not all) countries for HPV-based screening is 30 years and above. Nevertheless, the sensitivity analysis showed that accuracy in the age group recommended for screening closely resembled that of the entire study population. Another notable drawback was the categorization of women with normal colposcopy findings and without biopsy results as non CIN2+. This classification might have influenced the overall accuracy assessments, potentially leading to a slight overestimation of absolute sensitivity and an underestimation of absolute specificity for both sample types. Nonetheless, potential bias from such misclassification would affect both sample types equally, given that colposcopists were unaware of HPV test outcomes for either group. As a result, the impact on relative accuracy would likely be limited. Finally, order of the sample collection could have an impact on cell yield and influence the accuracy on vaginal self-samples. However, previous studies have reported no impact of the sample order on HPV positivity or agreement rates [23, 24]. In our study, contrast between first and second collection could not be assessed, since two different devices were used and different pre-analytical laboratory workflow were applied. Nevertheless, these variations in the pre-analytical workflow could be corrected through cut-off optimization, as our study successfully achieved.

In conclusion, sensitivity of Alinity m HPV assay was not different between self- and clinician-collected cervical samples, although post-hoc adjustment of signal threshold values resulted in accuracy improvement and a satisfactory balance was achieved between sensitivity and specificity

for all self-sampling specimens. Further research is required to finetune and standardise laboratory workflows on self-samples.

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Author contributions

Principal investigator and conceptualization of VALHUDES: MA.

Protocol development: MA, SVK, DVB, AV.

Funding acquisition: MA and AV.

Project administration: AL and MA.

Enrolment of patients: SW, GD, PS, WT, JD

Data Curation and formal analysis: AL, and MA.

Sample handling: DVB, SVK.

Drafting original manuscript: AL.

Critical review and editing of manuscript: all authors.

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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 a grant from Abbott Laboratories (Abbott GmbH, Wiesbaden, Germany), 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 co-founder and former board member of Novosanis (Belgium), a spin-off company of the University of Antwerp, and was minority shareholder until January 2019. 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.

Ethical Approval

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 prior to enrolment.

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Table 1. Relative accuracy of the Alinity m HR HPV 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
Manufacturer cut-offs[*]			
Urine	0.94 [0.85-1.03]	0.90 [0.78-1.04]	1.02 [0.95-1.10]
***m*C swab**	1.00 [0.94-1.06]	0.98 [0.88-1.09]	0.83 [0.76-0.90]
EB/QT[†]	0.92 [0.85-1.00]	0.93 [0.83-1.04]	1.08 [1.01-1.15]
EB	0.96 [0.87-1.05]	0.95 [0.87-1.05]	1.18 [1.06-1.31]
QT	0.88 [0.75-1.03]	0.90 [0.73-1.11]	1.00 [0.92-1.09]
Alternative cut-offs[‡]			
Urine	1.00 [0.93-1.07]	1.00 [0.91-1.10]	0.95 [0.88-1.03]
***m*C swab**	0.97 [0.90-1.05]	0.98 [0.88-1.09]	1.01 [0.95-1.08]
EB/QT	0.96 [0.90-1.03]	0.95 [0.86-1.05]	1.01 [0.95-1.08]
EB	1.00 [0.94-1.06]	1.00 [1.00-1.00]	1.11 [1.01-1.22]
QT	0.91 [0.79-1.04]	0.90 [0.73-1.11]	0.94 [0.86-1.02]

CI, confidence interval; CIN, cervical intraepithelial neoplasia; EB, Evalyn Brush; *m*C, *m*ulti-Collect; N, number; QT, Qvintip.

[*]Confidential manufacturer's fraction cycle number (FCN) cut-off established for cervical samples.

[†]Samples collected with EB or QT combined.

[‡]New a posterior defined cut-offs for urine and vaginal self-samples collected with EB or QT devices: ***HPV16 FCN<=32, Group A FCN<=31, Group B FCN<31.6.*** For *m*C swab samples following new cut-offs were defined: ***HPV16 FCN <=28, HPV18/45 FCN<=27, Group A FCN<=25.3, Group B FCN <=26.***

Relative sensitivity and specificity for women ≥ 30 years old are shown in Supplementary Table S3. Matched numbers of cases used to estimate relative accuracy are present in Supplementary Table S4.

Table 2. Type-specific agreement and test concordance between cervical and self-samples based on manufacturer cut-offs.

	HPV type	+/+*	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]†
Urine vs cervical							
Total population (n=492)	hrHPV‡	232	44	35	181	83.9	0.675 (0.610 - 0.741)
	HPV16	62	4	8	418	97.6	0.898 (0.841 - 0.955)
	HPV18	19	2	5	466	98.6	0.837 (0.719 - 0.956)
	HPV45	12	4	8	468	97.6	0.654 (0.472 - 0.837)
	Group A§	79	32	27	354	88.0	0.651 (0.570 - 0.733)
	Group B¶	105	25	35	327	87.8	0.694 (0.622 - 0.766)
mC swab vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=492)	hrHPV	258	18	53	163	85.6	0.702 (0.638 - 0.765)
	HPV16	64	2	16	410	96.4	0.855 (0.790 - 0.920)
	HPV18	18	2	9	463	97.8	0.754 (0.615 - 0.894)
	HPV45	11	4	7	470	97.8	0.655 (0.465 - 0.846)
	Group A	101	12	26	353	92.3	0.791 (0.727 - 0.854)
	Group B	117	13	44	318	88.4	0.723 (0.657 - 0.790)
EB/QT vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=493)	hrHPV	236	41	19	197	87.8	0.756 (0.698 - 0.813)
	HPV16	61	5	4	423	98.2	0.921 (0.870 - 0.972)
	HPV18	17	4	2	470	98.8	0.844 (0.721 - 0.967)
	HPV45	12	4	2	475	98.8	0.794 (0.633 - 0.955)
	Group A	88	25	10	370	92.9	0.789 (0.723 - 0.856)
	Group B	103	26	18	346	91.1	0.764 (0.698 - 0.830)
EB vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=233)	hrHPV	115	24	6	88	87.1	0.741 (0.655 - 0.826)
	HPV16	36	4	1	192	97.9	0.922 (0.855 - 0.990)
	HPV18	12	4	1	216	97.6	0.816 (0.659 - 0.973)
	HPV45	3	1	1	228	99.1	0.746 (0.406 - 1.000)
	Group A	41	14	1	177	93.6	0.806 (0.712 - 0.899)
	Group B	44	17	7	165	89.7	0.719 (0.614 - 0.823)
QT vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=260)	hrHPV	121	17	13	109	88.5	0.769 (0.691 - 0.847)
	HPV16	25	1	3	231	98.5	0.917 (0.837 - 0.998)
	HPV18	5	0	1	254	99.6	0.907 (0.726 - 1.000)
	HPV45	9	3	1	247	98.5	0.810 (0.629 - 0.992)
	Group A	47	11	9	193	92.3	0.775 (0.682 - 0.869)
	Group B	59	9	11	181	92.3	0.803 (0.720 - 0.885)

CI, confidence interval; EB, Evalyn Brush; hr, high risk; HPV, human papillomavirus; *m*C, *m*ulti-Collect; N, number; QT, Qvintip.

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

†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.

‡14 carcinogenic HPV genotypes.

§Group A: HPV31/33/52/58

¶Group B : HPV35/39/51/56/59/66/68

Concordance by disease status is shown in Supplementary Table S5.

Table 3. Type-specific agreement and test concordance between cervical and self-samples with alternative cut-offs.

	HPV type	+/+*	+/-	-/+	-/-	Concordance [%]	Kappa [95% CI]†
Urine vs cervical							
Total population (n=492)	hrHPV‡	247	29	39	177	86.2	0.718 (0.656 - 0.780)
	HPV16	63	3	10	416	97.4	0.891 (0.833 - 0.949)
	HPV18	19	2	5	466	98.6	0.837 (0.719 - 0.956)
	HPV45	12	4	8	468	97.6	0.654 (0.472 - 0.837)
	Group A§	91	20	28	353	90.0	0.728 (0.655 - 0.800)
	Group B¶	110	20	39	323	88.0	0.705 (0.636 - 0.775)
mC swab vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=492)	hrHPV	246	30	26	190	86.6	0.769 (0.713 - 0.826)
	HPV16	64	2	11	415	97.4	0.892 (0.835 - 0.950)
	HPV18	17	3	4	468	98.6	0.822 (0.693 - 0.951)
	HPV45	11	4	5	472	98.2	0.700 (0.514 - 0.886)
	Group A	95	18	21	358	92.1	0.778 (0.712 - 0.844)
	Group B	109	21	22	340	92.3	0.776 (0.712 - 0.839)
EB/QT vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=493)	hrHPV	248	29	23	193	89.5	0.786 (0.732 - 0.841)
	HPV16	62	4	5	422	98.2	0.922 (0.871 - 0.972)
	HPV18	17	4	2	470	98.8	0.844 (0.721 - 0.967)
	HPV45	12	4	2	475	98.8	0.794 (0.633 - 0.955)
	Group A	96	17	14	366	93.7	0.820 (0.759 - 0.881)
	Group B	106	23	23	341	90.7	0.759 (0.693 - 0.824)
EB vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=233)	hrHPV	123	16	6	88	90.6	0.807 (0.731 - 0.884)
	HPV16	37	3	2	191	97.9	0.924 (0.858 - 0.990)
	HPV18	12	4	1	216	97.6	0.816 (0.659 - 0.973)
	HPV45	3	1	1	228	99.1	0.746 (0.406 - 1.000)
	Group A	46	9	2	176	95.3	0.863 (0.785 - 0.942)
	Group B	47	14	9	163	90.1	0.738 (0.637 - 0.838)
QT vs cervical							
	HPV type	**+/+**	**+/-**	**-/+**	**-/-**	**Concordance [%]**	**Kappa [95% CI]**
Total population (n=260)	hrHPV	125	13	17	105	88.5	0.768 (0.690 - 0.846)
	HPV16	25	1	3	231	98.5	0.917 (0.837 - 0.998)
	HPV18	5	0	1	254	99.6	0.907 (0.726 - 1.000)
	HPV45	9	3	1	247	98.5	0.810 (0.629 - 0.992)
	Group A	50	8	12	190	92.3	0.783 (0.693 - 0.874)
	Group B	59	9	14	178	92.3	0.776 (0.690 - 0.863)

CI, confidence interval; EB, Evalyn Brush; hr, high risk; HPV, human papillomavirus; *m*C, *m*ulti-Collect; N, number; QT, Qvintip.

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

†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.

‡14 carcinogenic HPV genotypes.

§Group A: HPV31/33/52/58

¶Group B : HPV35/39/51/56/59/66/68

New post-hoc fraction cycle number (FCN) cut-offs defined for urine and vaginal samples collected with EB or QT: ***HPV16 FCN<=32, Group A FCN<=31, Group B FCN<=31.6.*** For *m*C swab samples following new cut-offs were defined: ***HPV16 FCN <=28, HPV18/45 FCN<=27, Group A FCN<=25.3, Group B FCN <=26***
Concordance by disease status is shown in Supplementary Table S6.

Figure Legends:

Figure 1. Flow chart of samples included in the VALHUDES trial tested with the Alinity m HR HPV assay. Grey boxes represent excluded samples. Detailed exclusions are reported elsewhere [13, 15].

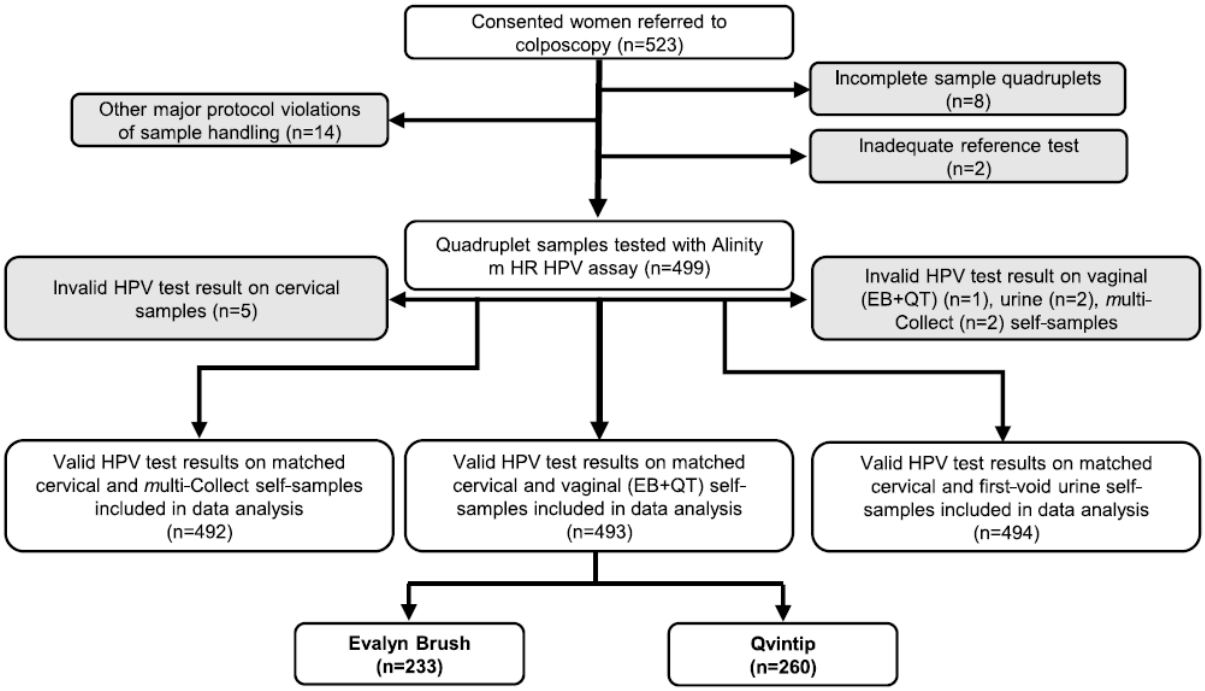
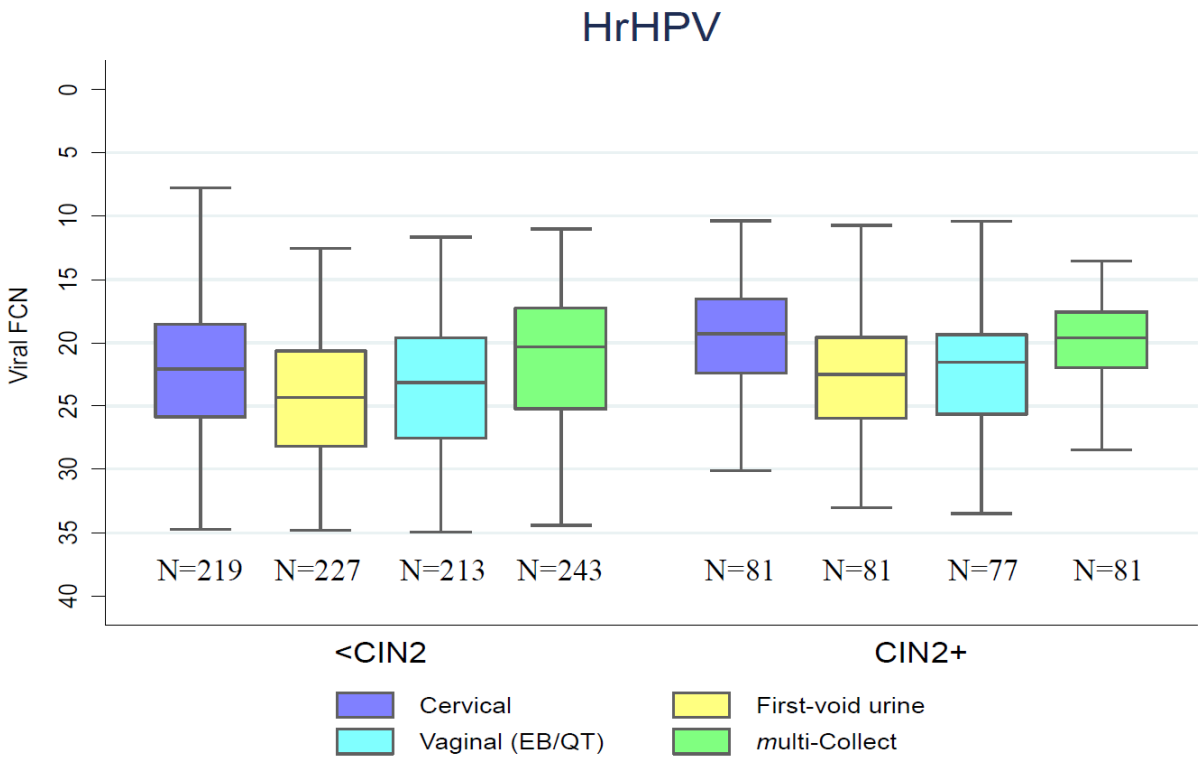


Figure 2. Alinity m HR HPV assay signal strength expressed by the fraction cycle number (FCN) stratified by CIN2+ and <CIN2 between specimen types.

Boxplots indicate median FCN values and interquartile ranges for HPV16, 18, 45, other hrHPV Group A, other hrHPV Group B. N indicates umber of samples with FCN value >0 in each category of matched 489 quadruplets.


HrHPV
Viral FCN
0
5
10
15
20
25
30
35
40
N=219
N=227
N=213
N=243
N=81
N=81
N=77
N=81
<CIN2
CIN2+
Cervical
Vaginal (EB/QT)
First-void urine
multi-Collect

613

614