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Clinical and analytical evaluation of the RealTime High Risk HPV assay in Colli-Pee collected first-void urine using the VALHUDES protocol

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KEYWORDS

Cervical cancer screening, HPV, VALHUDES, diagnostic test accuracy, self-sampling, first-void urine, Colli-Pee, HPV testing, Abbott RealTime High Risk HPV assay, clinical validation

ABSTRACT

Objective

Urine self-sampling has gained increasing interest for cervical cancer screening. In contrast to analytical performance, little information is available regarding the clinical accuracy for high-risk Human Papillomavirus (hrHPV) testing on urine.

Methods

VALHUDES is a diagnostic test accuracy study comparing clinical accuracy to detect high-grade cervical precancer (CIN2+) of HPV testing on self-collected compared to clinician-collected samples (NCT03064087). Disease outcome was assessed by colposcopy and histology. The Abbott RealTime High Risk HPV assay performance was evaluated on Colli-Pee collected first-void urine with cervical outcomes as comparator.

Results

As no assay cut-off for urine has been clinically validated, we used the predefined cut-off for cervical samples ($CN \leq 32$). Using this cut-off, hrHPV testing was similarly sensitive (relative sensitivity 0.95; 95% CI: 0.88-1.01) and specific (relative specificity 1.03; 95% CI: 0.95-1.13) for detection of CIN2+ compared to testing cervical samples. In the subgroup of women of 30 years and older, similar relative sensitivity (0.97; 95% CI: 0.89-1.05) and specificity (1.02; 95% CI: 0.93-1.12) was found. Additionally, an exploratory cut-off ($CN \leq 33.86$) was defined which further improved sensitivity and analytical test performance.

Conclusion

HrHPV-DNA based PCR testing on home-collected first-void urine has similar accuracy for detecting CIN2+ compared to cervical samples taken by a clinician.

INTRODUCTION

The identification of high-risk (hr) Human Papillomavirus (HPV) as the principal cause of cervical cancer has changed the paradigm of cervical cancer prevention [1-3]. Primary HPV-based screening offers a more effective strategy to protect against cervical precancer and cancer compared to cytology [4-6], and provides an alternative approach to reach screening non-responders by offering self-sampling kits. The latter is generally more effective in reaching under-screened women compared to sending invitations [7]. Meta-analyses have shown that hrHPV-based screening using a validated PCR-based HPV DNA test is similarly sensitive and slightly less specific to detect high-grade cervical intraepithelial neoplasia (CIN2+; CIN3+) in a vaginal self-sample compared to a clinician-collected cervical sample [7, 8]. Additionally, urine-based self-sampling provides a more preferred alternative [9-14], which is non-invasive and easily collected, however, data on the clinical accuracy of hrHPV detection in urine used in population-based screening settings are still scarce. Pathak (2014) reported an overall pooled virological sensitivity and specificity of HPV testing in urine (all possible fractions) of 77% (95% CI (confidence interval): 68-84%) and 88% (95% CI: 58-97%), respectively, to detect hrHPV in cervical samples. The diagnostic odds ratio was 22-fold when the first part of the urine void was collected compared to random or midstream samples [15]. Results from this meta-analysis and recent literature demonstrate the importance of using the first part, i.e. initial stream of urine defined as first-void (FV) urine. This initial urine stream washes away, and hence collects, HPV-containing mucus and debris from exfoliated cells from the female genital organs lining the urethra opening. Indeed, recent clinical trials

collecting urine using a standardized sample collection protocol which includes use of FV urine and DNA preservative demonstrate higher hrHPV test agreement [15-19] and clinical sensitivity [9, 12, 20-22] in self-collected urine versus cervical samples compared to trials where random or midstream urine was collected or not immediately preserved [23-25]. Although there are over 250 commercial HPV tests available on the global market to date, only few tests' performance are clinically validated for screening with an HPV assay according to either the Meijer guidelines (n=13), United States Food and Drug Administration (FDA) standards (n=2), or World Health Organization (WHO) prequalifications (n=3) [3]. Neither of these HPV assays are clinically validated for use with self-collected vaginal or urine samples. Several self-sampling devices are commercially available at present and have been evaluated separately in clinical studies [7]. A subgroup analysis did not reveal differences in relative clinical accuracy (self- versus clinician-collection) by self-collection device or preservative. Yet, since comparisons were indirect differences cannot be excluded [7].

Here, we report on the clinical accuracy of the Abbott RealTime High Risk HPV assay (Abbott GmbH, Wiesbaden, Germany) – hereafter referred to as RT hrHPV – in urine collected with Colli-Pee containing urine conservation medium (UCM) (Novosanis, Wijnegem, Belgium) using paired clinician-collected cervical samples and colposcopy(-directed biopsy) as reference following the VALHUDES protocol (VALidation of HUman papillomavirus assays and collection DEvices for Self-samples and urine samples) [26]. The RT hrHPV is a clinically validated [3, 27], automated, qualitative real-time PCR test detecting DNA from 14 hrHPV types (HPV16/18/31/33/35/39/45/51/52/56/58/59/66/68) in cervical specimens with concurrent partial genotyping (HPV16 and 18) [28]. The VALHUDES trial was set-up to address the scarcity of comparative clinical accuracy data of hrHPV-testing using validated PCR-based HPV DNA assays to detect CIN2+ in self-collected vaginal and FV urine samples – collected under standardized and optimized conditions – compared to cervical samples collected by a clinician from the same individuals.

This study primarily investigates the relative accuracy of RT hrHPV testing on FV urine versus clinician-collected cervical samples to detect CIN2+. Secondary objectives include evaluation of the analytical and clinical performance by age group and grading of reported cervical neoplasia.

METHODS

Study protocol and population

The study design of the VALHUDES trial (NCT03064087) has been formerly described [26]. In brief, VALHUDES was designed as a prospective diagnostic test accuracy study (according to STARD guidelines [29]) comparing HPV assays results in self-collected FV urine and vaginal samples (index test) with clinician-collected cervical samples (comparator test) using colposcopy applied to all women followed by colposcopy-directed biopsy for disease verification (reference standard). Negative colposcopy was accepted as providing sufficient ascertainment for absence of cervical disease. In case of multiple biopsy specimens, the worst histological outcome was used. Biopsies were assessed histologically as defined in routine pathology practice (blinded to index and comparator test outcomes) in the institutions of the participating colposcopy centers. Between December 2017 and January 2020, 523 women attending a colposcopy clinic referred due to previous abnormal screen test or follow-up result were enrolled in five Belgian colposcopy clinics; i.e. the University Hospitals of Antwerp (UZA), Brussels (UZ Brussels), Ghent (UZ Ghent), and Liège (CHU de Liège), and the General Regional Hospital Heilig Hart Tienen (RZ Tienen). Women with known pregnancy at consultation, hysterectomized women, non-consenting women, and women unable to understand patient information materials and informed consent form were excluded from the trial.

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee (central ethics committee UZA/University of Antwerp, Belgium (B300201733869)) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from study participants, who received a personal identifier to which all data were linked to ensure patient confidentiality.

Sample collection and processing

Women who met the inclusion criteria were informed about the study per phone call by a member of the colposcopy clinic's study team. A study package composed of the information brochure and informed consent form, instructions how to collect two FV urine samples, and two Colli-Pee devices was thereafter sent to the women's home address. The Colli-Pee device allows volumetric and standardized collection of ~13 ml of FV urine, which is automatically captured in a collector tube prefilled with 7 ml of non-toxic UCM. Women were asked to collect two FV urine samples at home, the day prior to colposcopy, and to not urinate at least two hours prior to each FV urine collection. In addition, instructions requested the women to not extensively wash their genitals prior to FV urine collection and store the samples at room

temperature between collection and receipt by the study nurse on the day of colposcopy. At the time of reception at the clinic, FV urine samples were stored at 2-8°C prior to transportation to the laboratory (Centre for the Evaluation of Vaccination (CEV), University of Antwerp). After vortexing for 15-20 seconds, FV urine was transferred to secondary tubes and stored at -35°C (Biobank Antwerp, Antwerp, Belgium; ID: BE 71030031000) [30]. Back-up aliquots in case of retesting were stored at -80°C.

Women also collected two dry vaginal self-samples at the clinic prior to colposcopy and filled in a questionnaire to assess attitudes and experiences about self-sampling [26]. These results will be reported in separate manuscripts.

In addition to the self-collected samples, a cervical specimen was taken by a trained gynecologist using the Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) prior to colposcopy and colposcopy-targeted biopsy if indicated. The cervical specimen was transferred in 20 ml PreservCyt solution (Hologic Inc., Bedford, Massachusetts, USA) according to European guidelines for preparation of cervical liquid-based cytology samples [31]. Upon arrival at AML Sonic Healthcare (Antwerp, Belgium), cervical samples were vortexed (15-20 seconds) and transferred to secondary tubes. Samples were stored between 2-8°C for a maximum of four months prior to HPV testing (Biobank, BB190002).

HPV testing

The RT hrHPV was performed at AML Sonic Healthcare, a routine laboratory which is part of the national Belgian reference centre for HPV. Laboratory personnel executing the RT hrHPV was blinded to the clinical endpoints. All quadruplets (cervical, FV urine, two dry vaginal self-samples) were tested in the same run on the automated Abbott *m2000* System, following manufacturer's instructions [28]. In brief, *m2000* reaction vessels were prefilled with 0.70 ml sample, from which DNA is extracted from 0.4 ml prior to setup of PCR reactions in a 96-well optical reaction plate by the *m2000sp* instrument. Amplification, result interpretation and reporting are performed by the *m2000rt* instrument. At the end of the real-time PCR, cycle number (CN) values for three HPV signals (HPV16, HPV18, and non-HPV16/18 hrHPV with target sequence located in the conserved L1 region of the HPV genome) are reported by the system. An endogenous human β -globin sequence for sample validity (cell adequacy, sample extraction and amplification efficiency), negative (DNA containing human β -globin) and positive controls (DNA containing human β -globin, HPV16, 18, and 58) are likewise reported to assess run validity. The system's software automatically reports when hrHPV DNA is detected at a CN cut-off of ≤ 32 . In case of

a test failure on one or more sample types from the same participant, a retest was performed for the entire quadruplet. If the comparator test had a test failure after the retest, the whole quadruplet was excluded. In case there was a test failure on the index test, then only this sample type was excluded from the subsequent analysis.

Data analysis

Analytical and clinical performance of the RT hrHPV test in FV urine and cervical samples were evaluated based on test results reported by the assay software. This implies application of the manufacturer's fixed assay cut-off of $CN \leq 32$. As no assay cut-off for RT hrHPV testing in FV urine has been clinically validated, we used the predefined cut-off for cervical samples ($CN \leq 32$) for FV urine. Additionally, an a posteriori defined exploratory cut-off ($CN \leq 33.86$) was defined for FV urine. This explorative FV urine cut-off was chosen using Receiver Operator Characteristic (ROC)-tables for each HPV channel. $CN 33.86$ was the cut-off in FV urine for which similar sensitivity for CIN2+ was obtained for all three HPV channels as in cervical samples evaluated based on the manufacturer's fixed assay cut-off ($CN 32$). Absolute and relative clinical performance were calculated with accompanying 95% CI. CI for relative measures were computed taking the matched design into account. The McNemar test was used to assess differences in sensitivity and specificity between paired FV urine and cervical samples. The agreement (expressed as Cohen's Kappa (κ)) was calculated to assess test result concordance between FV urine and cervical samples and was categorized as follows: $\kappa \leq 0.20$, poor; $0.21 \leq \kappa \leq 0.40$, fair; $0.41 \leq \kappa \leq 0.60$, moderate; $0.61 \leq \kappa \leq 0.80$, good; and $\kappa \geq 0.81$, excellent agreement [32]. Differences between normally or non-normally distributed continuous variables (age, CN-values) by levels of categorical variables were analyzed using the paired T-test or the Mann Whitney U-test, respectively. Relations between continuous variables were assessed by linear regression and Pearson's correlation (r). Sample size calculation was reported previously [26].

Statistical analyses were performed with the statistical software JMP Pro 14, Stata version 16 (College Station, Texas, USA), and R version 3.6.0. Statistical significance was accepted at a significance level of 0.05.

RESULTS

Study population and sample characteristics

After exclusions based on missing self- and clinician-collected samples (n=22) and colposcopy results (n=2) (Fig 1), 499 out of 523 samples from women attending colposcopy were available for RT hrHPV testing. From these 499, six FV urine and thirteen cervical samples had an initial test failure. After retesting, one out of six FV urine, and five out of thirteen cervical samples remained invalid. Majority of initial (n=13/19; 68%) test failures were due to technical errors whereas majority of failures after retesting (n=5/6; 83%) were due to insufficient β -globin. Providing valid test results for RT hrHPV on FV urine and cervical specimens from 493 women aged 19 to 72 years (median age 40; interquartile range (IQR): 31-50 years) for the current data-analysis (Fig 1). Histology results were available for 60% (n=294/493) of included subjects: CIN0 (n=108; 37%), CIN1 (n=98; 33%), CIN2 (n=43; 15%), and CIN3 (n=45; 15%). All CIN2+ cases provided valid FV urine and cervical test results. Two additional cases of low-grade glandular lesions (<CIN2; hrHPV negative in FV urine and cervical samples) but no cervical cancers were found. In the remaining 40% of women (n=199/493) no biopsy was taken because of normal or minor colposcopy findings and clinical context. Samples from these women were classified as <CIN2. Median age was higher in women with <CIN2 (41; IQR: 32-50 years) than with CIN2+ (35; IQR: 29-44 years) (p=0.003). In Table 1, age group prevalence of hrHPV in FV urine and cervical samples is shown for both the total study population and after stratification by disease outcome. Ninety-six percent (n=475/493) of FV urine samples were per protocol collected the day before colposcopy (min-max: 0-3 days). No significant linear correlation was found between age and β -globin CN nor HPV16/18/other hrHPV CN-values in both FV urine and cervical samples (p>0.05). Likewise, time between FV urine collection at the women's home and freezing the sample at the laboratory (mean 4.7 days; SE=0.1; minimum-maximum 2-9 days) had no impact on β -globin or HPV CN-values (p>0.05).

Clinical performance of the RealTime High Risk HPV assay

When applying the cut-off defined by the manufacturer for cervical samples (CN $\leq$ 32), absolute sensitivity for CIN2+, CIN3+, and specificity for <CIN2 of hrHPV testing was 88.6% (95% CI: 80.3-93.7%), 88.9% (95% CI: 76.5-95.2%), and 49.4% (95% CI: 44.5-54.2%) in FV urine, and 93.2% (95% CI: 85.9-96.8%), 97.8% (95% CI: 88.4-99.6%), and 47.9% (95% CI: 43.1-52.8%) in cervical samples, respectively. Two CIN2 cases were missed by hrHPV testing in cervical samples only, two CIN2 and four CIN3 cases were missed by hrHPV testing in FV urine only, whilst three CIN2 cases and one CIN3 were missed by hrHPV testing in both sample types. At the explorative CN cut-off of 33.86 for FV urine, one additional CIN2

and three CIN3 cases would have been identified, increasing the absolute sensitivity to detect CIN2+ and CIN3+ to 93.2 (95% CI: 85.9-96.8%) and 95.6% (95% CI: 85.2-98.8%), respectively at a specificity of 45.4% (95% CI: 40.6-50.3%) (Table 2). In women 30 years and older, two additional CIN3 cases were correctly identified in FV urine by increasing the cut-off to 33.86, at the cost of 15 true negatives becoming false-positive (Table 2). The single CIN3+ case missed by clinician-collected cervical samples was also missed by FV urine.

RT hrHPV testing in FV urine was found similarly sensitive (ratio=0.95; 95% CI: 0.88-1.01) and specific (1.03; 95% CI: 0.95-1.13) to detect CIN2+ in the total study population ($p>0.05$ and 95% CI included unity) (Table 3). Though, a slightly lower sensitivity for CIN3+ was observed in FV urine compared to cervical samples (0.91; 95% CI: 0.81-0.98; $p=0.0455$). Increasing the cut-off to 33.86 for FV urine resulted in equal sensitivity for both CIN2+ (1.00; 95% CI: 0.95-1.05) and CIN3+ (0.98; 95% CI: 0.93-1.00), while maintaining similar specificity for $<CIN2$ (0.95, 95% CI: 0.87-1.04) (Table 3). In women 30 years and older, similar sensitivity and specificity of RT hrHPV testing in FV urine was found with respect to cervical samples for CIN2+ and CIN3+ (Table 3).

Analytical high-risk HPV agreement for first-void urine and cervical samples

Mean CN-values were found to be higher in FV urine compared to cervical samples for all quantitative test outputs, resulting in positive differences in mean $CN_{FV\ urine} - CN_{cervical}$ of 0.99 for β -globin ($p<0.001$), 2.67 for HPV16 ($p<0.001$), 2.05 for HPV18 ($p=0.112$), and 2.32 for other hrHPV ($p<0.001$). Similarly, higher mean CN-values were observed in FV urine than in cervical samples for the disease endpoints $<CIN2$ and CIN2+ ($p<0.05$ for β -globin and other hrHPV). Median CN-values were lower in women with CIN2+ compared to $<CIN2$ for β -globin, HPV16, and other hrHPV in both FV urine and cervical samples (Fig 2). HPV18 CN-values did not differ significantly by disease endpoint in either sample type.

Table 4 shows good ($0.61 \leq \kappa \leq 0.80$) to excellent ($\kappa \geq 0.81$) agreement between FV urine and cervical samples, with moderate to fair overall hrHPV agreements for CIN2+ and CIN3+ at the predefined cut-off of CN 32.

When the FV urine cut-off was adjusted to 33.86, good to excellent agreement was observed for overall hrHPV positivity, and HPV16, HPV18, and other hrHPV (Table 4). Scatterplots displaying correlations in CN-values between FV urine and cervical specimens for each RT hrHPV channel are illustrated in the Supplementary figure S1.

DISCUSSION

Our study demonstrates that hrHPV testing with the Abbott RealTime High Risk HPV assay on FV urine collected at home using Colli-Pee shows similar clinical accuracy to detect high-grade cervical intraepithelial neoplasia as Abbott RealTime High Risk HPV testing on cervical samples collected by a clinician. This similar accuracy was observed for the total study population, as for women 30 years and older.

Validated self-sampling devices that are affordable, user-friendly, accepted by women, and accurate have the potential to play a key role in cervical cancer screening. In light of pandemics such as the current SARS-CoV-2 pandemic where standard care is suspended, offering self-sampling could facilitate timely screening and retesting whilst reducing the burden on health care personnel and risk of SARS-CoV-2 transmission. This increased interest in self-sampling in light of the pandemic was recently reported [33, 34]. Urine sampling using either a standard urine container or the Colli-Pee device is a non-invasive, easy to collect method that has been well accepted by women (Pauwels, *submitted*) [9-14, 17], and has been rolled out in population based pilot programs in a primary screening setting [23, 24] and as a tool to reach screening non-responders [35, 36]. In contrast to its acceptability, little evidence was available until now about the clinical performance of hrHPV testing using validated PCR-based assays in urine collected under standardized conditions.

The first results from VALHUDES reveal no statistically significant difference in sensitivity (CIN2+) nor specificity (<CIN2) of hrHPV testing with the Abbott RealTime High Risk HPV assay in FV urine collected with Colli-Pee, compared to clinician-collected cervical samples, to detect high-grade cervical lesions. This was confirmed by 95% CI of relative accuracy estimates including unity, demonstrating similarity of clinical accuracy in FV urine compared to cervical samples collected by a clinician. In women 30 years and older, no difference in sensitivity for CIN3+ was found either between FV urine and cervical hrHPV outcomes, confirmed by 95% CI's including unity. Adjusting the CN cut-off to CN 33.86 for FV urine was performed a posteriori as a clinical cut-off has only been validated in cervical samples to date. At this exploratory cut-off in FV urine, increasing sensitivity (CIN2+/CIN3+), no adverse change in significant differences for relative clinical specificity (<CIN2) was found compared to cervical samples, in women

30 years and older as well as for the total study population. Additionally, good to excellent agreements were observed at this explorative assay cut-off for both overall hrHPV positivity as for partial genotyping results (HPV16, 18, other hrHPV). This a posteriori exploratory analysis however requires validation in a new study to confirm that HPV testing with a given assay on FV urine is similar to testing on a cervical specimen.

Two other studies investigated clinical [13] and analytical performance [37] of the Abbott RealTime High Risk HPV assay in urine samples. In these studies, samples were not collected at home circumventing the impact of a real-life setting on for instance understanding self-sampling device user instructions by the participant, as well as storage and transportation of the samples to the clinic. In addition, a simple urine cup was used in both trials and a preservative was added to the sample after collection. Importantly, both studies used FV urine, which significantly impacts accuracy of hrHPV testing in urine [15, 18, 19]. Just recently, Cadman (2021) [22] and Ørnskov (2021) [38] reported on clinical accuracy of urinary hrHPV testing using the BD Onclarity and Roche Cobas 4800 HPV assay, respectively. The Predictors 5.1 study demonstrated similar sensitivity of hrHPV testing in Colli-Pee collected (and UCM preserved) FV urine to detect CIN2+ compared to the wet *digene* Female Swab (Qiagen GmbH) used as comparator, yet observed lower specificity for <CIN2 [22]. A disadvantage of this study however is that no paired cervical specimen was available to report relative clinical accuracy estimates compared to clinician-collected samples. In their cross-sectional study, Ørnskov (2021) demonstrated both non-inferior clinical sensitivity (CIN2+; CIN3+) and specificity (<CIN2) of hrHPV testing in urine collected in a simple urine cup compared to clinician-collected cervical samples [38]. This clinical validation is promising and first to demonstrate both non-inferior clinical sensitivity and specificity for urinary hrHPV testing compared to cervical samples collected by a physician. Yet, urine samples were not collected at home, were preserved in EDTA by laboratory staff, as well as requiring additional sample handling steps before being tested by Roche Cobas 4800.

Strengths of our study include the large sample size (n=493), use of an automated hrHPV-DNA based PCR-test clinically validated for primary cervical cancer screening, and use of a device that collects a standardized volume of the initial urine stream whilst immediately mixing with a non-toxic DNA preservative.

In contrast to vaginal self-samples collected with the Qvintip device within a Belgian population-based randomized trial [39], no impact of age on sample validity (β -globin) was observed for FV urine in VALHUDES. No effect of time between FV urine collection and storage (up to nine days) on sample validity was found, which suggests robustness of the presented sample collection and testing method. As VALHUDES was not designed to study the impact of age, nor time between sample collection and processing on sample validity, these results should be interpreted cautiously. Of note, FV urine was collected at home without in-person instructions how to collect the sample, representative of its use in a real-life health care setting.

The presented FV urine method is also complementary to high-throughput, automated Abbott RealTime High Risk HPV testing without the need for additional laborious sample handling steps such as preservative addition or centrifugation reported by others [13, 37, 38]. Colli-Pee collected, UCM preserved FV urine allows for sample handling following the same procedure applied for manufacturer validated cervical samples collected by a clinician.

The referral setting partakes the additional benefit of VALHUDES that all women were subjected to colposcopy (and biopsy if indicated) for disease verification based on clinical reasons rather than study-driven interventions [26].

Limitations of our study include the colposcopy setting itself as self-sampling has its utility essentially in primary screening, preventing us to translate absolute accuracy outcomes to a primary screening population. However, opposed to the absolute accuracy, in particular the specificity of hrHPV testing which depends on the clinical setting of the study population, relative sensitivity and specificity of hrHPV testing on self- versus on clinician-collected samples appear to be similar among screening and follow-up settings [7]. Furthermore, an update of the internationally established guidelines to validate HPV assays [40] is awaited and should include well-defined criteria to validate the clinical performance of HPV tests in self-collected vaginal and urine samples, including guidelines for sample collection, preservation, and nucleic acid extraction as well, being a valuable part of the HPV testing procedure[41]. Herewith, HPV assay accuracy estimates could be compared more easily between sample types. Secondly, in VALHUDES, vaginal and cervical samples were collected prior to colposcopy, which may have influenced colposcopy. However, it can be plausibly assumed that such a reference misclassification bias, if it would occur, would affect evaluation of accuracy of HPV testing on the diverse

self- and clinician-taken samples similarly, not affecting the evaluation of the relative accuracy. Additionally, no CIN2+ cases were missed due to invalid cervical test results, substantiating the quality of cervical samples in VALHUDES.

Thirdly, according to routine clinical practice, no formal expert review of colposcopy and histology was performed, accepting the quality control of participating colposcopy centers. Also here, it is reasonable to expect that relative accuracy estimates are similarly affected in case a misclassification would occur. Lastly, a proportion of FV urine samples was excluded because of not per protocol collection. This was due to more stringent rules applied for FV urine sample collection (e.g. minimal volume required, order and timing registry to make a distinction between the sample pair, quality of both and not only one sample). Though, only one FV urine sample was excluded after retesting compared to five cervical samples.

We can conclude that hrHPV-DNA based PCR testing with Abbott RealTime High Risk HPV in FV urine, collected at home with Colli-Pee allowing standardized FV urine collection and immediate DNA preservation, is similarly sensitive and specific to detect high-grade cervical intraepithelial neoplasia compared to cervical samples collected by a clinician.

CONTRIBUTORS

SVK, EP, DVB, AV and MA were involved in Conceptualization, Methodology, Validation, Resources, Data Curation, Project administration and Funding acquisition. MA is principal investigator of VALHUDES. SVK, EP and MA performed the Formal analysis of the results. SVK prepared the original and final manuscript with support from AV and MA. All authors contributed to the Investigation by collecting data and/or performing experiments, and reviewed the final version of the manuscript.

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ETHICS

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee (central ethics committee UZA/University of Antwerp, Belgium (B300201733869)) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from study participants, who received a personal identifier to which all data were linked to ensure patient confidentiality.

DISCLOSURE OF RELATIONSHIPS AND ACTIVITIES

VALHUDES is a researcher-induced study consortium initiated by Sciensano (Principal Investigator; Brussels, Belgium), CEV (University of Antwerp, Antwerp, Belgium), and AML (Antwerp, Belgium) with the aim to compare the clinical accuracy of particular high-risk HPV assays on self-collected vaginal and first-void urine samples in agreement with standardized protocols, with high-risk HPV testing on matched clinician-collected cervical samples. This research was supported by a grant from Abbott Laboratories (Wiesbaden, Germany) and Novosanis (Subsidiary of OraSure Technologies Inc, Wijnegem, Belgium). Other manufacturers can participate under the condition of a financial contribution to cover the costs for logistics, test kits/equipment, and statistical analysis.

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 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, a commercial lab performing cervical cytology and HPV testing. All other authors declare no competing interests.

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423

424 **APPENDIX A. SUPPLEMENTARY DATA**

425 Supplementary data to this article can be found online at *[insert doi]*.

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FIGURES LEGENDS

Fig 1. Flow diagram for the inclusion of participants, samples, and performed tests within the VALHUDES trial. Grey colored boxes mark the specific flow of index, comparator, and reference test outcomes reported within this manuscript to investigate relative clinical accuracy of RT hrHPV (Abbott RealTime High Risk HPV assay) testing in first-void urine compared to clinician-collected cervical samples to detect high-grade cervical intraepithelial neoplasia (CIN2+). In the VALHUDES trial, women also collected two dry vaginal self-samples at the clinic prior to colposcopy (grey colored boxes), and filled in a questionnaire to assess attitudes and experiences about self-sampling. These results will be reported in separate manuscripts. ^aNo valid first-void urine sample pair (Colli-Pee; n=4), no self-collected vaginal sample (n=1 [Evalyn Brush]; n=1 [Abbott multi-Collect]), no clinician-collected cervical sample (Cervex-Brush; n=2); ^bno colposcopy (n=2). RT hrHPV retest failure in ^cclinician-collected cervical samples only (n=5 <CIN2), ^dfirst-void urine only (n=1 <CIN2), ^evaginal self-samples only (n=3 <CIN2 and n=2 CIN2+ [Evalyn Brush]; n=1 <CIN2 and n=2 CIN2+ [Qvintip]). ^fTwo participants with <CIN2 were also diagnosed with glandular intraepithelial neoplasia.

Fig 2. Distribution of RealTime High Risk HPV cycle number (CN)-values between cervical (blue) and first-void urine samples (yellow) according to cervical intraepithelial neoplasia (CIN). Boxplots indicate median CN-values and according interquartile ranges (25th and 75th percentile) for β -globin (A), HPV16 (B), HPV18 (C), and other high-risk (hr)HPV (D). Significant lower median CN-values (indicated by an asterisk) were found in cervical samples from women diagnosed with high-grade CIN (CIN2+) compared to women with no or minor cervical abnormalities (<CIN2) for β -globin (n=493; CN_{Δ} =0.47; p=0.013), HPV16 (n=79; CN_{Δ} =3.46; p=0.005), and other hrHPV (n=264; CN_{Δ} =1.81; p=0.012). In first-void urine, lower median CN-values were likewise observed in CIN2+ compared to <CIN2 for β -globin (n=493; CN_{Δ} =1.11; p<0.001), HPV16 (n=86; CN_{Δ} =1.00; p=0.099), and other hrHPV (n=276; CN_{Δ} =0.34; p=0.140), yet only significant for β -globin. For HPV18, median CN-values were non-significantly higher for CIN2+ compared to <CIN2 in both cervical (n=23; CN_{Δ} =0.66; p=0.494) and first-void urine samples (n=28; CN_{Δ} =0.72; p=0.976).

565 **TABLES**

566

567 **Table 1. Prevalence of high-risk HPV and colposcopy (-directed biopsy) results according to age group and the total study population.**

Age group (yr)	No. of participants (%)		No. of RT hrHPV positive results (%) ^a			No. of participants by disease outcome (%)		
			First-void urine (CN ₃₂)	First-void urine (CN _{33.86})	Cervical samples (CN ₃₂)	<CIN2	CIN2	CIN3+
≤24	10	(2.03)	8 (80.0)	8 (80.0)	7 (70.0)	9 (90.0)	1 (10.0)	0 (0.0)
25-29	87	(17.7)	54 (62.1)	57 (65.5)	59 (67.8)	64 (73.6)	12 (13.8)	11 (12.6)
30-34	85	(17.2)	53 (62.4)	59 (69.4)	58 (68.2)	65 (76.5)	10 (11.8)	10 (11.8)
35-39	54	(11.0)	26 (48.1)	27 (50.0)	23 (42.6)	46 (85.2)	4 (7.4)	4 (7.4)
40-44	70	(14.2)	42 (60.0)	45 (64.3)	42 (60.0)	53 (75.7)	10 (14.3)	7 (10.0)
45-49	64	(13.0)	28 (43.8)	30 (46.9)	30 (46.9)	58 (90.6)	3 (4.7)	3 (4.7)
50-54	52	(10.6)	30 (57.7)	31 (59.6)	29 (55.8)	48 (92.3)	2 (3.8)	2 (3.8)
55-59	44	(8.9)	30 (68.2)	31 (70.5)	31 (70.5)	38 (86.4)	0 (0.0)	6 (13.6)
60-64	24	(4.9)	12 (50.0)	13 (54.2)	12 (50.0)	21 (87.5)	1 (4.2)	2 (8.3)
≥65	3	(0.4)	0 (0.0)	2 (66.7)	2 (66.7)	3 (100.0)	0 (0.0)	0 (0.0)
Total	493	(100.0)	283 (57.4)	303 (61.5)	293 (59.4)	405 (82.2)	43 (8.7)	45 (9.1)

568 ^aHigh-risk HPV positive for at least one genotype included in the Abbott RealTime High Risk HPV (RT hrHPV) assay: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.

569 RT hrHPV test positivity was calculated using the Abbott software cut-off clinically validated for cervical samples (cycle number (CN) cut-off ≤ 32), and CN cut-off ≤ 32 and

570 exploratory CN cut-off ≤ 33.86 for first-void urine samples. CINx: cervical intraepithelial neoplasia grade x.

571

Table 2. Absolute clinical sensitivity (CIN2+ and CIN3+) and specificity (<CIN2) of the RealTime High Risk HPV assay in first-void urine and cervical samples, in the total study population and women aged 30 years or older.

Study group and test ^a	Sensitivity % (no.); 95% CI		Specificity % (no.); 95% CI
	CIN2+	CIN3+	<CIN2
Total study population (n=493)			
Cervical (CN ₃₂)	93.2 (82/88); 85.9-96.8	97.8 (44/45); 88.4-99.6	47.9 (194/405); 43.1-52.8
First-void urine (CN ₃₂)	88.6 (78/88); 80.3-93.7	88.9 (40/45); 76.5-95.2	49.4 (200/405); 44.5-54.2
First-void urine (CN _{33.86})	93.2 (82/88); 85.9-96.8	95.6 (43/45); 85.2-98.8	45.4 (184/405); 40.6-50.3
Women ≥ 30 years old (n=396)			
Cervical (CN ₃₂)	92.2 (59/64); 83.0-96.6	97.1 (33/34); 85.1-99.5	49.4 (164/332); 44.1-54.8
First-void urine (CN ₃₂)	89.1 (57/64); 79.1-94.6	88.2 (30/34); 73.4-95.3	50.6 (168/332); 45.2-55.9
First-void urine (CN _{33.86})	92.2 (59/64); 83.0-96.6	94.1 (32/34); 80.9-98.4	46.1 (153/332); 40.8-51.5

^aHigh-risk HPV positive for at least one genotype included in the Abbott RealTime High Risk HPV assay: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68. Absolute sensitivity and specificity were calculated at a cycle number (CN) cut-off ≤ 32 for cervical samples, and CN cut-off ≤ 32 and exploratory CN cut-off ≤ 33.86 for first-void urine samples. 95% CI: 95% confidence interval; CINx: cervical intraepithelial neoplasia grade x.

580 **Table 3. Relative clinical sensitivity (for CIN2+ and CIN3+) and specificity (for <CIN2) of the RealTime High Risk HPV assay in first-void urine with respect to cervical**
581 **samples, in the total study population and women aged 30 years or older.**

Study group and test ^a	Relative sensitivity ^b						Relative specificity ^b		
	CIN2+ (95% CI)		p _{McN} ^c	CIN3+ (95% CI)		p _{McN}	<CIN2 (95% CI)		p _{McN}
Total study population (n=493)									
CN cut-off ≤ 32	0.95	0.88 - 1.01	0.1573	0.91	0.81 - 0.98*	0.0455**	1.03	0.95 - 1.13	0.4855
CN cut-off ≤ 33.86	1.00	0.95 - 1.05	1.000	0.98	0.93 - 1.00	0.3173	0.95	0.87 - 1.04	0.2320
Women ≥ 30 years old (n=396)									
CN cut-off ≤ 32	0.97	0.89 - 1.05	0.4142	0.91	0.79 - 1.00	0.0833	1.02	0.93 - 1.12	0.6056
CN cut-off ≤ 33.86	1.00	0.93 - 1.07	1.000	0.97	0.91 - 1.00	0.3173	0.93	0.85 - 1.02	0.1451

582 ^aHigh-risk HPV positive for at least one genotype included in the Abbott RealTime High Risk HPV (RT hrHPV) assay: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68.

583 ^bRelative accuracy was calculated using absolute sensitivity and specificity at a cycle number (CN) cut-off ≤ 32 for cervical samples (comparator test), and CN cut-off ≤ 32 and
584 exploratory CN cut-off ≤ 33.86 for first-void urine samples (index test). *Relative accuracy statistically significant different from unity; **p_{McN} ≤ 0.05 (McNemar test) indicate relative
585 accuracy estimates for which a statistically significant different mean outcome was found between first-void urine (index test) and cervical samples (comparator test) at a given
586 CN cut-off; 95% CI: 95% confidence interval; CINx: cervical intraepithelial neoplasia grade x.

587

588 **Table 4. High-risk HPV concordance between first-void urine and cervical samples in all participants and subgroups according to two cycle number cut-offs.**

Study group and test ^a	Cervical RT hrHPV test result (comparator)											
	All (n=493)			<CIN2 (n=405)			CIN2+ (n=88)			CIN3+ (n=45)		
First-void urine RT	+	-	κ-value (95% CI) ^b	+	-	κ-value (95% CI) ^b	+	-	κ-value (95% CI) ^b	+	-	κ-value (95% CI) ^b
hrHPV test result (index)												
CN cut-off ≤ 32												
High-risk HPV												
+	247	36	0.658 (0.590-0.725)	171	34	0.634 (0.559-0.710)	76	2	0.453 (0.138-0.768)	40	0	0.308 (-0.160-0.775)
-	46	164		40	160		6	4		4	1	
HPV16												
+	64	12	0.870 (0.808-0.932)	26	12	0.756 (0.638-0.874)	38	0	0.977 (0.932-1.000)	25	0	0.955 (0.867-1.000)
-	4	413		3	364		1	49		1	19	
HPV18												
+	16	5	0.751 (0.603-0.900)	13	4	0.731 (0.563-0.899)	3	1	0.851 (0.565-1.000)	2	1	0.789 (0.389-1.000)
-	5	467		5	383		0	84		0	42	
Other high-risk HPV												
+	196	37	0.663 (0.597-0.729)	146	31	0.641 (0.566-0.716)	50	6	0.732 (0.584-0.880)	21	4	0.687 (0.474-0.900)
-	46	214		41	187		5	27		3	17	

Exploratory CN cut-off ≤ 33.86

High-risk HPV

+	261	42	0.686 (0.621-0.752)	181	40	0.653 (0.579-0.727)	80	2	0.642 (0.317-0.967)	43	0	0.656 (0.031-1.000)
-	32	158		30	154		2	4		1	1	

HPV16

+	66	13	0.880 (0.821-0.940)	27	12	0.776 (0.663-0.889)	39	1	0.977 (0.932-1.000)	26	0	1.000 (NA)
-	2	412		2	364		0	48		0	19	

HPV18

+	18	8	0.754 (0.615-0.894)	15	6	0.758 (0.605-0.910)	3	2	0.739 (0.394-1.000)	2	1	0.789 (0.389-1.000)
-	3	464		3	381		0	83		0	42	

Other high-risk HPV

+	207	46	0.672 (0.606-0.737)	154	38	0.648 (0.574-0.722)	53	8	0.748 (0.604-0.893)	23	4	0.775 (0.590-0.959)
-	35	205		33	180		2	25		1	17	

589 ^aHigh-risk HPV positive for at least one genotype included in the Abbott RealTime High Risk HPV (RT hrHPV) assay: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68;
590 HPV16: positive for HPV16 only; HPV18: positive for HPV18 only; other high-risk HPV: positive for HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and/or 68. RT hrHPV test positivity
591 was calculated at a cycle number (CN) cut-off ≤ 32 for cervical samples, and CN cut-off ≤ 32 and exploratory CN cut-off ≤ 33.86 for first-void urine samples. ^bThe agreement
592 (expressed as Cohen's Kappa (κ)) was calculated to assess HPV agreement between paired samples and was judged as follows: κ ≤ 0.20, poor; 0.21 ≤ κ ≤ 0.40, fair (orange);

593 $0.41 \leq \kappa \leq 0.60$, moderate (yellow); $0.61 \leq \kappa \leq 0.80$, good (light green); and $\kappa \geq 0.81$, excellent (dark green) agreement [32]. 95% CI: 95% confidence interval; CINx: cervical
594 intraepithelial neoplasia grade x; NA: not available.