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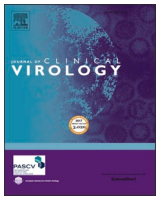

Clinical accuracy of OncoPredict HPV Quantitative Typing (QT) assay on self-samples

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ABSTRACT

Background: The VALHUDES initiative was established to assess the clinical accuracy of HPV assays to detect cervical precancers using urine and vaginal self-samples compared to cervical clinician-collected samples. Here, the clinical performance of OncoPredict HPV Quantitative Typing (QT) assay (OncoPredict QT) was evaluated.
Methods: 490 women referred to colposcopy self-collected a urine and a vaginal specimen using Colli-Pee and FLOQSwab, respectively. Subsequently, a colposcopy was performed, and a cervical sample was collected with Cervex-Brush, followed by biopsy if clinically indicated. Vaginal samples were transported dry and resuspended in 5 mL of eNAT medium, whilst cervical brushings were immediately transferred in 20 mL ThinPrep.
Results: The clinical sensitivity of OncoPredict HPV QT testing for CIN2+ in urine and vaginal self-samples was similar to cervical samples (ratios of 0.99 [95 % CI 0.94–1.05] and 1.00 [95 % CI 0.96–1.04]), respectively, when manufacturer's cut-offs were applied. The specificity for <CIN2 on both self-samples was lower than on cervical samples (urine/cervical ratio = 0.91 [95 % CI 0.84–0.98]; vaginal/cervical ratio = 0.90 [95 % CI 0.84–0.98]). Cut-off optimisation improved specificity without compromising sensitivity. Median viral load values adjusted for cellularity were significantly higher in cervical samples compared to urine or vaginal self-samples, in general for all 12 high-risk HPV and in particular for HPV16, 18, 31, 33, 35, 45, 51, 58 ($p < 0.05$). No difference was observed in median viral loads between urine and vaginal samples.
Conclusion: Following cut-off optimisation OncoPredict HPV QT assay demonstrated similar accuracy on self-collected versus cervical samples.

Introduction

High-risk (hr) HPV testing for detection of high-grade cervical intraepithelial neoplasia (CIN) has been successfully used as a primary cervical cancer screening tool [1]. In addition, HPV testing on self-samples offers opportunities to increase screening coverage in under-screened women [2,3]. Since self-samples are often well accepted among women and show similar sensitivity for the detection of cervical precancer, many countries are implementing self-sampling strategies within screening programmes routinely [4].

Meta-analyses have shown that polymerase chain reaction (PCR)-based HPV DNA tests, validated on clinician-taken samples are similarly

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accurate to detect high-grade CIN as testing on vaginal or urine self-samples [2,3,5]. These meta-analyses provided initial robust evidence demonstrating a high level of acceptance of self-sampling among women. Nevertheless, optimisation of pre- and post-analytical workflows is still lacking [6].

HPV testing using cervical samples for cervical cancer screening is well established. Performance criteria relevant for suitable HPV assays are incorporated within the Meijer 2009 or VALGENT criteria [7,8]. More than a dozen of HPV tests presently fulfil these international criteria and are considered clinically validated for cervical cancer screening [9]. Nevertheless, even if a test has been validated for cervical cancer screening using clinician-collected cervical samples, it does not automatically imply that this test can be applied to self-samples. Therefore, the VALHUDES framework was established to provide additional evidence of the HPV test accuracy on self-samples [10]. Several HPV tests were evaluated within the Belgian VALHUDES framework and have shown similar clinical accuracy on both first-void urine (FVU) and vaginal samples compared to clinician-taken samples using the manufacturer cut-off or after cut-off optimisation [11-16].

The second VALHUDES initiative was established with the support of the European Union (Grant No. 806551), bringing together the collaborative efforts of Italy, Ireland, and Scotland, however certain elements of the VALHUDES study protocols differed. In the first VALHUDES study, vaginal samples were collected with Evalyn Brush or Qvintip and resuspended in 20 mL ThinPrep, whereas in the European VALHUDES initiative a FLOQSwab was used in combination with 5 mL eNAT, an alcohol-free liquid-based medium which inactivates viruses and bacteria, preserving nucleic acids at room temperature. In the current VALHUDES report, we aimed to evaluate the accuracy of OncoPredict HPV Quantitative Typing (QT) assay to detect cervical precancer on FVU and vaginal self-samples collected with Colli-Pee and FLOQSwab devices, respectively. In addition, normalized type-specific HPV viral load was compared across specimen types. OncoPredict HPV QT assay is a quantitative full-genotyping assay targeting 12 hr HPV types and yielding normalized genotype-specific viral load. The assay has been validated within the VALGENT framework and can be used in cervical cancer screening based on clinician-collected samples [17].

Materials and methods

Study design

Matched cervical, vaginal and urine samples were collected from 500 patients recruited for the study within the European VALHUDES framework according to the STARD guidelines [18]. FVU was collected first using the Colli-Pee FV5000 collection device (Novosanis, Wijnegem, Belgium) followed by a vaginal self-collected sample with FLOQSwabs (Copan Italia Spa, Brescia, Italy), which was kept dry following collection. Thereafter, a cervical sample was taken by a gynaecologist using Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) prior to colposcopy. Colposcopy was performed as recommended by the European guidelines and a biopsy was taken if clinically indicated. Cervical samples were immediately transferred into 20 mL PreservCyt solution (Hologic, Marlborough, MA). All samples were labelled and sent to the laboratory connected with the colposcopy unit. After arrival in the laboratory, the dry vaginal samples were resuspended into 5 mL of eNAT medium (Copan Italia Spa, Brescia, Italy). The FVU samples were sent to the laboratory in the Colli-Pee container without further manipulation. At the laboratory, cervical and urine samples were divided into 1.5 mL aliquots, whereas vaginal samples were divided into 0.4 mL aliquots. All sample aliquots were properly labelled and stored at −20 °C until further dispatching to the MIRRI-IT Biobank of the University of Milano-Bicocca for storage at −80 °C. Study recruitment details are described in the supplementary methods.

HPV testing

HPV testing was performed using the OncoPredict HPV QT assay (Hiantis Srl, Milan, Italy), which is a quantitative full genotyping real-time PCR assay. The assay can detect and differentiate 12 hrHPV genotypes in four separate reaction wells (QT1: HPV 16/18/45; QT2: HPV 31/33/52; QT3: HPV 35/58/59; QT4: HPV 39/51/56). It targets *E6* and *E7* oncogenes using genotype-specific primers and probes. A fifth reaction well is used to quantitatively assess sample cellularity as well as the preanalytical nucleic acid extraction efficiency. Sample cellularity is calculated targeting the human —*C-C chemokine receptor type 5* (*CCR5*) gene. Both, sample cellularity and HPV viral loads are calculated using specific standard calibration curves. HPV type-specific viral loads are expressed as normalized viral genomic units/10^4 human cells based on the number of cells present in the sample determined by the quantitative CCR5 result. To assess DNA extraction efficiency, an external calibrator gene target is added to the sample prior to nucleic acid extraction and extraction efficiency is evaluated in terms of percentage recovery. Each reaction well also contains an exogenous amplification control, to independently assess PCR inhibition in each reaction mix. DNA extraction and quality control steps are described in the supplementary methods [17].

HPV positivity was defined using normalised genotype specific viral load expressed in viral copies/10^4 cells. Samples were considered HPV positive if $\geq$ 50 viral copies/10^4 cells were detected in cervical specimens and $\geq$ 10 copies/10^4 cells in vaginal and urine specimens, as recommended by the manufacturer. Cut-off optimization was subsequently performed based on the study results in order to achieve a balance between clinical specificity and sensitivity. Revised cut-offs were established at $\geq$15 HPV copies/10^4 cells for all HPV types on urine and vaginal samples. Additionally, normalised and non-normalised metrics of HPV16 positive samples were compared for their ability to identify CIN2+ cases. Normalised viral load per 10,000 cells of HPV16 was plotted against HPV16 Ct-value, as an approximation of "non-normalised viral load" per reaction, along with the clinical outcome. As the defined assay's cut-offs were 50 and 15 copies/10^4 cells for cervical and self-samples, respectively, we identified Ct-value cut-offs alternative to non-normalised 50 and 15 copies per reaction using standard calibration curves.

Statistical analysis

Given the matched design applied in VALHUDES studies, McNemar test was used to assess differences between index and comparator tests. McNemar standard errors were used to compute 95 % confidence intervals of the VALHUDES study. Statistical significance was accepted if p-values < 0.05 or when the 95 % confidence intervals around relative accuracy excluded unity.

Overall and type-specific HPV test agreement among self- and cervical samples was evaluated using Cohen's kappa values, 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). Concordance was assessed for the entire study population and according to disease outcome. Differences in median signal levels between samples were assessed with the matched Mann-Whitney test for paired comparisons, whereas for non-paired comparisons, the Wilcoxon signed-rank test was used. The natural logarithm of the viral load and cellularity was used in comparisons. The Spearman's correlation coefficient was used to assess the correlation between log (normalised viral load) of different sample types with application of the Benjamini–Hochberg (BH) false discovery correction [19]. All statistical analyses were conducted using Stata 16.1 (College Station, Texas, USA) and Jupyter Notebook 6.5.4.

Ethical approval

European VALHUDES study (NCT04312737) was approved by the

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. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants prior to enrolment.

Results

Study population

In total, 500 women were recruited (median age = 37 years; IQR: 31–47 years; range 25–64) from whom triplet samples were collected for the study. Ten patients were excluded due to major protocol violations as shown in Fig. 1. Twenty-seven cervical (27/490, 5.5 %), 13 vaginal (13/490, 2.7 %) and 14 urine (14/490, 2.9 %) samples were considered invalid. After excluding 27 participants due to invalid cervical samples, the dataset contained 463 participants: 452 matched cervical and vaginal and 450 matched cervical and urine samples were included in the evaluation of relative accuracy. Colposcopy was performed for 462 participants: 123 (123/462, 26.6 %) had negative colposcopy findings, 231 (231/462, 50.0 %) had minor colposcopy findings, 103 (103/462, 22.3 %) had major colposcopy findings and six (5/462, 1.1 %) had suspicion of cancer. Median age was higher in women without CIN (median age = 38; IQR 31–48) compared to CIN2 (median age = 34; IQR 30–41) or CIN3+ (median age = 33; IQR 30–40) ($P< 0.001$). Significantly more CIN2+ and CIN3+ cases were recruited in Milan than in the three other participating colposcopy clinics ($P< 0.001$). Characteristics of the study population are shown in Table 1.

Clinical accuracy of OncoPredict HPV QT assay

HPV testing with OncoPredict HPV QT assay was similarly sensitive for CIN2+ and CIN3+ on both urine and vaginal samples compared to cervical samples when manufacturer's cut-off were applied. Relative sensitivity was 0.99 [95 % CI 0.94–1.05] and 0.98 [95 %CI 0.91–1.06] for CIN2+ and CIN3+, respectively, on urine versus cervical samples. The relative sensitivity for CIN2+ on vaginal versus cervical samples was 1.00 [95 %CI 0.96–1.04] and for CIN3+ was 1.00 [95 %CI NA]. However, the specificity for <CIN2 was significantly lower on both urine (ratio = 0.91 [95 % CI 0.84–0.98], $P= 0.02$ and vaginal (ratio = 0.90 [95 %CI 0.84–0.98], $P= 0.01$) self-samples relative to cervical specimens (Table 2).

Following a posterior cut-off optimisation specificity improved to 0.94 [95 %CI 0.87–1.01] for urine and 0.94 [95 %CI 0.87–1.01] for vaginal samples, while relative sensitivity remained the same for vaginal samples (ratio = 1.00 [95 % CI 0.96–1.04]) and decreased by 0.02 for urine samples (ratio = 0.97 [95 %CI 0.90–1.03]) (Table 2). Absolute accuracy for the whole study population and for women 30 years and older is reported in Supplementary Tables S1 and S2. Relative accuracy for the study group of 30 years and older women resembled the accuracy of the whole study population (Supplementary Table S3).

Sample cellularity and HPV viral load

Median number of cells was higher in cervical samples (median = 3576 cells/reaction) compared to urine (median = 2540 cells/reaction), but lower than in vaginal samples (median = 41,347 cells/reaction) ($P< 0.001$) (Fig. 2).

Since, the amount of targeted DNA is inversely correlated with Ct-values, as expected, median Ct-values for overall hrHPV types and for HPV16, 18, 33, 35, 45, and *CCR5* gene were lower in cervical compared to urine samples (Fig. 3 and Supplementary Table S4). However, median Ct-values in vaginal samples were lower than in cervical samples considering all hrHPV types and individually HPV31, 51, 56 and *CCR5* gene, indicating higher amounts of DNA in vaginal samples (Fig. 3 and Supplementary Table S5). Comparison between urine and vaginal samples showed lower median Ct-values in vaginal samples for all hrHPV genotypes and for *CCR5* gene (Fig. 3 and Supplementary Table S6).

On the contrary, from the analysis of normalized viral load (viral copies/10^4 cells), median viral loads for overall hrHPV types and for HPV16, 18, 31, 33, 35, 45, 51, 52 and 58 were significantly higher in cervical samples compared to urine (Fig. 4 and Supplementary Table S7). Moreover, the median viral loads of overall hrHPV and HPV16, 18, 31, 33, 35, 39, 45, 51, 56 and 58 were also significantly higher in cervical compared to vaginal samples (Fig. 4 and Supplementary Table S8). In general, no differences (except for HPV35) were observed in median viral loads between urine and vaginal samples (Supplementary Table S9).

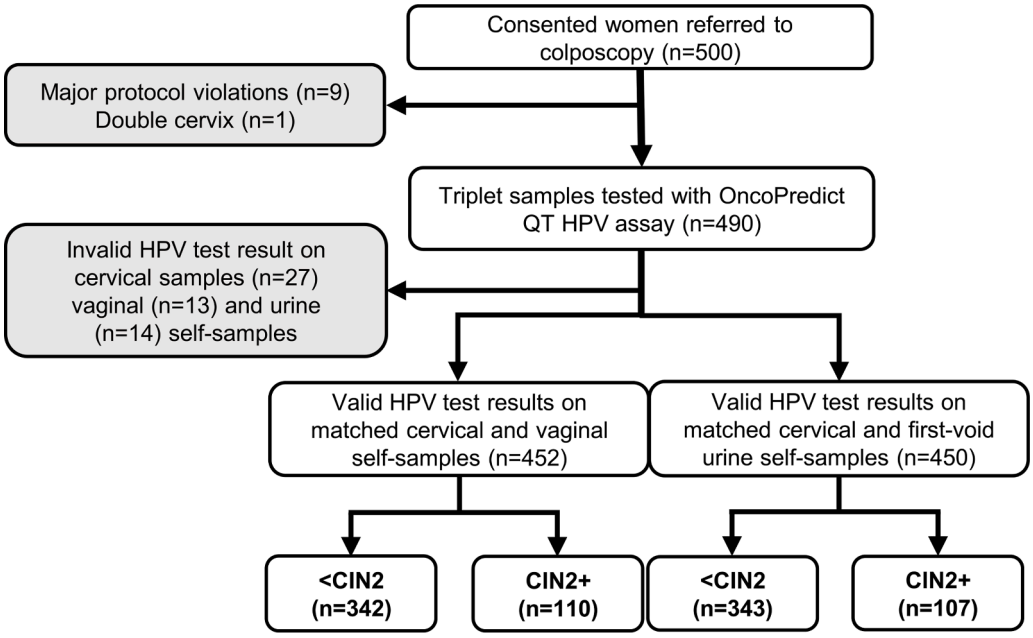

Fig. 1. Flow chart of samples included in the European VALHUDES trial tested with the OncoPredict HPV QT assay. Grey boxes represent excluded samples.

Table 1
Study population characteristics.

Age category (years)	Participants N (%)	Cervical hrHPV Pos N (%)	Vaginal hrHPV Pos N (%)	Urine hrHPV Pos N (%)	Disease outcome ≤CIN1 N (%)	≥CIN2 N (%)	≥CIN3 N (%)
<30	91 (19.7)	66 (25.2)	69 (24.8)	68 (25.0)	68 (19.3)	23 (20.9)	14 (20.3)
30–39	174 (37.6)	106 (40.5)	109 (39.2)	102 (37.5)	118 (33.4)	56 (50.9)	37 (53.6)
40–49	115 (24.8)	54 (20.6)	60 (21.6)	63 (23.2)	93 (26.4)	22 (20.0)	14 (20.3)
50–59	69 (14.9)	31 (11.8)	34 (12.2)	34 (12.5)	61 (17.3)	8 (7.3)	3 (4.4)
60+	14 (3.0)	5 (1.91)	6 (2.2)	5 (1.8)	13 (3.7)	1 (0.9)	1 (1.5)
Total	463 (100.0)	262 (100.0)	278 (100.0)	272 (100.0)	353 (100.0)	112 (100.0)	69 (100.0)
Colposcopy centre	Participants N (%)	Pos N (%)	Pos N (%)	Pos N (%)	≤CIN1 N (%)	≥CIN2 N (%)	≥CIN3 N (%)
Edinburgh	186 (40.2)	102 (38.9)	105 (37.8)	101 (37.1)	149 (42.2)	37 (33.6)	27 (39.1)
Brescia	47 (10.2)	19 (7.3)	23 (8.3)	23 (8.5)	42 (11.9)	5 (4.6)	0 (0.0)
Milan	137 (29.6)	98 (37.4)	102 (36.7)	101 (37.1)	76 (21.5)	61 (55.5)	40 (58.0)
Sassari	93 (20.1)	43 (16.4)	48 (17.3)	47 (17.3)	86 (24.6)	7 (6.4)	2 (2.9)

hrHPV, high-risk HPV; CIN, cervical intraepithelial neoplasia, Pos, HPV positivity.
Supplementary Fig. 1 shows number of overlapping HPV positive cases stratified by age category.

Table 2
Relative accuracy of OncoPredict HPV QT assay on cervical versus self-samples (urine or vaginal), observed in the whole study population.

	CIN2+sensitivity [95 %CI]	CIN3+sensitivity [95 %CI]	<CIN2 specificity [95 %CI]
Manufacturer cut-offs[1]			
Urine	0.99 [0.94–1.05]	0.98 [0.91–1.06]	0.91 [0.84–0.98]
Vaginal	1.00 [0.96–1.04]	1.00 [NA]	0.90 [0.84–0.98]
New cut-offs[2]			
Urine	0.97 [0.90–1.03]	0.97 [0.89–1.05]	0.94 [0.87–1.01]
Vaginal	1.00 [0.96–1.04]	1.00 [NA]	0.94 [0.87–1.01]

CI, confidence interval; CIN, cervical intraepithelial neoplasia;.
[1] Manufacturer's HPV positivity threshold for cervical samples: hrHPV ≥ 50 copies/10^4 cells; for urine or vaginal self-samples: hrHPV ≥ 10 copies/10^4 cells.
[2] New a posterior defined cut-offs for vaginal and urine samples at ≥15 copy/10^4 cells for all types.

Relative sensitivity and specificity for women ≥ 30 years old are shown in Supplementary Table S3.

HPV genotype-specific concordance and normalized viral load correlation between specimens

Good and excellent concordance with Kappa values between 0.68 and 0.93 was observed between both self- and cervical specimens (Supplementary Tables S10 and S11).

Supplementary Fig. S2 shows a correlation matrix of 630 total unique possible associations of the 12 individual hrHPV genotypes across three specimen types using normalised viral load. A significant positive normalised viral load correlation was observed across all three specimen types (cervical, urine and vaginal) for the 21 pairs of seven genotypes HPV16, 18, 31, 33, 45, 51 and 56, with correlation coefficients ranging from 0.49 to 0.92 ($P< 0.05$). For the remaining 5 individual genotypes (15 pairs) correlation was significant only for seven pairs. Of the remaining 594 unique possible associations across different HPV genotypes, 25 significantly positive and 18 inverse correlations were identified after BH false discovery correction (Supplementary Fig. S2, Supplementary Tables S12 and S13) ($P< 0.05$). For instance, HPV16 infections were positively correlated with HPV18 and HPV45, but inversely correlated with HPV39, HPV58 and HPV59 ($P< 0.05$).

Discussion

In this study, the performance of OncoPredict HPV QT genotyping assay, which incorporates normalized genotype-specific quantitative detection of 12 hrHPV, was evaluated on vaginal and urine self-samples. This is the first study to evaluate the clinical performance of OncoPredict HPV QT test on self-samples and the first, we believe, to detail performance of an assay which detects viral copy number in the context of self-

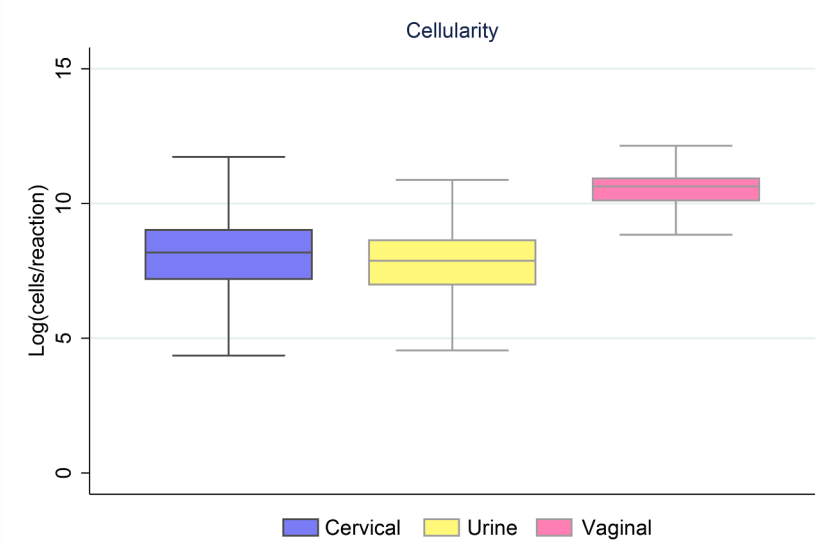

Fig. 2. Median log (cells/reaction) for all sample types. Boxplots indicate median log (cells/reaction) interquartile ranges, and extreme values (whiskers).

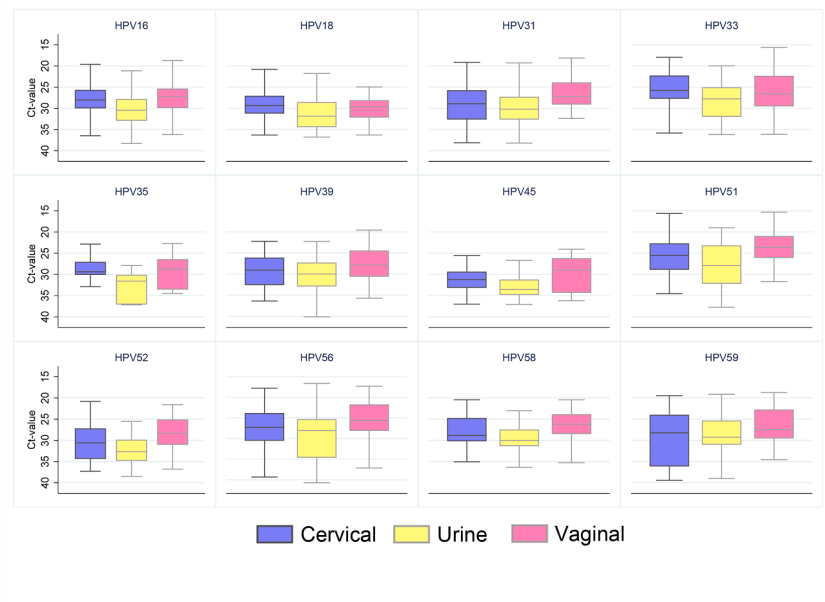

Fig. 3. Individual HPV median Ct-values for all sample types. In case of multiple infections, the highest viral load was considered. Boxplots indicate median Ct-values, interquartile ranges, and extreme values (whiskers). Note: Y-axis has been reversed so that lowest Ct values (corresponding with the highest amount of amplified HPV) are on top.

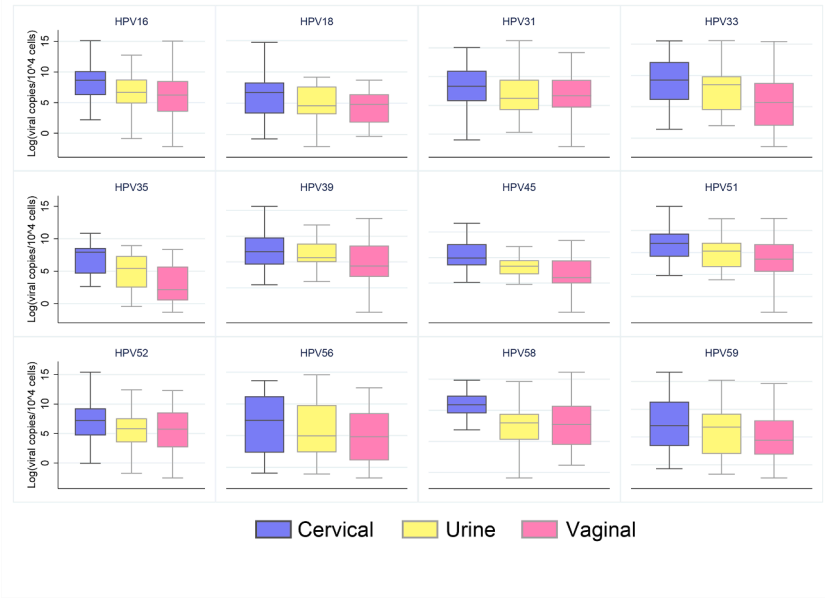

Fig. 4. Individual HPV median log (viral copies/10^4 cells) for all sample types. In case of multiple infections, the highest viral load was considered. Boxplots indicate median log (viral copies/10^4 cells), interquartile ranges, and extreme values (whiskers).

taken samples. In addition, the OncoPredict HPV QT assay is unique as compared to other more established HPV assays used for cervical screening in that it targets only the twelve HPV genotypes considered by IARC as carcinogenic.

The OncoPredict HPV QT test demonstrated similar sensitivity for precancerous lesions on both urine and vaginal samples compared to cervical samples, whereas specificity was lower in both types of self-collected samples, using manufacturer's cut-offs. Following cut-off optimisation specificity improved without compromising sensitivity on both types of self-collected specimens. Lower specificity on vaginal samples may be explained by the resuspension of the dry vaginal device in a small volume (5 mL) [6]. Consistent with our findings, previous studies have reported lower specificity when dry devices were placed in smaller media volumes [6,12,20]. In the Belgian VALHUDES project, the Alinity HPV assay was evaluated using Abbott multi-Collect swab resuspended in 2.5 mL of Abbott Cervi-Collect buffer [12]. In another validation study, BD HPV test was evaluated in combination with FLOQSwab resuspended in 3 mL of BD HPV self-collection diluent [20]. In both studies, sensitivity of the HPV assay on self-samples was similar to that on the cervical sample, whereas specificity was lower. The lower resuspension volumes may generate a more concentrated sample, which could lead to increased HPV detection and, therefore, lower specificity. Moreover, reduced specificity in both the urine and vaginal self-samples may also result from the higher rates of hrHPV infections in the lower vaginal tract not associated with CIN2+ as compared to the cervix [21, 22]. Nevertheless, an optimal balance between specificity and

sensitivity has been made possible through cut-off optimisation in both the present and previous reports [12,20].

To date four other HPV assays have been validated within the VALHUDES framework (Alinity m HR HPV, BD Onclarity HPV, RealTime High Risk HPV and Xpert HPV); of these Alinity m HR HPV and RealTime High Risk HPV also required cut-off optimisation [11-16]. A posterior cut-off optimisation is often required specifically when evaluating assays on self-samples even if those assays have been previously validated and used on cervical samples. Self-samples are collected from different gynaecological niches and often require different laboratory workflows as compared to clinician-collected samples. Given these differences, manufacturers of HPV assays should adapt their assays' cut-offs for self-samples based on the results of the relative validation studies. Or, for new products, consider and optimise specifications with self-samples in mind from the outset.

In the European VALHUDES project, the medium used for the resuspension of vaginal swabs was eNAT, whereas in the Belgian VALHUDES ThinPrep was used. eNAT is an alcohol-free medium, characterized by its capacity to preserve nucleic acids and inactivate microbial agents by cell lysis, which represents an important cost-effective alternative for self-samples resuspension in low-resource settings [6,23-25].

In general, this study showed a wide range of cellularity within the different sample types collected from participating women, pointing to potential differences in performing sample collection. The quantitative evaluation of sample cellularity by a single copy human gene as well as the inclusion of nucleic acids extraction and PCR amplification controls represent a unique feature of the OncoPredict HPV QT assay, providing a level of increased diagnostic confidence in reporting negative results. This is particularly important when evaluating sample adequacy on testing self-collected samples [26]. Presently, a major limitation of most commercially available HPV tests is either the lack of an internal quality control or the use of human controls that do not accurately assess sample cellularity [27,28]. The extensive quality control of the OncoPredict HPV assay has probably resulted in higher invalidity rates, which could be challenging in a real-world cervical cancer screening scenario as a significant number of women may need to be recalled to the clinic to collect another self-sample. Nevertheless, this strict quality control ensures that the valid samples are truly negative, reducing the risk of missing an underlying lesion.

In the present study, higher median viral loads were observed in cervical compared to both self-sample types when viral load was normalised by cellularity. However, Ct-value comparison revealed that median HPV Ct-values were highest in urine, followed by cervical and lowest in vaginal samples. As the Ct-value is inversely correlated with DNA concentration, this suggests a higher amount of viral DNA in vaginal samples before normalization compared to urine or cervical samples. Such differences could be expected, especially given the tenfold difference in cellularity observed in vaginal versus urine or cervical specimens, due in part to the different resuspension volumes used for vaginal and cervical samples. Although Ct-values reflect the initial quantity of viral DNA template in the reaction, it is important to underline that the measurement of viral load is uncertain without a sample's reference mass/volume unit. This is particularly the case for samples collected from mucosal surfaces where an intrinsic variability in cellularity can occur due to differences in the operator's collection technique or the patient's tolerance during the sampling process [29,30]. Moreover, viral load normalization based on sample cellularity can also account for preanalytical variables such as sample-collection, resuspension volumes as well as different nucleic acid extraction and amplification systems, particularly important for vaginal samples. Normalisation by cellularity could help mitigate these differences and provide more consistent results across various sample types. Furthermore, the normalization process in self-samples may also play a role in detecting clinically relevant infections. In order to demonstrate the potential advantages of viral load normalisation, normalised viral load and non-normalised Ct-values of HPV16 positive samples were compared to identify CIN2+ cases. On applying a threshold of 50 copies/10^4cells normalised viral load for HPV16 positivity on cervical samples versus HPV16 positivity with Ct-value threshold of 32.95, which corresponds to 50 copies per reaction non-normalised viral load, three additional CIN2+ cases were identified (Supplementary Fig. S3). Similarly, on self-samples, 15 viral copies/10^4 cells threshold for normalised viral load resulted in detection of four additional CIN2+ cases versus 34.51 Ct-value cut-off, corresponding to non-normalised 15 copies per reaction. Differences in the amount of DNA quantities have been previously reported with the highest viral signals in the upper genital tract decreasing towards the lower genital tract [21]. Moreover, cervical samples are collected by a physician from the transformation zone, where HPV infection and replication occur, while vaginal samples are collected from the lower vaginal environment by women themselves. In terms of urine collection, it contains cervical exfoliated epithelial cells, and free viral particles, accumulated between two urinations and washed out with the first urine stream. Therefore, it is not unexpected to observe these differences in normalised and non-normalised viral signals across different specimen types.

Additionally, we explored the correlation between individual HPV types within urine, vaginal and cervical specimens, as well as across different HPV types. Given that HPV concordance is estimated based on qualitative data, we aimed to explore alternative metrics using quantitative normalised viral load. Spearmen's correlation was applied to evaluate these associations which could be considered as an indicator of viral load concordance [31]. For example, the kappa concordance between cervical and vaginal samples for HPV16 was 0.90, indicating high agreement, while the correlation coefficient for normalised viral load between these specimens was 0.60. On the other hand, inverse and forward correlation was identified between different genotypes suggesting competition or co-occurrence. For instance, inverse HPV16 and HPV58 correlation was present in our study, which was previously reported by Mejlhede *et al.*, who employed a more complex model with OR as an indicator of co-occurrence or competition [32]. Such correlation studies could be useful in identifying HPV interactions in vaccinated and non-vaccinated populations. However, it is important to acknowledge that our study did not account for multiple infections, which could potentially introduce bias into our results.

In conclusion, this VALHUDES study demonstrated that HPV testing with OncoPredict HPV QT assay on self-vaginal and first-void urine samples, collected with FLOQSwab and Colli-Pee respectively, is as sensitive but less specific for precancerous lesions compared to cervical samples. Post-hoc cut-off optimisation enhanced specificity on self-samples without compromising sensitivity.

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CRediT authorship contribution statement

Ardashel Latsuzbaia: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Marianna Martinelli:** Writing – review & editing, Methodology, Data curation. **Chiara Giubbi:** Writing – review & editing, Methodology. **Kate Cuschieri:** Writing – review & editing. **Hana Elasifer:** Writing – review & editing. **Anna D. Iacobone:** Writing – review & editing, Methodology, Data curation. **Fabio Bottari:** Writing – review & editing, Methodology. **Andrea F. Piana:** Writing – review & editing, Methodology. **Roberto Pietri:** Writing – review & editing, Methodology. **Giancarlo Tisi:** Writing – review & editing. **Franco Odicino:** Writing – review & editing. **Clementina E. Cocuzza:** Writing – review & editing, Methodology, Resources, Supervision,

Project administration, Funding acquisition, Conceptualization. **Marc Arbyn:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The European VALHUDES is an investigator initiated study, coordinated by University of Milano-Bicocca (Milan, 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).

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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).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2024.105737.

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