Defining hrHPV genotypes in cervical intraepithelial neoplasia by laser capture microdissection supports reflex triage of self-samples using HPV16/18 and FAM19A4/miR124-2 methylation

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ABSTRACT

Objective: HPV16/18 genotyping and detection of hypermethylation of human cell genes involved in cervical oncogenesis have shown promising results in triage of high-risk HPV (hrHPV)-screen positive women on cervical smears. These tests can be performed on self-samples, which contain cervical and vaginal cells. We studied whether a self-sample represents the hrHPV type causing the worst cervical lesion and whether any differences in hypermethylation of FAM19A4/miR124-2 exist between CIN lesions caused by different hrHPV types. These results have important implications for reflex triage of self-samples.

Methods: Correlation between genotype found on self-sample using GP5+/6+-PCR-EIA-LMNX and causative hrHPV genotype in the worst lesion on histology was studied using laser capture microdissection (LCM)-SPF10-PCR (N=152). Hypermethylation of FAM19A4/miR124-2 in the self-sample was tested in a quantitative methylation specific PCR and compared between lesions caused by HPV16/18 and other hrHPV genotypes.

Results: Causative hrHPV genotype of the worst lesion (CIN1, CIN2, CIN3, invasive cervical cancer) was detected on self-sample in 93.4%. HPV16 was the most frequently found genotype on self-sampling (39.2%, 73/186) and causative genotype in CIN3+ (51.4%, 38/74, all detected on self-sample). There were no differences in the percentages of positive FAM19A4/miR124-2 methylation assays between lesions caused by HPV16/18 (73.8% in CIN3+) or other hrHPV genotypes (66.7% in CIN3+) (p=0.538).

Conclusions: Our results show that hrHPV genotypes found on self-sample were a good representation of hrHPV in the worst CIN lesion and that methylation testing on self-sample for detection of CIN3+ was not significantly different between lesions caused by HPV16/18 and other hrHPV genotypes.

INTRODUCTION

The introduction of population-based screening programs has significantly reduced the incidence of and mortality from cervical cancer in western countries [1, 2]. Over the past decades screening programs were based on cytology, however, high-risk human papillomavirus (hrHPV)-based screening is gaining ground in cervical cancer screening programs. Advantages of hrHPV testing are its high sensitivity for detection of cervical intraepithelial neoplasia (CIN)2+ and CIN3+, compared to cytology, and the possibility of performing the test not only on smears taken by a physician, but also on self-collected samples [3]. The introduction of self-sampling into the screening program can extend the reach of the program, as women who do not respond to the initial invitation are more likely to hand in a self-sample than to respond to a re-invitation for a physician-taken smear [4, 5]. This is important, since 50% of women diagnosed with invasive cervical carcinomas are non-responders [6]. A disadvantage of hrHPV screening is its lower specificity, compared with cytology screening, which results in unnecessary colposcopy referrals for women with a transient HPV infection who are not in need of treatment. Therefore, a sensitive and specific test is needed for triage of hrHPV positive women [7, 8].

Among the various triage strategies that have been evaluated, HPV genotyping and DNA methylation testing can be carried out on self-samples [7]. The risk of development of cancer and precancer differs substantially between HPV genotypes, and in ranking order HPV16 and HPV18 are most carcinogenic, causing 70% of cervical squamous cell carcinomas [9-11]. Therefore, triage of hrHPV positive women by hrHPV genotyping identifies a population of women with HPV 16/18 at the highest risk of cervical cancer [9].

Another triage strategy that can be performed on self-collected material and is non-inferior to cytology triage is hypermethylation testing of host cell genes involved in cervical carcinogenesis [12]. Hypermethylation levels of FAM19A4 and miR124-2 have been found to be very high in nearly all cervical carcinomas and to increase with severity of the CIN lesion [13], showing positivity in over 70% of CIN3+ lesions. Because CIN2/3 lesions associated with a duration of the preceding HPV infection of >5 year have increased methylation levels and have many more chromosomal aberrations compared to CIN2/3 lesions with a shorter duration of their associated HPV infection (<5 years) it has been argued that CIN2/3 lesions with a cancer-like methylation pattern are “advanced” CIN lesions in need of treatment [14, 15]. How methylation levels are correlated to genotype specific HPV infections has not been explored.

Several studies have shown good agreement between physician-taken smears and self-samples for the presence of hrHPV and specific HPV genotypes [16]. When a single

HPV genotype is present on a smear or self-sample, this is designated as the "causative" HPV type of the worst lesion present on biopsy. However, women can be infected with multiple hrHPV types and whether the causative type present in the worst lesion on biopsy is detected by the physician-taken smear or self-sample has only received limited attention. It is often assumed that the HPV genotype known to be associated with the highest risk of cervical cancer found on smear is responsible for the most severe lesion on biopsy. While van der Marel et al. found that in women with a CIN2/3 lesion who are positive for HPV16 on a physician-taken smear HPV16 is found in 96% of the CIN2/3 lesions [17], it is not known whether agreement between genotype on a self-sample, containing admixed many vaginal cells, and in the worst CIN lesion is similarly high.

For the present study, we selected all women with a hrHPV positive self-sample from the PROHTECT-3b trial of self-sampling in non-responders and correlated the causative HPV genotype found in the worst lesion on biopsy using LCM to the HPV genotypes found in the self-sample. In addition, we explored hypermethylation of FAM19A4 and miR124-2 in the self-sample between lesions caused by different HPV genotypes.

METHODS

PROHTECT-3B study

For the present study, a post-hoc analysis was performed on 456 women who participated in the PROHTECT-3B study and tested hrHPV positive on the initial self-sample. This selection was representative of the self-samples in the study. PROHTECT-3B is a device-comparative study that invited a large number of non-responders in the screening program to return a cervicovaginal self-sample for hrHPV testing [18]. The study was performed between October 2011 and February 2012. Both brush-based self-samples (Evalyn Brush, Rovers Medical Devices, Oss, The Netherlands) and lavage self-samples (Delphi Screener, Delphi Bioscience, Scherpenzeel, The Netherlands) were used in the study. Women who tested negative for hrHPV using the GP5+/6+-PCR-EIA test [19] on the self-sample returned to the screening program. Women who tested positive for hrHPV on the self-sample were invited for a physician-taken smear on which cytological examination was performed. Women with a cytology result of atypical squamous cells of undetermined significance or worse (≥ASC-US) either at baseline or after 6 months in case of a normal smear at baseline were referred for colposcopic examination with biopsies of the colposcopically worst region or 2 random biopsies in case of a normal colposcopic impression. HrHPV positive women with a negative cytology result were invited for a reflex physician-taken smear after 6 months, which was tested for cytological abnormalities and hrHPV positivity using GP5+/6+-PCR-EIA. If either of these tests was positive, a colposcopy with directed biopsies

as described above followed. Histology results were H&E based supported by p16 immunohistochemistry and were retrieved from the hospital systems and the nationwide histopathology and cytopathology registration network (PALGA, Utrecht, The Netherlands).

Laboratory testing

hrHPV testing of self-samples

After receiving a self-sampling kit with either a brush device or a lavage device, women collected and returned the cervicovaginal self-sample to the designated laboratory, for processing in Thinprep. Specimens were tested for hrHPV using the clinically validated GP5+/6+-PCR-EIA [19]. Participants were notified of the result and followed-up as described in the study protocol. For the present study, an aliquot of 1 ml of the Thinprep buffered self-sample was sent to DDL Diagnostic Laboratory, Rijswijk, The Netherlands, for additional hrHPV genotyping using GP5+/6+-PCR with genotyping (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) using the LMNX kit HPV GP HR test (Labo Bio-medical Products, Rijswijk, the Netherlands) [20, 21]. Samples were tested for DNA concentration and inhibition using a qPCR quantifying the human reference gene RNaseP and a Phocine Herpesvirus as an internal control.

Histology processing, HPV testing of whole tissue sections (WTS) and worst lesions selected for laser-capture microdissection (LCM)

Formalin fixed paraffin embedded (FFPE) tissue blocks were retrieved from the different hospitals and were sent to the Radboud university medical center, Nijmegen, The Netherlands, where they were processed according to the sandwich method. Slides for H&E staining were obtained, followed by a membrane slide for laser-capture microdissection, two tubes for HPV-PCR testing, one slide for p16 staining and three additional slides for any immunohistochemical staining, and finally one H&E after slide to confirm that the lesion of interest is present in all tested material [18].

H&E and p16 stained slides were reviewed by an expert gynaeco-pathologist and the worst lesion present on the slide was noted. This diagnosis was compared to the original local diagnosis, resulting in a consensus diagnosis when both diagnoses were the same. In case of a discrepancy, a third pathologist was consulted to reach a majority diagnosis.

DNA was isolated from the whole tissue sections (WTS) with the proteinase K procedure [22]. DNA was tested for HPV using the analytically sensitive SPF10-PCR-DEIA-LiPA version 1 system (LBP, Rijswijk, The Netherlands) which contains probes for 25 different HPV genotypes [HPV genotypes 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59,66, 68/73, 70, and 74] [23]. SPF10-PCR targets a small region on the L1 gene, allowing accurate HPV detection in fragmented DNA.

WTS that showed positivity for multiple HPV genotypes were selected for LCM. Worst lesions were annotated based on the H&E and p16 slide. In case of multiple regions with the same histological grade, multiple regions were selected for LCM. Slides were scanned using digital microscopy (Pannoramic Flash II 250, 3DHistech, Budapest, Hungary). Selected regions were excised with the Zeiss P.A.L.M. microbeam UV laser micro-dissection system and transferred to an AdhesiveCap500 opaque tube (Carl Zeiss B.V., Sliedrecht, The Netherlands). DNA isolation and genotyping of LCM regions was performed in the same way as for the WTS.

The HPV genotype present as a single infection in the WTS or the HPV genotype that was found in the worst lesion selected for LCM was considered the causative genotype.

Methylation testing

Methylation testing on self-samples was previously conducted as part of another study and the testing protocol is described elsewhere [24]. Testing results describing positivity for either FAM19A4 and/or miR124-2 were retrieved from the dataset. Methylation results were correlated to causative HPV genotype and to the presence of the causative genotype on self-sample.

Selection of patients

A flow-chart describing the selection and inclusion of women is shown in Figure 1.

Self-samples and the corresponding biopsies containing the worst lesions of women who tested hrHPV positive on the initial self-sample were selected for this study. A total of 125 women did not have a biopsy taken as they had normal cytology during the first and normal cytology and hrHPV negativity during the second visit to the physician. These women were considered to have no precursor lesions. Biopsies were sought for the remaining 331 women. Biopsies from 40 women were not available for testing, and 96 women had a histological endpoint of normal cervical squamous cell epithelium. Both these groups of women were excluded from the study.

Selection based on self-sample suitability

Self-samples of 195 remaining women were included in further analyses and consisted of 106 samples that were collected using the Delphi-Screener lavage device and 89 using the brush-based device Evalyn-Brush. Two self-samples could not be tested because there was less than 250µl of sample present upon arrival at DDL Diagnostic Laboratory and these samples were excluded. No hrHPV genotypes were found in 7 samples of which one also tested negative for the internal LMNX control implying that no human DNA was amplified. All remaining 186 samples tested positive for human DNA in the RNaseP qPCR.

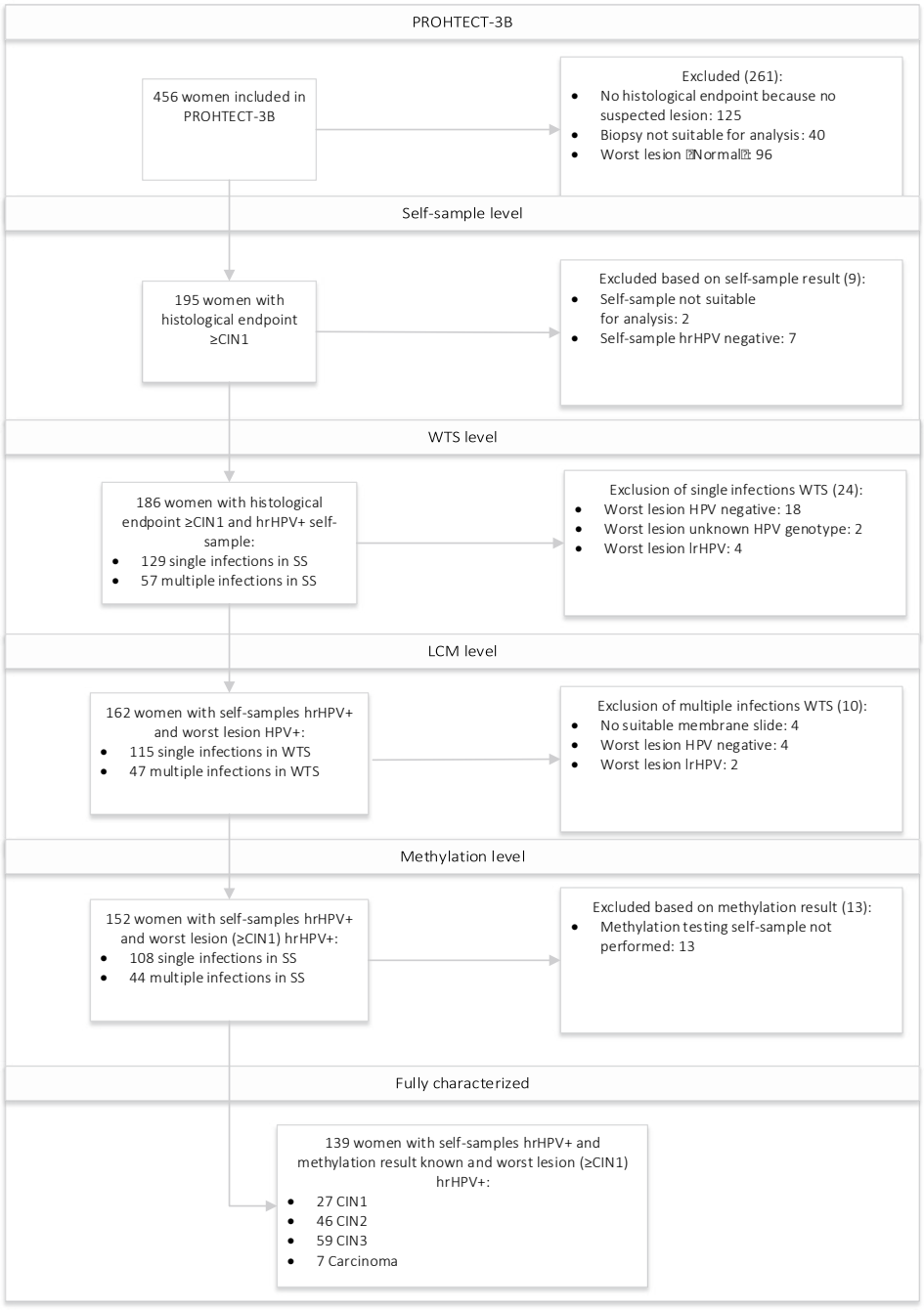

Figure 1. Flow-chart describing the selection of women for analyses, with reason for exclusion on study, self-sample, WTS, LCM and methylation level shown on the right.

Selection based on biopsy material suitability

Whole tissue sections of the study group of 186 women who had one or multiple genotypes detected on the self-sample were tested, but 18/186 WTS tested were HPV negative on SPF10-PCR-DEIA, 2 WTS were HPV positive on DEIA but could not be genotyped by SPF10 LiPA25 and 4 were positive for low-risk HPV genotypes only. In the remaining 162 WTS, 115/162 (71.0%) showed a single infection and 47/162 (29.0%) showed multiple infections.

Of the 47 WTS that were positive for multiple genotypes, no suitable membrane slide was available for 4 biopsies and LCM could not be performed. A causative hrHPV genotype was found in 37/43 biopsies; 4 worst lesions (all CIN1) were HPV negative and 2 were positive for lrHPV (HPV43 in CIN1 and HPV53 in CIN2).

Exclusion of these materials resulted in a group of 152 women with a hrHPV positive self-sample and a worst lesion (≥CIN1) which was also hrHPV positive.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 22.0 (Chicago, IL, USA) and R (Open Source). Results of comparisons are presented as percentages, with 95% confidence-intervals. P-values were calculated using the Chi-squared test and the level of significance was set at <0.05.

RESULTS

152 included women had a mean age of 39 (range 33-63) and were mainly from areas with more than 100,000 inhabitants (69.7% compared to 30.3% from areas with less than 100,000 habitants). No other demographics were available.

Self-samples

Figure 2 describes the relation between genotyping results of self-sample and biopsy material from the 152 women with a hrHPV+ self-sample and a worst lesion (≥CIN1) which was also caused by hrHPV. There were 108 women with a single hrHPV infection on self-sample and 44 with a multiple infection on self-sample. Most single infections on self-sample were HPV16 infection (42/108, 38.9%), followed by HPV31 (15/108, 13.9%). Most multiple infections were HPV16 infections in combination with another hrHPV genotype (20/44, 45.5%), followed by a combination containing HPV18 (12/44, 27.3%).

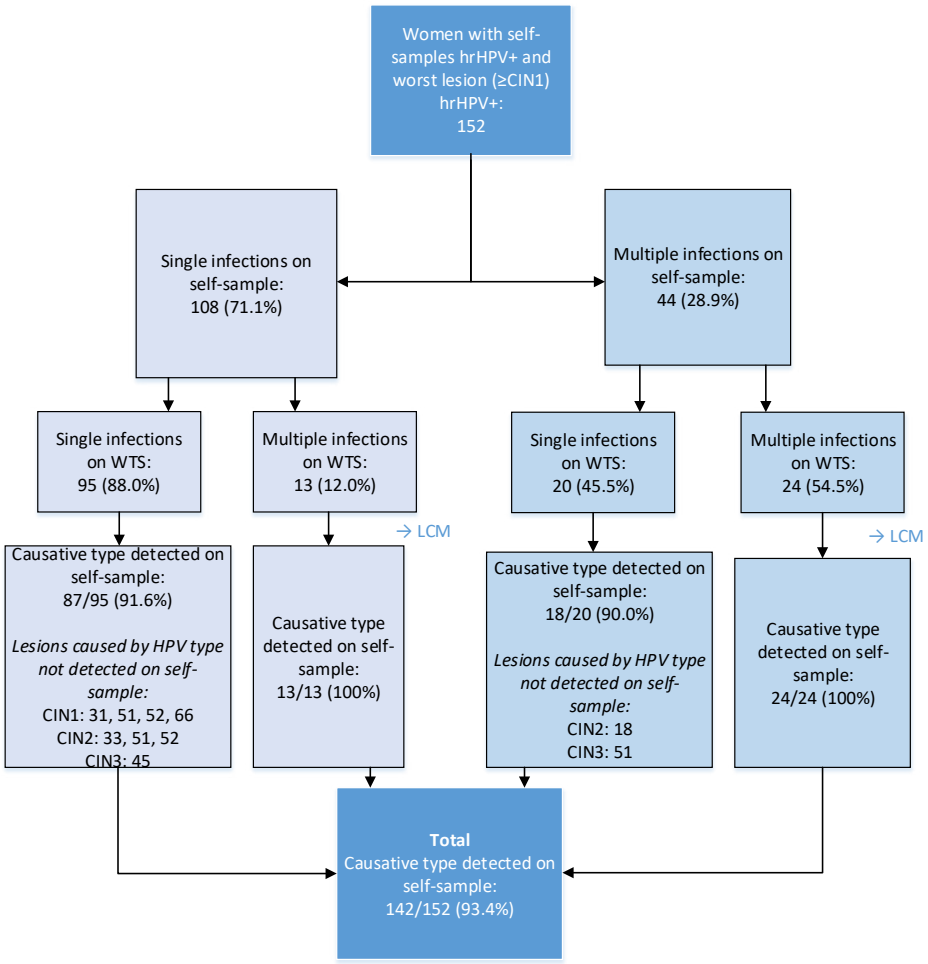

Figure 2. Flow chart describing the genotyping results of self-sample and biopsy material (single or multiple infection) in 152 women with a hrHPV positive self-sample and a ≥CIN1 lesion on biopsy caused by hrHPV.

Genotyping of WTS and LCM regions

Because some patients had multiple regions that were marked as the worst grade of lesion and which were caused by different hrHPV genotypes, a total of 157 lesions from 152 biopsies were included: 32 CIN1 lesions, 51 CIN2 lesions, 66 CIN3 lesions and 8 carcinomas.

The causative hrHPV genotype was defined as the hrHPV genotype detected in the worst lesion as a single infection on WTS or LCM level. Figure 3 shows an example of a biopsy with multiple HPV genotypes found in WTS in which the causative genotype of the worst lesion was detected using LCM-PCR.

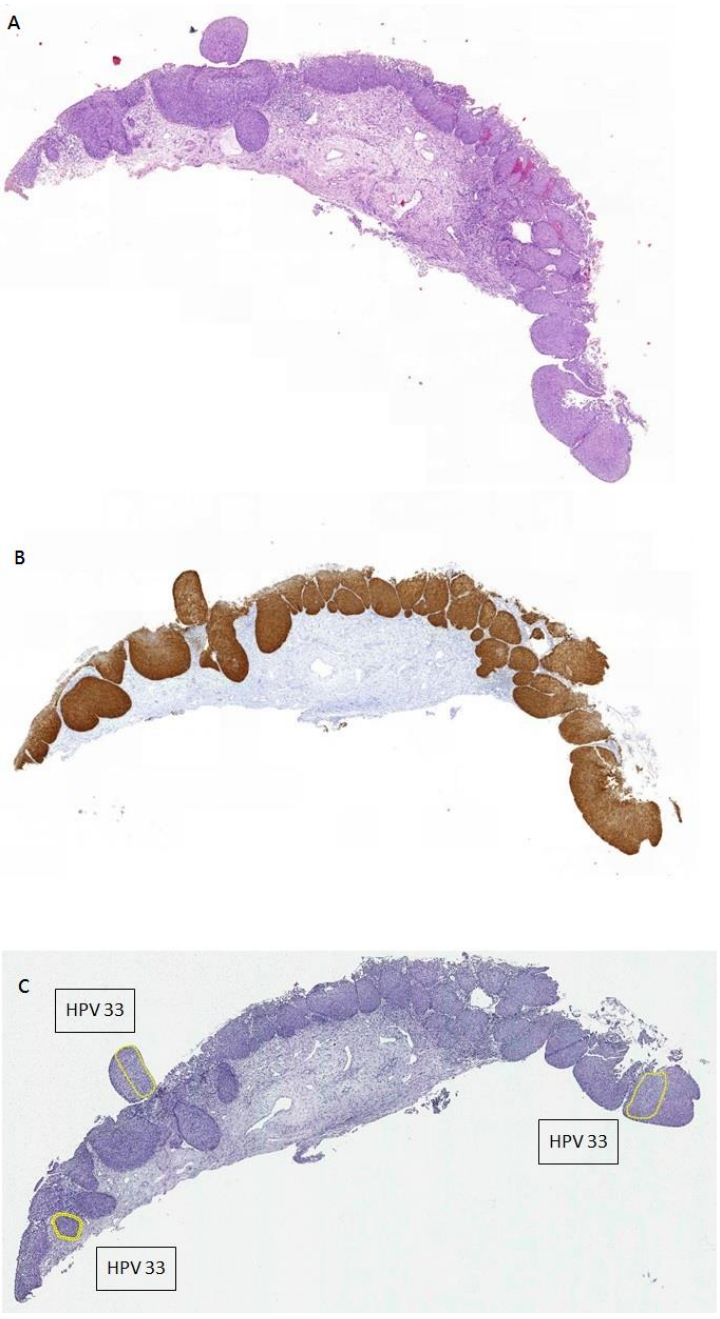

Figure 3. H&E slide (A), p16INK4A (B) slide and Haematoxylin slide (C) for LCM analysis of a multiple infection with hrHPV 33, 51, and 56 on self-sample, 33 and 51 on the whole tissue section, with worst diagnosis CIN3. Three regions of CIN3 were selected for laser capture microdissection to identify the causative genotype of CIN3. HPV33 was found in all three regions and was marked as the causative genotype of the worst lesion present.

The causative genotypes that were found in different grades of CIN and carcinomas are shown in Table 1. Overall, the most frequently found genotype was HPV16 (55/157, 35.0%) and this was also the most frequently found genotype in CIN3 (n=35) and carcinoma (n=5). HPV18 was the causative type of 11 lesions including 5 CIN3 lesions and 2 carcinomas. 45/74 (60.8%) CIN3+ lesions were caused by HPV16/18 and 29/74 (39.2%) were caused by other hrHPV genotypes.

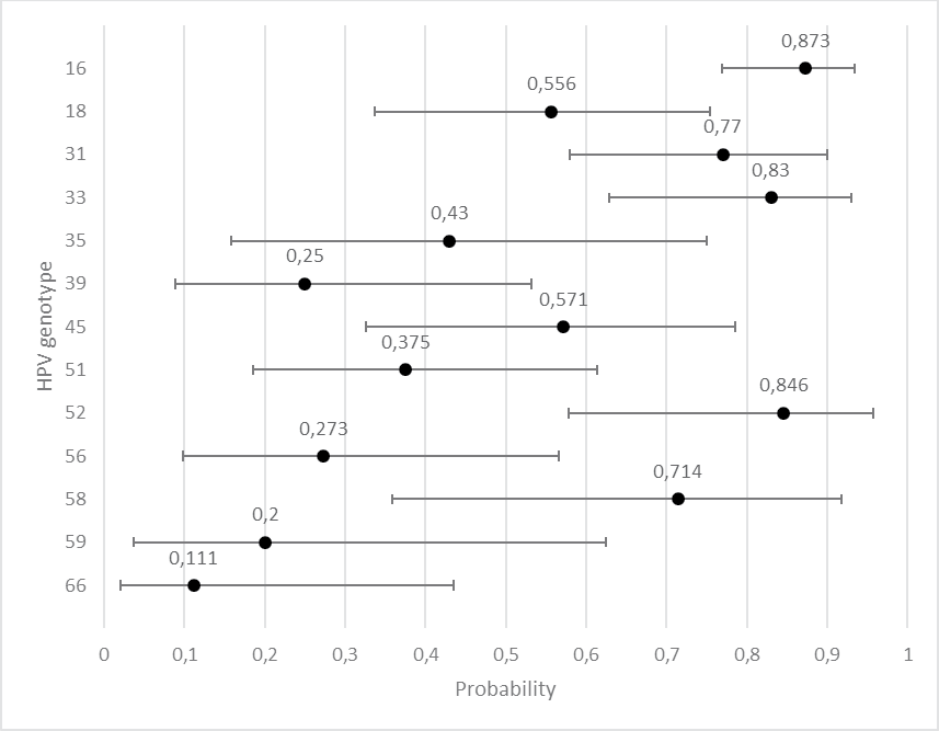

Figure 4. Probability of different hrHPV genotypes in self-sample being present in the worst lesion (≥CIN1).

Performance of self-sample for detection of the causative type of the worst lesion

Of the 152 patients of whom one or multiple causative genotypes for the worst lesion present on biopsy was found, the causative genotype was compared to the genotypes detected on self-sample.

The self-sample of 108/152 women included in this analysis contained a single HPV infection. This was also the causative genotype of the worst lesion in 100/108 (92.6%) women. Multiple infections on self-sample were found in 44/152 included women and one of the HPV- types found on self-sample was the causative type of the worst lesion in 42/44 (95.5%).

Table 1. Distribution of causative genotypes in different grades of CIN. Genotypes found in multiple infections on LCM were counted separately and multiple lesions per sample were found, resulting in 157 causative genotypes in 152 studied biopsies.

	Causative HPV genotypes found in WTS or by LCM												
Diagnosis	HPV16	HPV18	HPV31	HPV33	HPV35	HPV39	HPV45	HPV51	HPV52	HPV56	HPV58	HPV59	HPV66
CIN1	5	1	4	2	0	2	2	6	4	3	1	0	2
CIN2	12	3	9	9	2	1	3	2	6	0	3	1	0
CIN3	35	5	8	7	1	0	3	1	5	0	1	0	0
Carcinoma	3	2	0	2	0	0	1	0	0	0	0	0	0

At least one of the causative genotypes was detected in 142/152 (93.4%) self-samples. Ten self-samples contained an HPV genotype different from the causative genotype: four patients with worst lesions CIN1, four with CIN2 and two with CIN3. The causative type was found on self-sample in 27/31 (87.1%, 95% CI 71.2-94.9) CIN1 lesions; 47/51 (92.2%, 95% CI 81.5-96.9) CIN2 lesions; 60/62 (96.8%, 95% CI 90.0-99.1) CIN3 lesions and in all 8/8 (100%, 95% CI 67.6-100) carcinomas.

The probability of different hrHPV genotypes detected on self-sample being present in the worst lesion (≥CIN1) is shown in Figure 4. In self-samples in which HPV16 was found as a single or in a multiple infection from women with ≥CIN1, HPV16 was the causative type of the worst lesion in 55/63 (87.3%) women. In the 18 self-samples of women in which HPV18 was found with ≥CIN1 lesions, this was the causative type in 10 (55.6%), for HPV31 this was the case in 20/26 (77%) women, for HPV33 in 19/23 (83%), for HPV35 in 3/7 (43%), for HPV39 in 3/12 (25.0%), for HPV45 in 8/14 (57.1%), for HPV51 in 6/16 (37.5%), for HPV52 in 11/13 (84.6%), for HPV56 in 3/11 (27.3%), for HPV58 in 5/7 (71.4%), for HPV59 in 1/5 (20.0%) and for HPV66 in 1/9 (11.1%) women.

Association between hypermethylation of host-cell genes and HPV genotype

Methylation results on self-samples of 152 women of whom the self-sample was genotyped and a histological ≥CIN1 diagnosis was available, were used. Methylation results were available for 139/152 of these women with worst diagnoses: 27 CIN1, 46 CIN2, 59 CIN3 and 7 carcinomas. Methylation positivity was found in 67/139 (48.2%) self-samples, detecting 47/66 (71.2%) CIN3+ lesions, including all carcinomas, and 20/73 CIN1-2 (27.4%, 95% CI 18.5-38.6).

The relationship between hypermethylation in a self-sample and the causative HPV genotypes (HPV16/18 or other hrHPV) is shown in Table 2. No significant differences in positivity of the methylation assay were found between CIN3+ lesions caused by HPV16/18 and CIN3+ lesions caused by other hrHPV types (p=0.538). The levels of hypermethylation were compared between CIN3+ caused by HPV16/18 and other hrHPV

and did not show any significant differences; nor differences were observed for <CIN3 caused by HPV16/18 and other hrHPV (data not shown).

Results for lesions caused by HPV16 were also analysed separately. Of all tested self-samples of women with HPV16 as a causative genotype, 32/50 (64.0%) were methylation positive, with a positivity rate in CIN3+ of 80.0% (95%CI 64.1-90.0) and in CIN1-2 26.7% (4/15, 95% CI 10.9-52.0).

Table 2. Positivity rate of methylation testing on self-sample for different causative HPV genotypes found in WTS or LCM specimen of the worst lesion for the outcome of CIN3+.

Causative HPV genotype	Number of lesions	Number of CIN3+ lesions	Methylation positivity rate CIN3+ (%, 95% CI)	Methylation positivity rate CIN1-2 (%, 95% CI)
HPV16/18	60	42 (70.0%)	31/42 (73.8%, 58.9-84.7)	4/18 (22.2%, 9.0-45.2)
Other hrHPV	82	27 (32.1%)	18/27 (66.7%, 47.8-81.4)	16/55 (29.1%, 18.8-42.1)

DISCUSSION

This study explored the association between the HPV genotypes that can be found in self-samples taken for screening purposes, and the causative genotype found in the worst lesion on histology.

The causative genotype was found in the large majority of self-samples (93.4%), with a non-significant increase of association with increasing severity of the CIN lesion: 87.1% in CIN1, 92.2% in CIN2, and 96.8% in CIN3+. HPV16 was the most frequently found causative genotype in CIN3+ lesions, followed by HPV33, HPV31, and HPV18. There were no differences in methylation positivity between lesions caused by HPV16/18 or other hrHPV genotypes. Overall, the methylation positivity rate, irrespective of hrHPV genotype, for CIN3+ detection was 71.2% and for CIN1-2 was 27.4%.

This is the first study using a combination of WTS-PCR and LCM-PCR to study the agreement between HPV genotypes found on self-sample and HPV genotypes found in the worst lesion on biopsy in a population that was hrHPV screened using a self-sampling device. The results are in line with the results of a study performed by van der Marel et al., which compared the HPV genotypes found on physician-taken samples to the genotype found in the worst lesion [17]. We found multiple hrHPV genotypes in 30.6% (57/186) of the hrHPV positive self-samples, which again is consistent with findings for physician-taken samples [17]. With the growing use of HPV DNA detection in screening, our results argue for further support of the use of self-sampling devices in screening to

reach non-responders or women who prefer to collect a self-sample over visiting their physician.

On self-sample, HPV16 was the most frequently found genotype, and on biopsy HPV16 was the most frequently found causative genotype (35.0% of all lesions, 51.4% of CIN3+ lesions, respectively). In most cases where HPV16 was found on the self-sample, it was the causative type of the worst lesion on biopsy (87.3%). HPV18 was the causative type of 55.6% of the worst lesions of women with HPV18 on the self-sample. This partially supports previous studies that used the Cobas 4800 test (Roche) and attributed HPV16 or HPV18, when present, to the worst lesion on biopsy [9]. However, the relation between HPV16 on self-sample and the worst lesion present on biopsy seems stronger than for HPV18.

Partial genotyping for HPV16/18, in combination with cytology, has proven to be an attractive triage method for hrHPV positive women[9, 25, 26] since the prevalence of HPV16 and HPV18 in cervical cancers is high [27]. HPV genotyping can predict an increased risk of (high-grade) CIN but cannot differentiate between productive and transforming HPV infection. Detailed study of the relation between each HPV genotype and the specific lesions is important to understand the biology and natural history of each lesion and to evaluate the clinical importance of different HPV genotypes.

Hypermethylation levels of FAM19A4 and miR124-2 increase with severity of underlying lesion and duration of (hr)HPV infection and testing can be carried out on both physician-taken and self-collected material. However, differences in performance of methylation between lesions caused by different HPV genotypes were not previously studied. Based on our results, methylation testing seems to be a reliable test for lesions caused by all hrHPV genotypes.

This study shows that self-sampling for HPV-screening detects the causative genotype in the large majority of hrHPV positive cases with an underlying lesion. Comparison of HPV16/18 caused lesions with lesions caused by another hrHPV genotype in relation to the performance of FAM19A4/miR124-2 methylation indicate that methylation detects CIN3+ lesions caused by any hrHPV genotype. This study represents a sub analysis of a large screening trial which was not designed or powered to address this specific issue. Our results suggest an interesting new approach to using molecular biomarkers in triage after self-screening. Given that HPV16 and 18 have an increased relative risk for CIN3 and cancer, and hypermethylation of FAM19A4 and miR124-2 detects all carcinomas and advanced CIN lesions, our results support the combined use of HPV16/18 genotyping and hypermethylation of FAM19A4/miR124-2 as triage test for HPV positive women,

resulting in a high sensitivity for CIN3 and strong reassurance against cancer [25, 28]. Used in this way on cervico-vaginal self-samples, full molecular cervical self-screening is in range.

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