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Cytology and Human Papillomavirus Testing 6 to 12 Months after ASCUS or LSIL Cytology in Organized Screening To Predict High-Grade Cervical Neoplasia between Screening Rounds

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We carried out a prospective study comparing the performance of human papillomavirus (HPV) E6/E7 mRNA (PreTect HPV-Proofer; NorChip, Klokkarstua, Norway) and DNA (Amplicor HPV test; Roche Diagnostics, Basel, Switzerland) triage testing of women 6 to 12 months after atypical-squamous-cells-of-undetermined-significance (ASCUS) or low-grade-squamous-intraepithelial-lesion (LSIL) cytology in organized screening to predict high-grade cervical intraepithelial neoplasia of grade 2 or worse (CIN2+) between screening rounds. Between January 2005 and April 2008, 692 study women with screening-detected ASCUS/LSIL cytology 6 to 12 months earlier returned for HPV mRNA and DNA testing and repeat cytology. The median follow-up time was 3 years, using existing health care facilities. Follow-up test results were available for 625 women. Of the 145 CIN2+ cases detected during the study period, 95 (65.5%) were HPV mRNA positive 6 to 12 months after screening-detected ASCUS/LSIL, 44 (30.4%) were HPV mRNA negative, and 6 (4.1%) were invalid. The corresponding HPV DNA results were 139 (95.9%), 5 (3.4%), and 1 (0.7%), respectively. The cumulative incidences of CIN2+ 3 years after a negative HPV mRNA and DNA test were 10.3% (95% confidence interval [CI], 7.2 to 13.3%) and 1.8% (95% CI, 0.0 to 3.6%), respectively. The cumulative incidences of CIN2+ 3 years after positive HPV mRNA and DNA tests were 52.8% (95% CI, 40.1 to 60.1%) and 41.3% (95% CI, 35.5 to 46.6%), respectively. In conclusion, both positive HPV mRNA and DNA test results have a high enough long-term prediction of CIN2+ risk to consider referral to colposcopy as good practice when performed in delayed triage of women with ASCUS/LSIL cytology. In addition, the low CIN2+ risk among women with a negative Amplicor HPV test in our study confirms its safe use in a clinical setting.

The 2-year cumulative risk of invasive cervical cancer for women with cytological results of atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL) has been reported to be in the range of 0.10% to 0.25% (20). High-risk human papillomavirus (HPV) is recognized as the main cause of cervical cancer (40), and HPV DNA testing in the follow-up of women with ASCUS/LSIL cytology has proven to be a very sensitive method to further identify those at increased risk of cervical cancer, albeit at the cost of low specificity (3). Immediate high-risk HPV DNA testing (i.e., reflex testing) has proven useful in identifying women with ASCUS cytology who should be referred promptly to colposcopy. However, application of reflex testing among women with LSIL cytology is disputed, as three of four women generally test positive for high-risk HPV DNA (3, 15).

European guidelines for quality assurance in cervical cancer screening recommend several different follow-up algorithms for women with ASCUS and LSIL cytology, but there is no consensus on the use of any one algorithm for a given cytological diagnosis. The guidelines recommend that women with LSIL or ASCUS be referred either to direct colposcopy or repeat cytology after 6 months, while reflex high-risk HPV DNA testing is preferred for the latter when liquid-based cytology is used. The guideline authors recognize that high-risk HPV DNA testing 12 months after LSIL cytology has been shown to be as sensitive at detecting high-grade cervical neoplasia as direct colposcopy and is more sensitive and specific, leading to fewer colposcopy referrals than repeat cytology (1, 13, 15). Nevertheless, further research with more specific tools, such as mRNA tests that detect viral oncogene expression, HPV DNA genotype persistence, type-specific viral load targeting essentially HPV type 16/18 and P16 among others, is needed to improve our ability to identify which women with LSIL cytology are at increased risk of malignant progression.

HPV testing was introduced in Norway in 2005 as part of the organized screening program and is currently utilized in the follow-up of women with screening-detected ASCUS/LSIL, in addition to repeat cytology after 6 to 12 months. At follow-up, women with ASCUS/LSIL cytology and a positive HPV test, or with high-grade cytology regardless of HPV test result, are referred to colposcopy. It is considered safe to return women with a negative HPV test result and normal or ASCUS/LSIL follow-up cytology to the 3-year schedule of the organized screening program. Women who are HPV positive with normal cytology are recalled again after another 6 months (23).

Reports from controlled research settings have shown that both mRNA- and DNA-based HPV detection methods are useful to detect cervical intraepithelial neoplasia grade 2 or worse (CIN2+) (21, 26, 34). In addition, the performance of different HPV mRNA and DNA tests, applied according to Norwegian tri-

Received 31 January 2012 **Returned for modification** 6 March 2012
Accepted 5 April 2012
Published ahead of print 18 April 2012
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doi:10.1128/JCM.00265-12

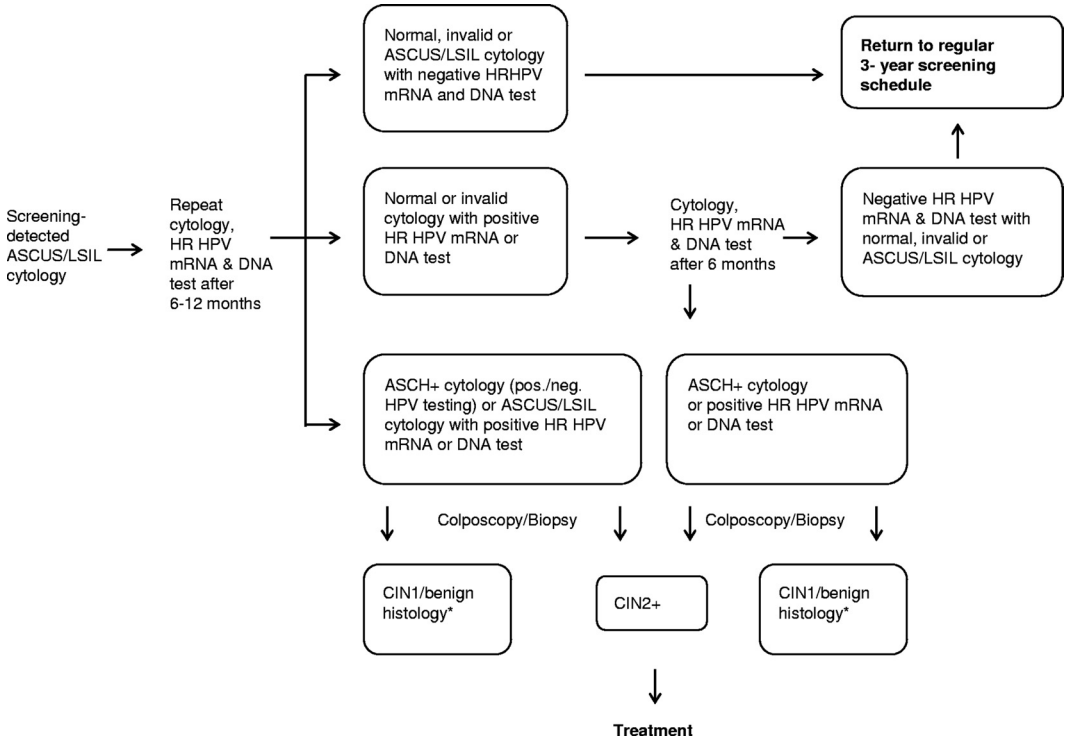

FIG 1 Follow-up algorithm for women who underwent the PreTect HPV-Proofer, Amplicor HPV test, and repeat cytology 6 to 12 months after screening-detected ASCUS/LSIL cytology. *, repeat cytology and high-risk (HR) HPV testing after 6 to 12 months.

age guidelines, has recently been reported (25). However, the use of HPV testing in the follow-up management of women with screening-detected ASCUS/LSIL cytology in the 3-year interval between screening rounds in Norway has not yet been published. Real-life clinical follow-up does not necessarily have the same outcome as a carefully controlled research trial, due to differences in sampling and analysis of specimens. Also, diagnostic practices vary between countries and laboratories, and compliance with follow-up guidelines in a research study group is different from that in a population-based screening referral group (12, 29).

The aim of this study was to compare the levels of performance of high-risk HPV DNA and mRNA testing performed simultaneously according to the guidelines of the Norwegian organized cervical cancer screening program, using existing health care facilities for long-term follow-up. We report here the results of an HPV E6/E7 mRNA test (PreTect HPV-Proofer; NorChip, Klokkarstua, Norway) detecting five high-risk HPV types, an HPV DNA test (Amplicor HPV test; Roche Diagnostics, Basel, Switzerland), and cytology performed 6 to 12 months after screening-detected ASCUS/LSIL cytology, with a median follow-up time of 3 years. The cumulative incidences of CIN2+ were estimated 1 and 3 years after HPV testing and repeat cytology by type of test.

MATERIALS AND METHODS

Study population, recruitment, and follow-up procedure. Between January 2005 and April 2008, women attending the national cervical cancer screening program in Norway were invited to participate in a large prospective follow-up study coordinated at Akershus University Hospital. In the current study, general practitioners and private gynecologists in the Akershus District located Northeast of Oslo, served by the Akershus University Hospital, recruited women who had ASCUS or LSIL cytology 6 to 12 months earlier (i.e., screening-detected ASCUS/LSIL). These study women were invited to undergo the HPV mRNA test (PreTect HPV-Proofer), the DNA test (Amplicor HPV test), and repeat cytology; the date of sample collection for the aforementioned tests was recorded and defined as the study inclusion date.

Data from the population-based Cancer Registry of Norway were used to confirm that screening-detected ASCUS/LSIL cytology was not a result of follow-up after detection of a cervical abnormality. Repeat cytology results from study inclusion were linked to data from the Cancer Registry of Norway, which records cytological and histological data on all women in Norway; completeness is considered to be close to 100% (17). Women were excluded if they had a histological result of CIN2+ or a cytological result more severe than ASCUS/LSIL in the year preceding the study inclusion date. After application of these exclusion criteria, 692 women (age range, 18 to 83 years; mean age, 39.6 years; median age, 39.0 years; interquartile range, 30 to 48 years) were finally included: 66.3% (459/692) with screening-detected ASCUS cytology, and 33.7% (233/692) with screening-detected LSIL cytology preceding the study inclusion date. The median time between screening-detected ASCUS/LSIL and HPV testing or repeat cytology was 212 days (7 months; minimum, 59 days; maximum, 365 days).

Physicians were recommended to refer participants to further follow-up or to return them to the 3-year schedule of the organized screening program, based on the results of HPV testing and repeat cytology (Fig. 1). Private and hospital gynecologists performed colposcopy and biopsy when indicated. Norwegian guidelines recommend the use of Reid's colposcopy index (23), which was not reviewed.

Histologically verified CIN2+ among women with screening-detected ASCUS/LSIL cytology during the follow-up period after study inclusion was the main endpoint of our study. To reflect the real-life situation, no review was made to confirm the histological endpoint; the community-based histological diagnoses were used as they appeared in the routine clinical setting. Based on the World Health Organization classification of

cervical neoplasia, CIN grades I, II, and III (CIN1, -2, and 3, respectively) are routinely applied in Norway for cervical histology (30). To ensure complete cytological and histological follow-up also for those women who were followed up by physicians who did not send their Pap smears to the Akershus University Hospital for analysis, data from the Cancer Registry of Norway were obtained for all 692 subjects until 31 December 2010. Most study women had several follow-up events registered during the follow-up period.

HPV mRNA and DNA testing and repeat cytology. A conventional cytology sample was taken with a wooden spatula (ectocervix) and a Cytobrush Plus (Medscan Medical AB, Malmo, Sweden) (endocervix). The brush was placed in PreservCyt medium (Hologic, Bedford, MA) and stored for up to 21 days at room temperature or at 4°C before HPV testing. Cytology samples were evaluated blinded to HPV results and classified according to the 2001 Bethesda classification (31).

HPV testing was performed using both high-risk HPV mRNA and DNA methods, on nucleic acid extracted with easyMag (bioMérieux), as previously validated and described (37). The PreTect HPV-Proofer mRNA test detects and genotypes E6/E7 full-length mRNA transcripts of five high-risk HPV types 16, 18, 31, 33, and 45. The Amplicor HPV test detects the DNA of 13 high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

Statistical analysis. As previously mentioned, participants were included at the time of repeat cytology and HPV testing, and all histological and cytological data for these participants were retrieved through the Cancer Registry of Norway until 31 December 2010. We censored all observations at the date of the last cytological or histological result registered to account for the short follow-up time of subjects enrolled at the end of the study. Repeat cytology results from samples taken on the study inclusion date were divided into several categories: invalid, normal, ASCUS/LSIL, or ASCUS or worse (ASCUS+), as well as cases in which atypical cells cannot exclude high-grade squamocolumnar lesion or a more severe finding (ASCH+). ASCUS+ was chosen to look at the risk of negative cytology alone, and ASCUS/LSIL results together with HPV test results were chosen to analyze risk in the test algorithm arms.

Statistical analyses were performed using a Kaplan-Meier estimator calculating the main outcome: cumulative incidence of CIN2+ and CIN grade 3 or worse (CIN3+) 1 and 3 years after the study inclusion date, with 95% confidence intervals (95% CIs), stratified by HPV mRNA test, HPV DNA test, and repeat cytology results of normal or ASCUS+ at study inclusion. Cumulative incidence curves are only given up to 4 years after study inclusion since the statistical uncertainty increased significantly toward the end of the total follow-up period.

The negative predictive value (NPV) was calculated as 1 − the cumulative incidence of CIN2+ 3 years after a negative test result. For the positive predictive value (PPV), the cumulative incidence of CIN2+ 3 years after a positive test result was used. Analyses were further stratified by screening-detected ASCUS and LSIL and by normal or ASCUS/LSIL repeat cytology at study inclusion. We calculated the crude hazard ratio between positive and negative test results for the two HPV tests as from 1 year after HPV testing and repeat cytology by the Cox proportional hazard model. *P* values of <0.05 were considered statistically significant. Statistical analyses were performed using SPSS 18 and R 2.12.2.

Ethics. The Regional Committee for Ethics in Medical Research, East Region, Norway (676-04239), and Southeast Region, Norway (10/849), the Norwegian Health Directorate (05/163), and the Norwegian Data Inspectorate (07/00975-2/SVE) approved the study. Written informed consent was obtained from all study participants.

RESULTS

Test results at study inclusion. Of the 459 women with screening-detected ASCUS cytology, 118 (25.7%) tested HPV mRNA positive and 206 (44.9%) tested HPV DNA positive 6 to 12 months later. The corresponding results for the 233 women with screening-detected LSIL cytology were 77 (33.0%) and 151 (64.8%). When stratifying by severity of the repeat cytology result, HPV mRNA was positive in 15.9% (62/390) of women with normal cytology, 40.9% (56/137) of women with ASCUS, 43.6% (44/101) of women with LSIL, 53.7% (29/54) of women with ASCH+, and 40.0% (4/10) of women with invalid cytology. Corresponding percentages of HPV DNA positivity were 36.2% (141/390), 62.8% (86/137), 72.3% (73/101), 94.4% (51/54), and 60.0% (6/10). One HPV DNA-negative woman and two women with invalid HPV DNA results were positive by the HPV mRNA test.

Follow-up events observed for all 692 study participants in the first year of follow-up and during the total follow-up period are shown in Table 1 by test results at study inclusion. Benign and CIN1 biopsy results are shown in the footnotes. Histological and/or cytological test results were available for 625 of the 692 study women. The median follow-up time between study inclusion date (i.e., from HPV testing and repeat cytology) and the last follow-up event was 3 years. The maximum follow-up time was 5 years and 244 days. During the follow-up period, a total of 142 women were diagnosed with CIN2, CIN3, or adenocarcinoma *in situ*, and 3 were diagnosed with cervical carcinoma. The median time to CIN2+ diagnosis was 93 days. One hundred four of the CIN2+ cases were detected within 1 year of HPV testing and repeat cytology at study inclusion.

At study inclusion, the HPV mRNA test was positive in 95 (65.5%), negative in 44 (30.3%), and invalid in 6 (4.1%) of the 145 women diagnosed with CIN2+ during the follow-up period. The HPV DNA test was positive in 139 (95.9%), negative in 5 (3.4%), and invalid in 1 (0.7%) of these women. Repeat cytology at study inclusion showed ASCUS/LSIL in 67 (46.2%), ASCH+ in 41 (28.3%), normal in 33 (22.8%), and invalid in 4 (2.8%) of the 145 CIN2+ cases. The percentage of positive tests for all three testing methods in CIN2+ cases was higher in the first year than for the total follow-up period.

Cumulative incidence of CIN2+ by HPV test and repeat cytology result at study inclusion. The cumulative incidence of CIN2+ and CIN3+ 1 and 3 years after study inclusion, by HPV test and repeat cytology, is shown in Tables 2, 3, and 4. Both ASCUS+ repeat cytology and a positive HPV DNA test showed a 10% lower cumulative incidence of CIN2+ and CIN3+ 3 years after study inclusion than a positive HPV mRNA test (52.8% [95% CI, 44.1 to 60.1%] and 40.5% [95% CI, 31.3 to 48.5%], respectively) (Table 2). The cumulative incidences of CIN2+ 1 and 3 years after a negative HPV DNA test at study inclusion were low: 0.4% and 1.8%, respectively. The corresponding numbers for CIN3+ were 0% (95% CI, 0.0 to 0.0%) and 0.5% (95% CI, 0.0 to 1.4%), respectively. Comparatively, the percentages of risk of CIN2+ were 6.6% and 10.3% among women with negative HPV mRNA and 5.1% and 8.8% among those with normal repeat cytology. The corresponding numbers for CIN3+ were 4.7% and 6.0% among HPV mRNA-negative women and 3.6% and 5.9% among those with normal repeat cytology (Table 2). A combination of a negative HPV mRNA test and normal cytology resulted in a 2.3% and 5.0% risk of CIN2+ and in a 2.3% and 3.3% risk of CIN3+ at 0 to 1 and 0 to 3 years, respectively. This was about 2 times lower than the corresponding risks of CIN2+ and CIN3+ in women with a negative HPV mRNA test alone. The combination of a negative HPV DNA test and normal cytology had little impact on the risk of CIN2+ and CIN3+ compared to that in women with a negative HPV DNA test alone (Tables 2 to 4). An

TABLE 1 PreTect HPV-Proofer (mRNA), Amplicor HPV test (DNA), and repeat cytology results among 692 women at study inclusion[a]

Test result at inclusion	No. with follow-up test result:									
		No cytology		Last cytology normal[b]		Last cytology ASCUS/LSIL		ASCH+ cytology		CIN2+ (CIN3+) histology	
	Total (n = 692 [100%])	First yr (n = 344)	Total period (n = 67)[c]	First yr (n = 182)	Total period (n = 426)[d]	First yr (n = 48)	Total period (n = 34)[e]	First yr (n = 14)	Total period (n = 20)[f]	First yr (n = 104 [64])	Total period (n = 145 [97])
PreTect HPV-Proofer											
Negative	470 (67.9%)	300	59	109	333	28	23	6	11	27 (18)	44 (26)
Positive	195 (28.2%)	28	4	66	77	20	10	8	9	73 (42)	95 (65)
Invalid	27 (3.9%)	16	4	7	16	0	1	0	0	4 (4)	6 (6)
Amplicor HPV test											
Negative	331 (47.8%)	285	56	38	252	6	12	1	6	1 (0)	5 (1)
Positive	357 (51.6%)	59	11	142	172	42	22	12	13	102 (64)	139 (96)
Invalid	4 (0.6%)	0	0	2	2	0	0	1	1	1 (0)	1 (0)
Repeat cytology											
Normal	390 (56.4%)	244	45	106	292	17	9	6	11	17 (9)	33 (23)
ASCUS/LSIL	238 (34.4%)	90	18	66	123	27	23	6	7	49 (28)	67 (41)
ASCH+	54 (7.8%)	7	2	5	7	3	2	2	2	37 (26)	41 (30)
Invalid	10 (1.4%)	3	2	5	4	1	0	0	0	1 (1)	4 (3)

[a] Study inclusion represents 6 to 12 months after screening-detected ASCUS/LSIL cytology. Follow-up CIN2+ and CIN3+ events occurring 0 to 1 and 0 to 5.5 years after study inclusion are presented separately for each test result at study inclusion.
[b] All normal cytology or ASCUS/LSIL cytology with last cytology normal.
[c] Twenty-three biopsy specimens had benign or CIN1 histology.
[d] Seventy-seven biopsy specimens had benign or CIN1 histology.
[e] Thirty-five biopsy specimens had benign or CIN1 histology.
[f] Eighteen biopsy specimens had benign or CIN1 histology.

immediate increase in the incidence of CIN2+ was observed among women with ASCUS/LSIL repeat cytology and a positive HPV test (Fig. 2), reflecting the current recommendations for an immediate diagnostic workup (Fig. 1). Women with a positive HPV test combined with normal repeat cytology in our study were recommended to undergo follow-up again in another 6 months, and consequently an increase in the risk of CIN2+ was observed 6 months after study inclusion. No increase was seen in women with normal repeat cytology in combination with a negative HPV DNA test, while for the combination of normal repeat cytology and a negative mRNA test, an increase at 6 months was observed. Among women with normal and ASCUS/LSIL repeat cytology, a continuously increasing risk of CIN2+ was observed among HPV-positive women (Fig. 2).

HPV DNA testing showed the highest NPV for detecting CIN2+ and CIN3+, with a PPV similar to that of cytology 3 years after study inclusion. HPV mRNA testing had the lowest NPV and highest PPV for detection of CIN2+ and CIN3+ (Table 5). The hazard ratio for CIN2+ (excluding diagnoses in the first year) associated with a positive HPV mRNA test was 5.3 (95% CI, 2.8 to 10.0) ($P < 0.0001$) compared to HPV mRNA-negative women. The corresponding numbers for HPV DNA testing and repeat cytology were 10.6 (95% CI, 3.7 to 29.8) ($P < 0.0001$) and 2.2 (95% CI, 1.2 to 4.2) ($P = 0.017$), respectively.

TABLE 2 Cumulative incidence of CIN2+ and CIN3+ 1 and 3 years after study inclusion, stratified by PreTect HPV-Proofer (mRNA), Amplicor HPV test (DNA), and repeat cytology results[a]

Test result at inclusion	% (95% CI) cumulative incidence of:			
	CIN2+		CIN3+	
	0–1 yr	0–3 yr	0–1 yr	0–3 yr
PreTect HPV-Proofer				
Negative	6.6 (4.2–9.0)	10.3 (7.2–13.3)	4.7 (2.6–6.8)	6.0 (3.6–8.4)
Positive	38.6 (31.2–45.1)	52.8 (44.1–60.1)	26.4 (19.5–32.6)	40.5 (31.3–48.5)
Amplicor HPV test				
Negative	0.4 (0.0–1.1)	1.8 (0.0–3.6)	0 (0.0–0.0)	0.5 (0.0–1.4)
Positive	29.7 (24.7–34.4)	41.3 (35.5–46.6)	21.4 (16.8–25.7)	30.5 (24.7–35.8)
Repeat cytology				
Normal	5.1 (2.7–7.4)	8.8 (5.5–12.0)	3.6 (1.6–5.6)	5.9 (3.2–8.6)
ASCUS+	31.6 (25.8–36.9)	41.0 (34.6–46.8)	22.0 (16.8–27.0)	29.3 (23.0–35.0)

[a] Study inclusion represents 6 to 12 months after screening-detected ASCUS/LSIL cytology.

Table 3 Cumulative incidence of CIN2+ 1 and 3 years after study inclusion, stratified by PreTect HPV-Proofer (mRNA) and Amplicor HPV test (DNA) results in combination with repeat normal or ASCUS/LSIL cytology[a]

Test result at inclusion	% (95% CI) cumulative incidence of CIN2+ at:			
	0–1 yr		0–3 yr	
	Normal repeat cytology	ASCUS or LSIL repeat cytology	Normal repeat cytology	ASCUS or LSIL repeat cytology
PreTect HPV-Proofer				
Negative	2.3 (0.5–4.1)	5.1 (1.0–9.0)	5.0 (2.2–7.8)	10.9 (4.9– 16.6)
Positive	19.3 (8.4–29.0)	41.6 (31.0–50.6)	26.3 (13.1–37.5)	57.5 (44.6–67.4)
Amplicor HPV test				
Negative	0.5 (0.0–1.4)	0 (0.0–1.0)	1.9 (0.0–4.0)	2.0 (0.0–5.6)
Positive	12.6 (6.6–18.2)	30.8 (23.2–37.7)	20.3 (12.5–27.4)	43.4 (34.5–51.1)

[a] Study inclusion represents 6 to 12 months after screening-detected ASCUS/LSIL cytology.

When looking separately at CIN2+ cases with screening-detected ASCUS cytology, the percentages of risk of CIN2+ 3 years after study inclusion were 7.3% (95% CI, 4.0 to 10.4%) for a negative HPV mRNA test, 52.3% (95% CI, 41.1 to 61.4%) for a positive HPV mRNA test, 1.8% (95% CI, 0.0 to 3.8%) for a negative HPV DNA test, and 38.7% (95% CI, 30.9 to 45.6%) for a positive HPV DNA test. The corresponding results for screening-detected LSIL cytology were 17.1% (95% CI, 10.1 to 23.5%) and 53.8% (95% CI, 39.1 to 64.9%) for negative and positive HPV mRNA tests, respectively, and 2.0% (95% CI, 0.0 to 5.8%) and 45.0% (95% CI, 35.6 to 52.7%) for negative and positive HPV DNA tests, respectively.

DISCUSSION

Our study shows that a negative mRNA test result by PreTect HPV-Proofer 6 to 12 months after screening-detected ASCUS/LSIL cytology confers a risk of CIN2+ that is 5 times higher than that of a negative HPV DNA test result by the Amplicor HPV test. Accompanied by normal cytology at the time of the HPV test, the risk of CIN2+ was still 2.6 times higher among women negative by the PreTect HPV-Proofer than among those negative by the Amplicor HPV test. On the other hand, the risk of CIN2+ was 10% higher for women with a positive PreTect HPV Proofer result than for those with a positive Amplicor HPV test result.

According to Norwegian screening guidelines, a delayed HPV test (methodology not specified) together with repeat cytology 6 to 12 months after screening-detected ASCUS/LSIL cytology serves to identify both women who should be referred to colposcopy and those who can be safely returned to the regular 3-year schedule of the organized screening program. A risk model for CIN3+ has been introduced as a guide for clinical management to prevent cervical cancer in a safe and cost-effective manner and can be used as a tool to monitor whether a given test fulfills the criteria for clinical utility (11). Using the same model for CIN2+, Arbyn et al. indicated that if the risk of CIN2+ is below 2%, it can be considered safe to return the patient to the regular screening schedule. If the risk of CIN2+ is between 2 and 10%, repeat cytology after 1 year is considered safe practice, while for a risk of CIN2+ above 10%, referral to colposcopy is usually considered good practice (2, 11). We chose to focus on risk of CIN2+ because CIN2 is the threshold for treatment in Norway.

When using HPV testing as a "negative-triage test" that is to determine if a woman can be safely returned to the regular 3-year screening schedule after a negative HPV result and normal, inadequate, or ASCUS/LSIL repeat cytology, a test with a very high clinical sensitivity for CIN2+ and a high NPV in long-term follow-up is important (27). In such "negative-triage settings," use of an internal control in the HPV test ensures that there are enough cells present in the samples to make them suitable for analysis. Both HPV tests in our study had an internal control. PreTect HPV-Proofer was invalid in 3.9% of the samples, and the Amplicor HPV test was invalid in 0.5%. This was most likely attributable to the fact that RNA is more prone to degradation than DNA, indicating a need for more recall for analysis when using RNA-

Table 4 Cumulative incidence of CIN3+ 1 and 3 years after study inclusion, stratified by PreTect HPV-Proofer (mRNA) and Amplicor HPV test (DNA) results in combination with repeat normal or ASCUS/LSIL cytology[a]

Test result at inclusion	% (95% CI) cumulative incidence of CIN3+ at			
	0–1 yr		0–3 yr	
	Normal repeat cytology	ASCUS or LSIL repeat cytology	Normal repeat cytology	ASCUS or LSIL repeat cytology
PreTect HPV-Proofer				
Negative	2.3 (0.5–4.1)	3.4 (0.1–6.7)	3.3 (1.0–5.5)	4.5 (0.5–8.3)
Positive	11.1 (2.3–19.1)	27.4 (17.5–36.1)	18.8 (6.5–29.4)	43.4 (28.9–55.0)
Amplicor HPV test				
Negative	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.6 (0–1.8)	0.0 (0.0–0.0)
Positive	9.6 (4.3–14.6)	21.0 (14.1–27.2)	15.0 (8.0–21.6)	30.0 (21.3–37.8)

[a] Study inclusion represents 6 to 12 months after screening-detected ASCUS/LSIL cytology.

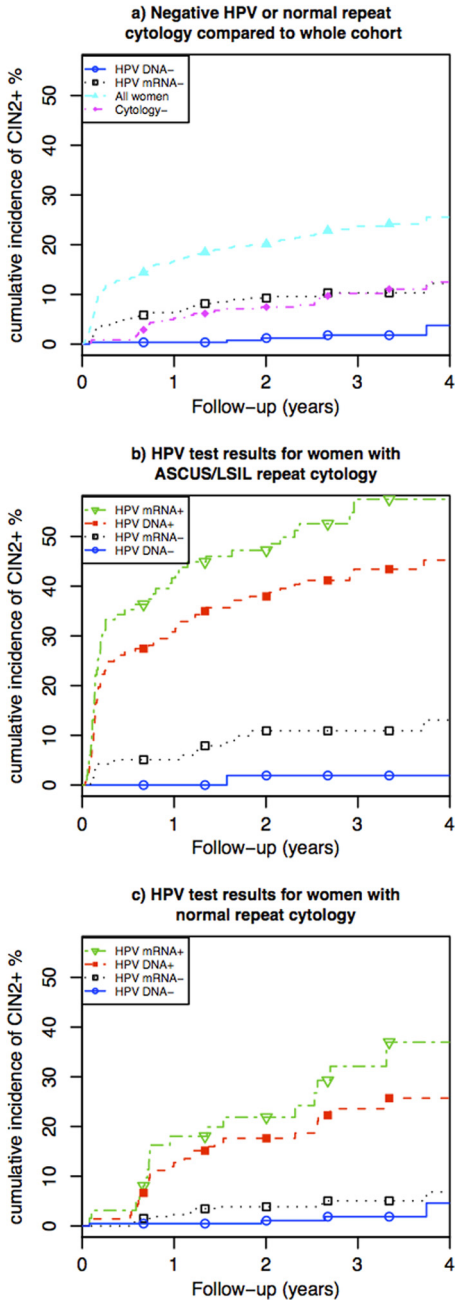

FIG 2 Cumulative incidence of CIN2+ up to 4 years after study inclusion (6 to 12 months after screening-detected ASCUS/LSIL cytology). (a) Women with a negative HPV DNA (Amplicor HPV test) test, RNA (PreTect HPV-Proofer) test, and normal repeat cytology. For comparison, the cumulative incidence of disease in the whole cohort is also shown. (b) HPV test results for women with ASCUS/LSIL repeat cytology. (c) HPV test results for women with normal repeat cytology.

based testing and showing the need for internal controls in HPV DNA-based tests as well, which should be considered when choosing an HPV test. When evaluating only valid test results, the Amplicor HPV test detected 96.5% (139/144) of CIN2+ cases diagnosed during the follow-up period, with a median follow-up time of 3 years. These results are in agreement with earlier studies and a recent report from the Cancer Registry of Norway describing 15,000 women with the same follow-up regimen as our study with the PreTect HPV-Proofer, Amplicor HPV test, or Hybrid Capture II (HC2) (8, 14, 37). The risk of CIN2+ in our study was 1.8% (95% CI, 0.0 to 3.6%) 3 years after a negative Amplicor HPV test, which is considered acceptably low to return women to the normal screening schedule (2, 11). Cytology in combination with the Amplicor HPV test did not detect more CIN2+ cases than the Amplicor HPV test alone, indicating a good discriminative power of the HPV DNA test in our study context.

When evaluating only valid test results, PreTect HPV-Proofer 6 to 12 months after screening-detected ASCUS/LSIL detected 68.3% (95/139) of CIN2+ cases diagnosed during the follow-up period (Table 1). These results are somewhat lower than earlier results from delayed mRNA testing alone for the management and triage of women with ASCUS/LSIL cytology (14, 32). This is probably due to the fact that in our study, a positive DNA test led to a more aggressive application of a diagnostic procedure that identified more CIN2+ cases, whereas some of these CIN2+ cases might have regressed with time. On the other hand, a lower proportion of CIN2+ was detected by the PreTect HPV-Proofer in another study, with a multiple-test comparison similar to ours but with less than 1 year of follow-up (25). Another HPV mRNA test used in that study was the Aptima HPV mRNA assay, which detects 14 high-risk HPV types, compared to the 5 types included in the PreTect HPV-Proofer. It reported the sensitivity of the Aptima test to detect CIN2+ in short-term follow-up to be comparable to that of HPV DNA testing (96 to 100%) (25). The much lower detection rate of CIN2+ by PreTect HPV-Proofer may be explained by the limited spectrum of genotypes detected by this assay, as it would not detect high-grade lesions caused by HPV types other than 16, 18, 31, 33, and 45. However, even after restriction of the analysis to women positive for these five genotypes, the PreTect HPV-Proofer has been shown to be less sensitive than the Amplicor HPV test in detecting women with CIN2+ (36). Thus, another explanation for the lower CIN2+ detection by PreTect HPV-Proofer could be the lower sensitivity of the test or low levels of E6/E7 mRNA transcripts in the sampled cells, which might be indicative of lesions that are more prone to spontaneously regress without treatment. On the other hand, as viral oncogenes are also expressed in benign lesions, the usefulness of mRNA testing has some limitations. In our study, the cumulative incidence of CIN2+ 3 years after a negative HPV mRNA test by PreTect HPV-Proofer alone was 10.3% (95% CI, 7.2 to 13.3%), and the incidence was 5.0% (95% CI, 2.2 to 7.8%) when the PreTect HPV-Proofer was combined with normal repeat cytology. Risk above 2% has been considered too high to safely return a patient to the regular screening schedule (11). The cumulative incidence of CIN2+ 3 years after a negative PreTect HPV-Proofer test and ASCUS/LSIL repeat cytology was 10.9% (95% CI, 4.9 to 16.6%). Therefore, ASCUS/LSIL repeat cytology, even with a negative HPV mRNA test by PreTect HPV-Proofer, may justify immediate colposcopy, whereas the current practice of laboratories using this test as a triage tool in Norway is to recommend repeat cytology again after another 6 months (33). Since cytology itself has a low sensitivity, this could result in women with undiagnosed CIN2+ being returned to the regular screening schedule.

Moreover, in a population with a high HPV prevalence, the use of a very sensitive test involves a trade-off for a lower specificity and PPV. The Amplicor HPV test is more analytically sensitive for the presence of high-risk-HPV DNA than HC2 (39), which has

TABLE 5 NPV and PPV with 95% CIs to predict CIN2+ and CIN3+ at 3 years, for PreTect HPV-Proofer (mRNA), Amplicor HPV test (DNA), and repeat cytology tests performed at study inclusion[a]

Test	% (95% CI) predictive value			
	CIN2+		CIN3+	
	NPV	PPV	NPV	PPV
PreTect HPV-Proofer	89.7 (86.7–92.8)	52.8 (44.1–60.1)	94.0 (91.6–96.4)	40.5 (31.3–48.5)
Amplicor HPV test	98.2 (96.4–1.00)	41.3 (35.5–46.6)	99.5 (98.6–1.0)	30.5 (24.7–35.8)
Repeat cytology[b]	91.2 (88.0–94.5)	41.0 (34.6–46.8)	94.1 (91.4–96.8)	29.3 (23.0–35.0)

[a] Study inclusion represents 6 to 12 months after screening-detected ASCUS/LSIL cytology.
[b] ASCUS+ threshold.

been extensively performed in both reflex testing for triage and primary screening settings. Using a test with a clinical sensitivity equal to or higher than that of HC2 (34, 38) in a delayed manner has been shown to be more specific than reflex testing (7). The PPV for the Amplicor HPV test in delayed testing 6 to 12 months after screening-detected ASCUS/LSIL cytology in our study was 41.3% (95% CI, 35.5 to 46.6%), which is more than twice the PPV found by Kelly et al. when performing reflex high-risk HPV DNA testing with HC2 among women with borderline or mild dyskaryosis (16). The corresponding 3-year PPV for CIN2+ was 52.8% with PreTect HPV-Proofer, which is in agreement with other studies when performed as a reflex test (34, 35), but lower than earlier results presented in a study with the same follow-up regimen as ours (32). A positive PreTect HPV-Proofer result in combination with ASCUS+ repeat cytology was the best indicator of CIN2+ in the present study.

As delayed HPV testing allows for regression of HPV infection, an HPV-positive test 6 to 12 months after ASCUS/LSIL cytology may indicate a persistent infection, which confers a higher risk of CIN2+, resulting in a higher PPV, even when analytically highly sensitive tests are used. In addition, the high PPV for both HPV mRNA and DNA testing that we observed might be due to the fact that we censored participants after the last follow-up cytology or histology registered, making incidence of CIN2+ higher than if cases were censored at the end of study, immigration, or death. The continuous increase in risk of CIN2+ for HPV-positive women during the total follow-up period shows that high-grade neoplasia is either being missed by further diagnostic procedures or that these women are at risk of rapidly developing CIN2+, which reflects the importance of a longer follow-up period when evaluating HPV tests and cytology. Prevalence of high-risk HPV types varies greatly among women with ASCUS/LSIL cytology at different study centers, suggesting heterogeneity in cytological interpretation at different laboratories (2, 16). We chose to analyze predictive values for ASCUS and LSIL cytology combined because of our small study group, large interobserver variety in cytological results, and because there has been no significant difference in risk of CIN2+ reported in long-term follow-up studies of HPV-positive women when stratified by ASCUS or LSIL cytology (10). Our study had too few participants to do extensive analysis of age effects. We only performed log rank tests to assess the effect of age within each group defined by test result (DNA^+, DNA^-, RNA^+, or RNA^-). There were no significant differences in the cumulative incidence of CIN2+ during the follow-up between the different age groups (older and younger than 32 years) (data not shown).

One of the major strengths of our study is the linkage to the Cancer Registry of Norway, from which we retrieved all follow-up data and to which all cytological and histological results from the uterine cervix are reported. Furthermore, cytology and both HPV tests were performed for all women at study inclusion, so that test comparisons could be conducted in a manner that avoided variations between women. However, our study cannot reproduce the results of a randomized trial in which women would receive only one of the tests or a combination of two and was not designed as a head-to-head study with a blinded/randomized follow-up. Cytology and histology were not reviewed, and general gynecologists in private practices and hospitals performed colposcopy. Our results therefore reflect a real-life community-based setting. Along the same lines, an ASCUS+/CIN2+-free period longer than 12 months prior to study inclusion was not chosen. This fact might be reflected in the slightly higher HPV mRNA and DNA positivity rates and ASCUS+ repeat cytology rates compared to the large national report from the Cancer Registry of Norway, which had more strict inclusion criteria (14).

Delayed HPV DNA testing has been considered safe (4, 6), but this conclusion is based on small studies. In our study, three cervical carcinomas were detected, all of which had normal cytological results up to 15 years before invitation to our study, the criterion for which was screening-detected ASCUS/LSIL cytology. These cases could possibly have been detected earlier if reflex HPV testing had been performed. Another concern is the possibility of loss to follow-up when delaying HPV DNA testing and repeat cytology 6 to 12 months. Indeed, women with ASCUS/LSIL cytology results are followed up less often in Norway than those with cytological results indicating a high-grade lesion (24), despite the fact that a reminder system for women lost to follow-up after triage ASCUS/LSIL cytology is a part of the Norwegian organized screening program (9). One study from The Netherlands estimated that delayed HPV DNA testing 6 months after borderline or mild dyskaryosis cytology is more cost-effective than reflex HPV DNA testing when using conventional cytology at 5-year screening intervals (6). Although no cost-effectiveness analysis has been done for reflex versus delayed HPV testing in Norway, the Dutch example may well hold true. Indeed, most laboratories in Norway still use conventional cytology, meaning that any type of HPV testing would necessitate the recall of women. Reflex HPV DNA testing may entail less loss to follow-up and earlier disease diagnostics than delayed testing, but there is a trade-off. Reflex HPV DNA testing leads to higher HPV positivity due to transient HPV infections, with all its implications: unnecessary worry, overtreatment, and additional work.

With the introduction of new biomarkers for cervical cancer, more screening options are available. When studying different screening algorithms, Naucler et al. found that, compared to cy-

tology alone, HPV DNA testing in primary screening with cytology triage, or if cytology is normal, triage by persistence of the same HPV genotype after 1 year, was the regimen with highest sensitivity and highest PPV, while leading to a decrease in screening tests (22). PreTect HPV-Proofer is the only commercial HPV test that includes less than 13 high-risk HPV types, and it may lack sensitivity to detect CIN2+ in primary screening. Neither sensitivity nor specificity for CIN2+ should be inferior to HC2, and guidelines for high-risk HPV DNA tests to be used in primary screening have proposed about 14 types to be included in these tests (18, 19). Although biomarkers measuring the interaction of HPV with cervical cells may one day become the basis of primary screening tests, they will first be used for triage of women with positive cytology and/or HPV tests (28). If HPV DNA testing is used in primary screening, the PreTect HPV-Proofer (32, 33) might perform better than cytology, as a reflex test, due to its high PPV in identifying women who should promptly be referred to colposcopy. Indeed, Benevolo et al. showed that PreTect HPV-Proofer is a good reflex test for triage to reduce colposcopy referral, but with a sensitivity comparable to that of repeat cytology, and they therefore recommended a strict follow-up despite a negative test result (5). Persistence of the same genotype may give an even better PPV than testing positive once by PreTect HPV-Proofer, but this was not investigated in the present study. Newer HPV DNA tests, including direct genotyping with 16/18 or CINtec p16INK4a, have been shown to be other promising triage test methods (34).

We conclude that it is safe to return women with a negative HPV DNA result by Amplicor HPV test in delayed testing to the regular 3-year screening schedule in Norway. The PPV for the Amplicor HPV test was fairly high, and therefore it performs adequately in a positive-triage setting 6 to 12 months after screening-detected ASCUS/LSIL cytology. A more specific HPV mRNA test like PreTect HPV-Proofer might perform better to detect women with an increased short-term risk of CIN2+; however, the corresponding NPV is considered too low to safely return women with a negative PreTect HPV-Proofer result to the regular 3-year screening schedule.

ACKNOWLEDGMENTS

We thank Katrine Lie for initiating the study, Mona Hansen and Grete Rutgerson for excellent technical assistance, Unni Westerhagen for managing the data collection, Håvard Hagavei for IT assistance, the gynecology clinic at Akershus University Hospital, and the local gynecologists and general practitioners who helped with recruiting and collecting samples. We also thank Trudy Perdrix-Thoma for editing assistance and language review.

This study was financially supported by grants from Health Region East (reference no. 2769112).

We report no conflicts of interest for this study.

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