Increased risk for development of severe cervical dysplasia among postmenopausal women with normal cytology and presence of HPV mRNA

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Katrin Christine Asciutto christine.asciutto@yahoo.com
Skåne University Hospital
Corresponding Author

Christer Borgfeldt
Klinikum rechts der Isar der Technischen Universität München Klinik und Poliklinik für Kinder und Jugendmedizin Kinderklinik München Schwabing

Ola Forslund
Laboratory Medicine, Region Skåne

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Abstract

Background: During 2013 and 2016 the Region of Skåne, Sweden, started to analyse human papillomavirus (HPV) and cytology in postmenopausal women 60-65 years of age. Our aim was to evaluate if the presence of high-risk (HR) HPV mRNA could predict the development of cervical abnormalities among HR-HPV DNA positive women with normal cytology. Methods: A total of 271 women, 60-65 years of age, underwent liquid based cytology (LBC) and HPV testing by using the HR-HPV DNA MGP-PCR-Luminex assay. HR-HPV DNA-positive women with normal cytology underwent complimentary HPV mRNA testing (Aptima, Hologic). Over a period of 49 months (SD 11.0) the women received regular follow-up at intervals of 12-18 months. Women with abnormal cytology and/or a positive HR-HPV result at two subsequent visits were scheduled for colposcopy and clinical examination. Results: Over the surveillance period, 3.6% (10/271) of the HR-HPV DNA positive women developed histologically confirmed high-grade squamous intraepithelial lesions (HSILs) or worse and 13.3% (36/271) were diagnosed with cervical atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSILs). The cumulative incidence rates (CIR) were 29.7% (CI 24.8-30.1) for HSIL or worse among HPV mRNA-positive women at enrolment (39.4% 107/271) and 0% among HPV mRNA negative women (60.5%, 164/271), (p=0.002). The corresponding CIRs for ASCUS and LSIL was 59.9% (95% CI 59.3-66.2) and 26.1% (95 %CI 16.5-35.9), (p=0.001). Conclusions: Postmenopausal women with normal cytology along with presence of HR-HPV mRNA are at increased risk for development of severe cervical dysplasia, in contrast to those women with negative HR-HPV mRNA.

Background

International consensus when to stop cervical cancer screening among postmenopausal
women does not exist (1). In Sweden 30% of cervical cancer cases are diagnosed in women older than 60 years of age (The Board of Health and Welfare (2015) Cancer incidence in Sweden 2014).

In a recent audit in the region of Skåne (southern Sweden) it was observed that 24% (31/177) of squamous cervical cancer (SCC) or cervical adenocarcinoma cases between 2016 and 2017 were over 65 years of age (Personal communication Gunilla Thorn, Department of Clinical Pathology and Genetics, Lund Sweden).

Most of the affected older women have symptoms at the time of diagnosis due to an advanced cancer stage and the mortality is as high as 70% (2). This data indicate that there is a need to identify postmenopausal women who are at risk to develop cervical intraepithelial neoplasia (CIN) or cancer before they leave the screening program.

Among postmenopausal women it has been shown that combined screening of high risk (HR) human papillomavirus (HPV) DNA testing and cytology offers a higher sensitivity than screening with cytology alone (3,4). Therefore the organized cervical cancer screening program performed between 2013 and 2016 in the Skåne region a double test consisting of both high-risk (HR) HPV testing and cytology in postmenopausal women aged 60-65 years as a last test before leaving the screening program. The double test consisted of a liquid based cytology (LBC) specimen which was co-tested for HR-HPV DNA. In HR-HPV DNA positive women with normal cytology the LBC specimen were further investigated for the presence of HR-HPV mRNA.

The aim of this study was to prospectively evaluate if the presence of HR-HPV mRNA at enrolment could predict the future development of cervical abnormalities among HPV- DNA positive, postmenopausal women with normal cytology over a four year follow-up period.

Methods

Between 2013 and 2016, women 60-65 years of age, with normal cytology in the southern
region of Sweden (Skåne) (n=5925) were tested for the presence of high-risk (HR) human papillomavirus (HPV).

Cervical HPV DNA positivity was found in 286 (4.8%) individuals with a mean age of 61.9 years (SD +/- 1.7).

Exclusion criteria from further follow-up were history of cervical neoplasia and/or treatment of cervical disease such as the loop electrical excision procedure (LEEP), hysterectomy or trachelectomy and ongoing oncological treatment at the time the double test was performed. A total of 271 HR-HPV DNA positive women with normal cervical cytology were eligible for inclusion in this prospectively follow-up study.

The double test consisted of a liquid based cytology (LBC) sample (Thinprep) that was analyzed for HR-HPV DNA by using the MGP-PCR-Luminex assay (5, 6). In women testing positive for HR-HPV DNA, a concomitant HPV E6/E7 mRNA assay (APTIMA) was performed. Women with normal cytology and a positive HR-HPV DNA result were scheduled for a new follow-up examination after 12 months including a new LBC specimen and HPV-testing with both HPV assays. All women diagnosed with cervical pathology or a positive HR-HPV result, were planned for a further clinical evaluation with colposcopic assessment. The same accounts for women with a positive HR-HPV outcome on two subsequent controls. The next routine co-testing procedure was scheduled after twelve months including even those women who underwent a clinical examination.

At all further follow-up controls which were performed at intervals of 12 to 18 months, the same selection criteria were applied to determine which women were in need of a further clinical investigation. During our surveillance period at least three consecutive follow-ups could be documented.

Women presenting with normal cytology and negative HR-HPV DNA results left the routine screening service.
Classification of LBC and Histology Results

Pathological LBC results were defined as low-grade squamous intraepithelial lesions (LSILs), atypical squamous cells of undetermined significance (ASCUS), high-grade intraepithelial lesions (HSILs), and atypical glandular cells (AGCs) according to the Bethesda classification (7). Histopathological results were defined as LSIL and HSIL lesions using a two-tiered classification system (8).

In case of a discrepancy between findings on LBC and the corresponding histopathological results, the most severe diagnosis was taken into account for final evaluation. Recurrent cytological abnormalities of the same severity level were considered as one incident case.

In women with HSIL lesions on LBC and corresponding colposcopic findings, a loop electrical excision procedure (LEEP) was performed for therapeutic management. Also patients with cytological ASCUS or LSIL but a colposcopic picture suggestive of an underlying precancer lesion were scheduled for a LEEP procedure.

In case of an inaccessible transformation zone located within the cervical channel, cervical biopsy or conisation specimen were obtained for diagnostic reasons.

HR-HPV testing

The MGP-PCR Luminex HPV DNA assay detects several HPV types simultaneously (5, 6). Initially, sample DNA was purified by MagnaPure LC (Roche) and then HPV DNA was amplified by PCR with modified GP5+/6+ (MGP) primers (6).

After amplification, the Luminex-based HPV genotyping allows the identification of the following HR-HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and the probable high-risk type 68 (A and B) as well as the possibly high-risk types 26, 53, 66, 67, 69, 73 and 82 as described by IARC classification from year 2012 (9). In the present study, probable and possible HR-HPV types were classified as HR-HPV types.

The HPV E6/E7 mRNA (APTIMA) assay (Hologic, Inc.) detects qualitatively E6/E7 mRNA from
14 HR-HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

We calculated the proportion of HPV E6/E7 mRNA positivity for each of these 14 HR-HPV types as well as for HPV67 (APTIMA is known to cross-react with HPV67, Kit insert, APTIMA HPV Assay, nr 503744), as determined by the MGP-PCR Luminex HPV DNA assay.

The presence of the same HR-HPV genotype at inclusion and at follow-up was defined as a persistent infection. At follow-up, women with benign cytology who tested negative for HR-HPV DNA but were positive for low-risk (LR) HPV DNA according to the Luminex assay, the HR-HPV status was considered as negative or as cleared infection.

**Endpoints**

The endpoint was the development of cervical atypia/dysplasia or worse over a follow-up period of four years.

**Statistical evaluation**

Statistical comparisons were based on two-sided chi-square tests. All comparisons were two-sided, and a 5% level of significance was applied. The strength of association between the HPV mRNA results and the presence of cervical abnormalities was measured using the odds ratio and the corresponding 95% confidence intervals.

Cumulative incident rates (CIR) during the follow-up period were calculated according to Kaplan-Meier survival analysis and presented as percentages with the corresponding 95% Confidence Interval (CI).

The statistical analyses were performed using SPSS version 19.0 or higher (IBM Corp., Amonk, NY, USA) and Omnistat (SBU, Trelleborg, Sweden).

**Results**

Over a total surveillance period of 49 months (SD 11.0), abnormal cytological findings were encountered in 17% (46/271) of the women, including 10 cases of high-grade intraepithelial lesions (HSILs) and 36 cases of atypical squamous cells of undetermined
significance (ASCUS) or low-grade intraepithelial lesions (LSILs).

The cumulative incidence rate (CIR) for HSIL or worse was 29.7% (95% CI 24.8-30.0) in the subgroup of HR-HPV mRNA positive individuals and 0% in those women with a negative HR-HPV mRNA result (p=0.002) (Figure 1). The corresponding CIRs for ASCUS or worse were 59.9% (95% CI 57.3-66.2) and 26.1% (95% CI 16.5-35.9), respectively (p=0.001). Among HR-HPV mRNA positive women, the relative risk to be diagnosed with HSIL or worse was 32.1 (95% CI 1.9-542) and 4.3 (95% CI 2.4-8.0) for the development of ASCUS or worse. At baseline HR-HPV mRNA was present in all cases (10/10) who were found to have histologically confirmed HSIL and in 67% (24/36) of the women diagnosed with cytological ASCUS or LSIL.

Among women testing positive for HR-HPV types 16/18 the CIR for HSIL or worse was 14.6 % (95% CI 13.3-22.7) and among those with other HR-HPV types the corresponding figure was 18.5% (95% CI 13.2-23.7, p=7.58). The CIRs for ASCUS or worse were 64.9% (95% CI 47.05-72.9) for women infected with HR-HPV 16/18 and 51.1% (95% CI 43.05-63.8, p=7.68) in the other subgroup.

After one year, 41% (112/271) of the HR-HPV DNA positive women had cleared their infection, whereas 59% (159/271) were still persistently infected with the same HR-HPV type(s). Over the entire follow-up period persistent HR HPV type(s) were observed among 17.3% (47/271) of the women whereas clearance of the HR-HPV infection could be detected in 70.1% (190/271) of the cases. Thirty-four women were lost during follow-up after two years.

HPV16, 68A, 31, 52 were the most common HR-HPV type at enrolment (Table I).

Out of the 164 women with a negative HR-HPV mRNA outcome at baseline 85% had no detectible HPV DNA at two years follow-up (137/164, 95%CI 77.1-88.5), whereas the corresponding clearance rate for HPV mRNA positive women was 49.5%; (53/107, 95% CI
LEEP procedure was performed in all 10 patients with cytological HSIL and in seven patients with ASCUS or LSIL, due to abnormal findings on colposcopy. Histopathological evaluation revealed seven cases of HSIL, two glandular precancer lesions and one case of cervical adenocarcinoma. In other 22 patients with cytological ASCUS or LSIL, a diagnostic conisation procedure could confirm the presence of histopathological LSIL in three cases and benign tissue conditions in the remaining 19 patients. In seven patients with cytological low-grade lesions no tissue material was obtained due to normal findings on colposcopy. Worst histology/cytology findings were taken into account for final analysis.

Discussion

In our cohort of high-risk (HR) human papillomavirus (HPV) DNA positive postmenopausal women with normal cytology we found significantly increased risk for high-grade squamous intraepithelial lesions (HSIL) or worse (CIR 29.7%) and for atypical squamous cells of undetermined significance (ASCUS) or worse (CIR 59.9%) among women who tested HR-HPV mRNA positive at enrolment and were followed for up to four years. In contrast, none of the HPV-DNA positive women with a negative HR-HPV mRNA result at baseline developed HSIL or worse (CIR 0%) during follow-up, whereas a risk regarding the development of low grade lesions such as ASCUS or low-grade squamous lesions (LSIL) was still evident (CIR 26.1%). Concerning the persistency of HR-HPV types we observed that after four years of follow-up 17.3% of the women were still diagnosed with a persistent HR-HPV infection while 70.1% had cleared their infection spontaneously.

It is already shown in the literature that the HR-HPV testing is a safe screening option in postmenopausal women (10) as it increases the likelihood of identifying cervical precancer lesions while cytology alone is known to have a relatively low sensitivity in this age group (3,11). Accordingly, we could also observe in our series a certain discrepancy of about 20
% between LBC results and the corresponding histological findings. In three women with
ASCUS or LSIL, histopathological analysis of the matching loop electrical excision
procedure (LEEP) specimen revealed the presence of underlying HSIL lesions, while in
three other women with high-grade cytology no histopathology could be confirmed on
conisation material. According to literature, the discrepancy level between cytology
findings and the corresponding histological outcomes varies between 5 to 55%. Factors
that may cause those elevated rates of false negative results are the subjective
interpretation of the specimen and/or the absence of diagnostic cells (12,13).
Clinical circumstances that may further contribute to the limited sensitivity of cervical
cytology is the higher probability of sampling errors as the transformation zone tends to
be located higher up in the cervical channel. Also aging effects like a decline in estrogen
can lead to cellular changes that are mistakenly interpreted as ASCUS or LSIL lesions
(13,14). The described difficulties to obtain an adequate LBC sample in postmenopausal
women underline the need of an objective screening tool i.e. HPV analyses in this age
group offering a higher sensitivity.
Our data show that HR-HPV mRNA positive women aged 60 years or older are at risk of
developing cervical abnormalities and are therefore in need of regular follow-up controls
including HPV analyses. Additional gynecological examination is indicated if a persistent
HR-HPV infection is found at two subsequent annual controls and/or cytology shows
abnormal results. Also Johannson et al could demonstrate that among HPV DNA positive
women aged 35 years or older with either cytological ASCUS or LSIL at baseline, a positive
HR-HPV mRNA result could predict the development of a high -grade cervical
intraepithelial neoplasia (CIN 3) or worse with a sensitivity of 100% within the following
four years (15). Like in our study, all women who were diagnosed with CIN 3 or worse were
HPV mRNA positive at baseline.
Postmenopausal women represent a special patient group, as they tend to have a higher risk for persistent HR-HPV infections than younger individuals, who have a higher acquisition frequency but also a faster clearance rate (16,17). Furthermore a type-specific HR-HPV persistence, especially for HR-HPV types 16, 18 and 31 appears to be associated with the future development of cervical precancer or worse in this age group (10). It is of clinical importance to distinguish between those individuals with active viral replication who are at risk to develop cervical precancer lesions and those with latent HR-HPV infections lacking any clinical significance.

There is evidence in the literature that the level of the mRNA copies increases proportionally to the severity of the cervical lesion (18). On the contrary, a negative HPV mRNA result in combination with a positive HPV-DNA outcome seems to reflect the presence of an inactive HR-HPV infection with low or absent viral replication. According to our data, a negative HR-HPV mRNA result at baseline and at the one year follow-up control was associated with a high probability to clear an existing HPV infection spontaneously.

The overall clearance rate in our cohort was 70.1% (190/271) and 137 (72%) healed their HR-HPV infection within the first twelve months. Those data are comparable to the results of other studies reporting clearance rates of about 40% in older women within an average time span of four months (4, 10, 19). The data of recent studies indicate that the long-term protective effect of a negative HPV mRNA result is comparable to that of negative HPV-DNA test (15, 20, 21).

A cohort study with longterm follow-up could demonstrate that the five year cumulative risk of developing CIN 3 or worse was comparable between the cohort of HPV DNA negative and HPV mRNA negative women (22). Those clinical data lead to the assumption that the implementation of four year screening intervals is a safe strategy in HR-HPV mRNA negative women like it is already reported for HR-HPV DNA negative women aged
40 years or older (20, 23, 24).

Regarding the risk stratification of cytological ASCUS and LSIL lesions, it is stated in the literature that the APTIMA assay offers a higher specificity when compared to other DNA based HR-HPV detection methods (25-27). This is in agreement with the observation that in our series none of the twelve ASUCS lesions detected in HPV mRNA negative women was of clinical significance. In all cases the following LBC results were classified as benign and cervical biopsy could confirm the presence of normal underlying tissue conditions. Furthermore the results of a long-term follow-up study could demonstrate that a negative APTIMA co-testing result among HPV-DNA positive women with minor cytological abnormalities at baseline was associated with a high negative predictive value of 100% for CIN 3 or worse within the following four years (15). Even though it seems that HPV mRNA negative women with cytological ASCUS or LSIL might be re-screened safely at four year intervals, further long-term studies are necessary to evaluate if these assumptions can also be applied to the postmenopausal patient cohort.

Regarding the distribution of the HR-HPV genotypes, we could observe that HR-HPV types 16, 68A and 31 were the most frequent ones in our cohort. Those data are in line with other publications indicating that the HR-HPV profile in postmenopausal women to some extent differs from those encountered in younger individuals (28,29). Another study investigating the HR-HPV profile in postmenopausal women could confirm that HR-HPV type 31 contributed more to the development of cervical dysplasia than HR-HPV types 16/18 (30).

As also other studies indicate that a type-specific HR-HPV persistence may be predictive for the development of precancer lesions in postmenopausal women (10), HR-HPV DNA genotyping could be another complementary screening strategy in this age cohort. HR-HPV genotyping has already been found to be a successful triage method for the middle
Conclusions

Our data favour the use of an HPV mRNA assay as screening tool in postmenopausal women.

While its sensitivity is comparable to that of other DNA based HPV assays, it offers a higher specificity and a higher negative predictive value reducing the number of unnecessary colposcopies and allowing a safe prolongation of screening intervals for up to six years (20, 21, 24).

Since postmenopausal women testing positive for HR-HPV mRNA and normal cytology have a substantial risk of developing cervical abnormalities we believe that these women should be scheduled for regular, annual or bi-annual follow-up examinations. In case of a persisting HR-HPV infection at two subsequent controls and/or the presence of cytological abnormalities a cervical tissue biopsy/curettage or diagnostic LEEP specimen should be obtained especially if the transformation zone is inaccessible for colposcopic evaluation.

On the other hand our data indicate, that postmenopausal women with a negative outcome for HR-HPV mRNA and normal results on LBC are no longer in need of further follow-up for at least four years.

Abbreviations

ASCUS: atypical squamous cells of undetermined significance

CIN: cervical intraepithelial neoplasia

CIR: cumulative incidence rate

HR-HPV: high-risk human papilloma virus

HSIL: high-grade squamous intraepithelial lesion

LBC: liquid based cytology
LEEP: loop electrical excision procedure

LSIL: low-grade squamous intraepithelial lesion

Declarations

**Ethics approval and consens to participate:**

The study was approved by the Regional Ethics Board of the Lund University, Lund, Sweden (Reference number DNR 390:2013). The collection of a consent to participate was non applicable due to the retrospective character of this study.

**Availability of data and material:**

The data belong to the screening - register for cervical cancer stored at the department for microbiology, Lund university

**Competing interests:**

None of the authors have declared any competing interest

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**Authors contribution:**

All authors have read and approved the manuscript.

KCA: study design, interpretation of data; manuscript drafting.

OF: responsible for the HPV analyses, revising the manuscript critically.

CB: study design, revising the manuscript critically.

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Not Applicable

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Tables

Table 1: Frequencies of Human papillomavirus genotypes in women with HPV DNA positives and normal cytology at baseline in relation to worst findings on cytology/histology at follow-up.

| Cytology/Histology at follow-up (n) | HPV types baseline | Normal | LSIL/ASCUS | HSIL | Total |
|-----------------------------------|--------------------|--------|------------|------|-------|
| 16                                |                    | 42     | 5          | 2    | 49    |
| 68A                               |                    | 22     | 3          | 1    | 26    |
| 31                                |                    | 19     | 3          | 2    | 24    |
| 52                                |                    | 19     | 4          | 0    | 23    |
| 51                                |                    | 16     | 1          | 1    | 18    |
| 66                                |                    | 15     | 7          | 0    | 22    |
| 56                                |                    | 13     | 3          | 1    | 17    |
| 39                                |                    | 13     | 1          | 1    | 15    |
| 18                                |                    | 13     | 2          | 1    | 16    |
| 45                                |                    | 11     | 2          | 0    | 13    |
| 58                                |                    | 6      | 1          | 0    | 7     |
| 35                                |                    | 5      | 0          | 0    | 5     |
| 59                                |                    | 4      | 0          | 0    | 4     |
| 68B                               |                    | 2      | 0          | 0    | 2     |
| 33                                |                    | 1      | 0          | 0    | 1     |
| Other                             |                    | 24     | 4          | 1*   | 29    |
| Total                             |                    | 225    | 36         | 10   | 271   |
ASCUS: Atypical squamous cells of undetermined significance; HSIL: high-grade squamous intraepithelial lesion; LSIL: low-grade intraepithelial lesion.

* Among the group of other HR-HPV types, HR-HPV 67 was detected in the case diagnosed with a HSIL lesion

Figures

![Graph showing cumulative incidence rate](image)

Figure 1

Cumulative Incident cases of high-grade squamous intraepithelial lesions (HSIL) or worse over a mean follow-up period of 49 months (SD 11.0) in correlation to the HPV mRNA status obtained with the APTIMA assay at baseline in HPV DNA positive women with normal cytology