Can viral load, semi-quantitatively evaluated, of human papillomavirus predict cytological or histological outcome in women with atypical squamous or glandular cells of undetermined significance cytology?

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Summary

Objective: 1) To assess the regression to normal cytology in women with cervical smears diagnosed as atypical squamous or glandular cells of undetermined significance (ASCUS/ AGUS) and absence or clearance of human papillomavirus (HPV) infection; 2) To evaluate the association between viral load, semi-quantitatively evaluated, and cytological or histological outcome.

Material and methods: In this cohort study HPV test and biopsy was taken in 148 women with ASCUS/AGUS cytology. After 12-18 months a HPV test and cervical smear were repeated in 121 women.

Results: Absence or clearance of HPV showed significantly more regression to normal cytology than persistent or newly acquired infected women, odds ratio 27 (95% confidence interval; 7-103). The viral load of the HPV test at enrollment was not correlated with the follow-up cytological outcome (Spearman correlation coefficient 0.2, $p = 0.2$). A marked association between viral load and histological outcome at enrollment was shown (Spearman correlation coefficient 0.43, $p < 0.0001$).

Conclusion: Absence or clearance of HPV can predict regression to normal cytology. Viral load at enrollment cannot predict cytological regression. There was a marked association between viral load and the underlying CIN at enrollment. However, there was large overlapping of viral loads among the grades of CIN. Therefore, viral load is not a useful parameter to predict high-grade lesions in women with ASCUS/AGUS cytology

Key words: Atypical squamous cells of undetermined significance; Atypical glandular cells of undetermined significance; Human papillomavirus; Viral load; Cervical intraepithelial neoplasia.

Introduction

The introduction of the atypical squamous or glandular cells of undetermined significance (ASCUS/AGUS) category has created a management dilemma for clinicians, as a number of studies have shown that 5-30% of women with this diagnosis harbor undetected cervical cancer precursors or even, cervical cancer [1-6]. Although the majority of women with ASCUS/AGUS diagnoses will have trivial lesions, some will have significant lesions that warrant either closer surveillance or further investigation. In a previous cohort study we assessed the prevalence of cervical intraepithelial neoplasia (CIN) and evaluated the role of human papillomavirus (HPV) in detecting underlying CIN. We found 7% CIN II or more in women with ASCUS/AGUS cytology [7]. Like others we concluded that HPV DNA testing could be used to triage women with ASCUS/AGUS cytology. Especially, because of the high sensitivity and negative predictive value of the HPV test (90% and 99%, respectively) [7].

Epidemiological and molecular data suggest that persistent infections with high-risk human papillomavirus (HPV) are the intermediate endpoints, leading to cervical intraepithelial neoplasia and cervical cancer [8]. No progression to CIN III was seen among women without high-risk human papillomavirus infections [9].

Some propose to use the high-risk HPV DNA test in screening and triaging ASCUS cytology to improve the sensitivity of cytology to predict high-grade CIN. Sensitivity rates for cytology of only 40%-80% and specificity rates of 83-92% for high-grade CIN (II and III) have been reported [10, 11]. In patients with Papanicolaou smears reported as ASCUS, the sensitivity of the HPV Hybrid Capture II (HC II) test was increased to 60-96%, but with lower specificity rates of 40-68% [2, 7, 12-15]. The positive predictive value of the HPV test in women with ASCUS/AGUS cytology may be improved by changing the threshold of a positive test. Therefore, we explored alternative human papillomavirus test cut-off points for detecting high-grade lesions.

Because of the serious risk of the underlying CIN, the percentage of loss to follow-up, the costs and the anxiety in women with ASCUS cytology, we need a better strategy to detect women at risk for high-grade lesions. In this study we compared women with persistent HPV infection with women who cleared their infection for predicting regression to normal cytology, and we explored the association between viral load, semi-quantitatively evaluated by the relative light units (RLU) of the HC II HPV test, and underlying CIN.

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Material and Methods

Patients

The colposcopic, histological and virological results of women with ASCUS/AGUS cytology are reported elsewhere [7]. A cohort of patients with cytology diagnosed as ASCUS/AGUS (Pap II, borderline dyskaryosis) were included from April 1997 to March 2000 at the Gynecological Outpatient Clinic of the Medical Center Haaglanden, the Hague, the Netherlands. Pregnant women and women with HIV were excluded. After informed consent was obtained all women underwent a colposcopic examination which included a biopsy and HPV test. During the study, patients were treated according to current protocols, irrespective of their HPV status. Two women were operated on because of the underlying cervical cancer. Twenty-five women discontinued the study despite several recalls. The cause of discontinuation is unknown. One hundred and twenty-one women of the 148 at enrollment continued this study for cytological and virological follow-up. A Pap smear and HPV DNA test was repeated after 12 to 18 months. The medical ethical committee of the hospital approved this study.

Cytology

Cervical smears were collected using a Cervix-Brush (Rovers BV, Oss, the Netherlands). All cervical smears were Papanicolaou stained, screened routinely, and reviewed without knowledge of the HPV status. The initial classifications were made according to the current Dutch cytological classification system (KOPAC) [16], a modification of the commonly accepted Papanicolaou procedure used in the Netherlands [17]. Atypical squamous or glandular cells of undetermined significance are classified as Pap II [17].

Of the 121 smears with Pap II or ASCUS/AGUS cytology, 102 smears showed only atypical squamous cells (ASCUS). One showed only atypical squamous metaplastic cells (atypical repair), nine only atypical glandular cells (AGUS), one a combination of all three kinds of abnormalities, and eight a combination of atypical squamous and glandular cells.

Clinical regression was defined as normal cytological outcome.

Histology

Histological tissues were classified according to CIN classification (WHO). Biopsy specimens revealing koilocytotic atypia were included in the CIN I classification. Histological diagnoses of squamous metaplasia, immature squamous metaplasia, reactive changes, or inflammatory atypia were classified as no CIN. All specimens were reviewed by two of the authors (RV and CW).

Detection of HPV

Specimens for HPV were collected from the cervix. High/intermediate-risk types of HPV DNA (16/18/31/33/35/39/45/51/52/56/58/59/68) were detected using the Hybrid Capture II™ technology, which is a signal amplified hybridisation antibody capture microplate assay using chemiluminescence for the quantitative detection of human papillomavirus DNA in cervical specimens (Digene Corporation, Gaithersburg, MD).

Signal strengths in relative light units were compared to 1 pg/ml HPV positive controls (RLU/PC) and specimens with ratios of one or greater were positive (as described in detail previously) [18]. Viral load was semi-quantitatively evaluated by the relative light units (RLUs) of the HC II HPV assay.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences version 10.0 (SPSS Inc, Chicago, IL). Logistic regression was used to test the association between HPV at enrollment or follow-up and cytology at 12-18 months follow-up; p values of ≤ 0.05 were considered significant.

The Spearman correlation coefficient was calculated to explore the strength of the association between viral load and cytological or histological outcome.

The sensitivity and specificity of the HPV test at different cut-off points of RLU/PC were compared to the standard of ≥ 1.0 RLU/PC cut-off point, the manufacturing threshold of a positive test. The receiver operating characteristic curve (ROC) was used to explore alternative cut-off points for the Hybrid Capture test to predict cytological or histological outcome.

Results

The Pap smear and HPV test were repeated in 121 (82%) women of the 148 with ASCUS/AGUS cytology at enrollment. The mean age of these 121 women was 36 years (range 16-66 years). Two women of the 148 with ASCUS/AGUS cytology at the first visit were treated for cervical cancer. The seven women with histological CIN II and the one with CIN III underwent a loop excision of the transformation zone (LLETZ). All eight continued the study and had a repeated Pap smear and HPV DNA after 12-18 months. Thirteen women with ASCUS/AGUS cytology showed histological CIN I and 100 showed no CIN at the first visit. In total 121 women continued the study and they showed similar characteristics as the 25 cases lost to follow-up, except for age (Table 1). The

Table 1. — *Characteristics of 146 women with ASCUS/AGUS cytology.*

Characteristics	Loss to follow-up N = 25	Follow-up N = 121	p value* < 0.05
Mean age	30	36	< 0.05
Cigarette smoking	12 (48%)	50 (41%)	n.s.
Oral contraceptives†	8 (32%)	43 (36%)	n.s.
Parity ≥ 4	2 (8%)	19 (16%)	n.s.
First intercourse ≤ 18	15 (60%)	75 (63%)§	n.s.
Sexual partners ≥ 5	7 (30%)¶	24 (20%)#	n.s.
History of STD‡	5 (20%)	19 (16%)	n.s.
HPV test positive	14 (56%)	51 (42%)	n.s.
≥ 2 quadrants abnormal	9 (36%)	51 (42%)	n.s.
Colposcopic ≥ CIN II	4 (16%)	15 (12%)	n.s.
Histological ≥ CIN II	0 (0%)	8 (7%)	n.s.

Logistic regression. Adjusting for age did not change the estimates of interests; * A p value of 0.05 = significant; n.s. = not significant; † Current use; ‡ STD = sexually transmitted disease; § N = 120; ¶ N = 24; # N = 118.

women who discontinued the study were only significantly younger. Excluding the eight cases of CIN II or more and adjusting for age did not change the estimates of interests.

The results of the HPV test in women with ASCUS/AGUS cytology at enrollment, the repeated HPV test at 12-18 months, and the follow-up cytology are listed in Table 2. After 12-18 months 83% (100/121) of the women with ASCUS/AGUS showed regression to normal cytology. Absence (neg-neg HPV test) or clear-

ance (pos-neg) of the virus (82/85) showed significantly more regression to normal cytology than persistent (pos-pos) HPV or newly acquired (neg-pos) infected women (18/36), odds ratio (OR) 27 (95% confidence interval (CI), 7-103). This was even higher when we compared absence (55/56) of the virus with persistent infection (12/22); OR 46 (95% CI, 5-393). No cytological LSIL/HSIL was seen in absence or clearance of HPV infection (Table 2).

Table 2. — *Data of 121 women with ASCUS/AGUS: result of HPV test at first visit, repeated HPV and cytological outcome after 12-18 months.*

HPV first visit-repeated	Follow-up cytology Normal	ASCUS/ AGNUS	LSIL	HSIL	Total
neg-neg	55	1	0	0	56
neg-pos	6	4	3	1	14
pos-neg	27	2	0	0	29
pos-pos	12	5	3	2	22
Total	100	12	6	3	121

HPV at enrollment did not significantly predict cytology at 12-18 months of follow-up, although there seemed to be a trend, OR 2.1 (95% CI, 0.8-5.4).

In order to assess the viral load (semi-quantitatively) as a potential predictor of cytological and histological outcome, we estimated the sensitivity and specificity of the HPV test at different cut-off points, both at enrollment and 12-18 months of follow-up. In the previous study of 148 women with ASCUS/AGUS we estimated a sensitivity of 90% and a specificity of 58% of the high/intermediate risk HPV DNA test at enrollment to predict the underlying high-grade CIN [7]. A specimen of ≥ 1, the manufacturing standard cut-off point, was regarded as positive. The HPV test was significantly more often positive in women with ≥ CIN II than in women with ≤ CIN I ($p < 0.005$) [7]. A cut-off point of 0.8 did not improve the sensitivity, but decreased specificity. Reducing the cut-off to 0.2 improved the sensitivity to 100%, but the specificity decreased tremendously (4%). The HPV test with a cut-off level of ≥ 0.2 was not significantly more positive in women with ≥ CIN II than in women with ≤ CIN I. The sensitivity of the HPV test decreased to 70% at a cut-off level of ≥ 2 and even to 60% when a cut-off point of ≥ 10 was used (ROC-curve, Figure 1). We could not estimate an optimal cut-off point to improve the HPV test for predicting high-grade lesions in women with ASCUS/AGUS cytology at enrollment. There was a large overlapping of viral loads among the grades of CIN (Figure 2). However, more high-grade lesions were seen when the viral load increased (Spearman correlation coefficient 0.43, $p < 0.0001$, Figure 2).

The viral load at enrollment could not predict the cytological outcome 12-18 months later (Spearman correlation coefficient 0.2, $p = 0.2$). Nevertheless, when the follow-up HPV test was negative (i.e. < 1 RLU/PC) cytological regression to normal was significantly more frequently seen than in women with a positive follow-up HPV test (i.e. ≥ 1 RLU/PC), $p < 0.001$. We also found that the probability of cytological regression decreased with viral load of the repeated HPV test (Spearman correlation coefficient 0.45, $p < 0.0001$, Figure 3).

Discussion

The overall regression to normal cytology at 12-18 months of follow-up in women with ASCUS/AGUS cytology was 83% (100/121). Regression to normal cytology was significantly more often seen in absence or

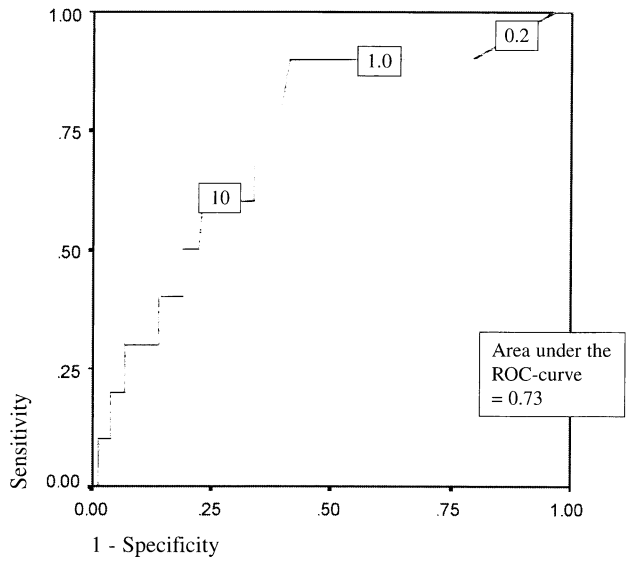

Figure 1. — ROC-Curve for Hybrid Capture II test, for detection of ≥ CIN II or more at enrolment.

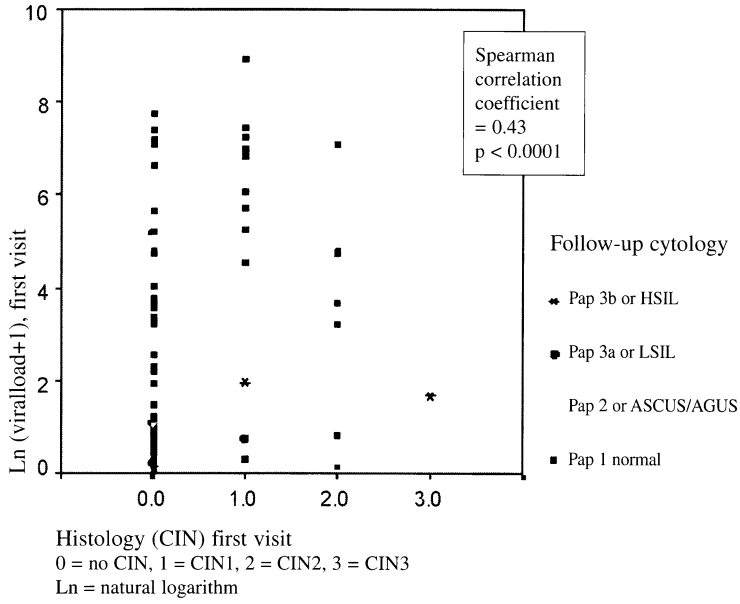

Figure 2. — Correlation between viral load at enrolment and histology at enrolment and between viral load at enrolment and cytology after 12-18 months.

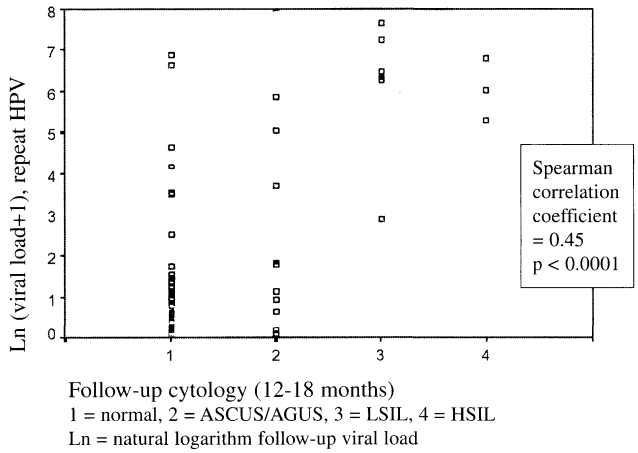

Figure 3. — Correlation between viral load of repeated HPV test and follow-up cytology.

clearance of HPV infection (96%) than in women with persistent or acquired HPV infection (50%), OR 27 (95% CI, 7-103).

All women with ASCUS/AGUS cytology included in this study from April 1997 to March 2000 had the same intervention, viz. colposcopy and biopsy. A LLETZ was done in eight women with CIN II [7] or CIN III [1]. The removal of tissue for diagnostic purposes might affect the natural history of the disease. However, in a randomised controlled trial Chenoy and colleagues have shown that the trauma associated with colposcopic directed punch biopsies, such as were taken in this study, does not have a significant effect on the immediate natural history of cytology and CIN [19]. When we excluded the eight women with CIN II-III at enrollment, the estimates of interest did not change significantly.

Two cohort studies that compared the effectiveness of testing high-risk human papillomavirus and repeated cervical smears showed no clinical progression or histological CIN III in women without high-risk human papillomavirus infection [9, 20]. In our study HSIL cytology was also not seen when the HPV virus was absent or cleared after 12-18 months.

Persistence of high-risk HPV infection in women with ASCUS/AGUS cytology showed significantly less regression to normal cytology compared to absence of HPV, OR 46 (95% CI, 5-393). Only three women showed progression to cytological high-grade lesions. Thus, we could not accurately compare cytological progression in HPV positive women at enrollment to cytological progression in HPV negative women at enrollment.

Bory *et al.* estimated the relative risk (RR) of incident high-grade CIN in a large prospective study of women with normal cytology. The RR of incident high-grade CIN was 97 (95% CI, 96-98) in positive high-risk HPV women compared to negative high-risk HPV women at enrollment. This RR increased even to 237 (95% CI, 223-252) when the positive HPV test persisted at two controls (10-18 months) [21].

As the HC-II assay cannot detect specific HPV types, it is difficult to distinguish persistent infection from re-infection (with same or new type HPV). Especially when the interval between the tests is long relative to the median duration of a high-risk HPV infection of 8-13.5 months [21, 22]. Franco *et al.* showed that the median retention time of a high-risk HPV infection was 8.1 months (95% CI, 7.8-8.3 months) [22]. The long interval between the tests in our study may explain the apparent absence of association between the viral load at enrollment and the cytological outcome 12-18 months later.

Viral load may be considered as a risk factor for persistence of HPV infection and progression to high-grade lesions. Cuzick *et al.* showed that in women with cytological abnormalities, HPV positivity at a high-level detected by a semi-quantitative PCR was strongly related to high-grade CIN [23]. In this study of women with ASCUS/AGUS cytology more high-grade lesions were also seen in women with high-levels of viral load. However, large overlapping of viral loads was seen among the grades of CIN and, therefore, viral load can not predict high-grade lesions. Recently, Van Duin *et al.* have suggested that in women with normal cytology, an increased HPV type 16 load conferred an increased risk of developing a cervical lesion [24]. Nevertheless, Bory *et al.* concluded in a large prospective study of 3,091 women with normal cytology at enrollment that the quantitative approach provided by the HC-II assay for the assessment of the viral load was not reliable for predicting HSIL in normal smears [21].

Conclusion

We showed a marked association between viral load and the underlying CIN. However, viral load does not seem to be a useful parameter to predict high-grade lesions. Either absence or clearance of the virus showed significantly more regression to normal cytology. Improving the specificity of the HPV test by increasing the cut-off point would lead to unacceptable low sensitivity rates in this cohort of women with ASCUS/AGUS cytology at enrollment. At least temporarily the recommended cut-off point of ≥ 1 RLU/PC seems appropriate for women with ASCUS/AGUS cytology.

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