

## ORIGINAL ARTICLE

# Prospective study on cervical neoplasia: presence of HPV DNA in cytological smears precedes the development of cervical neoplastic lesions

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**Objectives:** The principal aim of the study was to verify whether HPV infection in healthy women, as determined by HPV DNA detection, was associated with an increased risk of development of cervical lesions.

**Methods:** Cervical smears collected at enrolment into the prospective study conducted in Prague during 1975–83 were tested for the presence of HPV DNA by means of a polymerase chain reaction (PCR) using the general GP5/6 primers and a mixture of primers specific for the *E6* gene. 120 smears from patients in whom cervical neoplasia had been detected in the course of the prospective study and 208 smears from control women who had remained healthy throughout the observation period were analysed. Patients and controls were matched by age, number of sexual partners, age at first intercourse, and smoking habit. Patients were divided into three groups, A, B, and C, according to their cytological, colposcopic, and histological findings at enrolment. Group A consisted of 67 women found ill at enrolment, group B of 26 women with slightly suspicious findings, while group C comprised 27 women with normal findings at enrolment. In addition, sera taken at enrolment from these patients and controls were tested for the presence of antibodies reactive with virus-like particles (VLPs) of HPV 16, 18, and 33.

**Results:** For the whole cohort, there was a statistically highly significant difference in the presence of HPV DNA between patients and controls. Furthermore, the difference in the presence of HPV DNA between patients and controls was highly significant not only in those who had been found ill at enrolment (group A) but, most importantly, also in women who had developed the disease in the course of the follow up (groups B and C). Women positive for HPV DNA possessed HPV antibodies to VLP16, 18 and 33 significantly more often than those who were free of HPV DNA.

**Conclusion:** This indicated that healthy women who were positive for HPV DNA at enrolment were at an increased risk of developing cervical neoplasia (OR = 18.5; CI 5.9 to 57.6).

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A large prospective study conducted on more than 10 000 women in Prague in 1975–83 failed to sustain herpes simplex virus (HSV-2) as an aetiological factor in cervical carcinogenesis.<sup>1–3</sup> At nearly the same time the first human papillomavirus (HPV) was detected in invasive cervical carcinoma (INCA).<sup>4</sup> As the sensitivity of HPV DNA detection methods increased, so did readings of the prevalence of HPV in INCA and cervical intraepithelial neoplasia (CIN) lesions. Recently, it has been shown that the prevalence of HPV, if properly examined, in high grade CIN lesions and in INCA reaches 100%.<sup>5</sup> Up to now, more than 35 HPV genotypes have been detected in genital lesions, and the prevalence of so called high risk (HR) HPV has been shown to increase with severity of the disease.<sup>6</sup> Furthermore, numerous studies have suggested that the presence of HR HPV types is indispensable for the persistence and progression of cervical lesions.<sup>7–9</sup> Besides molecular biological and epidemiological data, the results of seroepidemiological studies also suggest an aetiological role of HPV in the pathogenesis of CIN and INCA. HPV specific antibodies are more frequent in the sera of women with CIN or INCA than in healthy controls<sup>10</sup> and HPV DNA positive women show a significantly higher prevalence of HPV specific antibodies than HPV DNA negative women.<sup>11–13</sup> It has also been shown that women with anti-HPV 16 VLP antibodies are in higher risk of developing CIN or INCA than women without them.<sup>14–17</sup> Moreover, a relation has been found between the presence of antibody and the severity and prognosis of the disease.<sup>18–21</sup> Furthermore, HPV is capable of immortalising human and rodent cells in vitro and the

expression of the viral oncogenes *E6/E7* is necessary for the malignant phenotype of the cells to be maintained (for review see Stoppler *et al*<sup>22</sup>). Despite all these findings, which substantiate the hypothesis that HPV is a likely aetiological factor in CIN and INCA development, direct evidence from prospective studies is still limited. Prospective studies can be of substantially higher value for the confirmation or disproof of aetiological inferences than case-control (retrospective) studies; they could clearly show whether healthy women infected with HPV were at increased risk of developing cervical neoplasia. However, such studies are expensive and time consuming. Thanks to the improved methods of molecular biology, valuable materials that were collected in prospective studies in the past can now be examined.

We accordingly tested HPV DNA presence in Giemsa stained cervical smears taken at enrolment of women into the 1975–83 prospective study, which had been undertaken to test the hypothesis that HSV-2 was aetiological involved in INCA development. We tested smears collected from subjects displaying cervical lesions at enrolment, from those developing the disease during the subsequent follow up period, and from controls matched with patients by age and by certain factors found to increase the risk of cervical neoplasia development in the population.<sup>1</sup>

The principal aim of our study was to verify whether HPV infection in healthy women, as determined by HPV DNA detection, was associated with an increased risk of development of cervical lesions.

## MATERIALS AND METHODS

### Population studied

The archival cervical smears used in the present study were collected at enrolment of more than 10 000 women aged 25–45 years in a prospective study conducted in Prague during the 1975–83 period.<sup>1</sup> None of these women had previously been treated for cervical neoplasia. At their enrolment, all were examined cytologically and colposcopically, their blood samples were taken, and a detailed and structured questionnaire concerning the medical history, education, socioeconomic status, present health status and lifestyle, including sex and reproduction associated attributes, was completed by each of them. At the enrolment, 93 cases of moderate to severe dysplasia (MSD), 54 cases of carcinoma in situ (CIS), and 15 invasive carcinomas (INCA) were detected histologically. The women with lesions were not included in the prospective part of the study. Healthy subjects were followed by cytology and colposcopy at 2 year intervals—that is, 2 years and 4 years after their enrolment. During the follow up, 57 new cases of MSD, 27 of CIS, and six of INCA were detected. All the biopsy diagnoses were done by a single pathologist.

In the present study, we examined all the samples available—that is, 67 smears from patients in whom cervical neoplasia had been found at their enrolment, and 53 smears from patients in whom cervical neoplasia had been detected in the course of the above prospective study. In addition, 208 smears from control women who remained healthy throughout the observation period were tested. Based on the findings at enrolment, the cases were divided into three groups: group A included 67 women with biopsy proved cervical neoplasia; group B comprised 26 women with borderline or suspicious colposcopic and/or cytological findings at the enrolment; and group C from 27 women with normal findings at enrolment. Of the 67 group A cases, 45 had had MSD, 18 CIS, and four had had INCA at enrolment. Among the 26 group B follow up cases, 17 had developed MSD, eight CIS, and one INCA, and among the 27 group C follow up cases, 18 had developed MSD, six CIS, and three INCA. Controls were matched with the patients (1–3 per patient) by age, number of sexual partners, age at first intercourse, smoking habits, and history of DKG (diathermoelectrocoagulation). This rigorous matching was achieved in 80% patients. There were 147 matched controls for group A, 56 for group B, and 55 for group C. Some controls were used for two or three groups (43 controls for two groups and three controls for all three groups). When data from different groups were combined, each of these controls was represented once only. The archival smears had been processed by Giemsa staining techniques and had been stored at room temperature. All sera had been kept frozen at –20°C.

### DNA extraction

DNA isolation from archival smears was performed using the modified proteinase K lysis method. Smears were overlaid with 100 µl of xylene and scraped off the glass slide with a sterile razor blade into a microtube containing 1 ml xylene. The cells so obtained were incubated at room temperature for 30 minutes and pelleted by centrifugation. The supernatant was removed, the pellet was washed with 1 ml absolute ethanol for 30 minutes, the cells were pelleted again and dried at 55°C for 15 minutes. The pellets were then resuspended in 300 µl lysis buffer (50 mM TRIS-HCl, pH 8; 5 mM EDTA, pH 8; 1% Tween 20, and 100 µg/ml proteinase K (Sigma, St Louis, MO, USA)) and the lysates were kept at 55°C overnight. Proteinase K was inactivated at 95°C for 10 minutes and the samples were stored at –20°C.

### Detection of HPV DNA by PCR

Five µl of the crude lysates were analysed in a PCR thermocycler type PTC 100 (MJ Research, Inc). In the first reaction, primers PC03 and PC04,<sup>23</sup> which amplify a 110 bp β globin

gene fragment, were used to confirm the integrity of DNA in the samples. Each 25 µl reaction mixture contained 10 mM TRIS-HCl, pH 8.5; 50 mM KCl, 4 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.5 pmol primers and 2.5 U Taq polymerase (Promega, Madison, WI, USA). The PCR was performed in 40 cycles; each cycle consisting of 1 minute of denaturation at 95°C, 2 minutes of annealing at 55°C, and 2 minutes of elongation at 72°C. The first cycle was preceded by a 5 minute denaturation step at 95°C, and the last cycle was followed by an incubation at 72°C for 3 minutes. From each amplification reaction, an aliquot of 10 µl was separated electrophoretically on a 3% agarose gel (NuSieve 3:1, FMC BioProduct, Rockland, ME, USA). The bands, when present were stained with ethidium bromide, visualised, and photographed under ultraviolet transillumination.

For HPV detection, the general primers GP5/6, which amplify a 150 bp long fragment in the conserved region of the *L1* gene of HPV, were used.<sup>24</sup> Each 50 µl reaction mixture contained 10 mM TRIS-HCl, pH 8.5; 50 mM KCl, 3.5 mM MgCl<sub>2</sub>, 200 µM each dNTP, 50 pmol primers GP5 and GP6, and 1 U Taq polymerase (Promega). Initial denaturation at 94°C for 5 minutes was followed by 40 cycles, each consisting of 1 minute at 94°C, 2 minutes at 40°C, 1.5 minutes at 72°C, and a final extension at 72°C for 4 minutes. Sterile water and HPV negative DNA of LEP cells, a human lung embryonic fibroblast cell line,<sup>25</sup> were used as negative controls. DNA from SiHa, a human cervical cancer cell line containing one or two copies of the HPV 16 genome per cell,<sup>26</sup> was included in every run as a positive control. An aliquot of 10 µl from each amplification reaction was separated electrophoretically on a 3% agarose gel (NuSieve 3:1, FMC BioProduct). The gels were stained with ethidium bromide, visualised, and photographed under ultraviolet transillumination.

A 5 µl volume of each PCR product was dot blotted on a Biotodyne B nylon membrane (Pall Biosupport). The membranes were neutralised for 15 minutes in 1 M TRIS-HCl, pH 7.4; 1.2 M NaCl and equilibrated in 3X SSC for 10 minutes. DNA was fixed to the membranes at 80°C for 2 hours.

The GP5/6 PCR products were analysed at 50°C overnight by non-radioactive hybridisation with a cocktail of probes. The membranes were saturated with 2X SSC and incubated at 50°C for 2 hours in hybridisation buffer (5X high grade SSC, 5% formamide, 0.1% Ficoll, 0.1% BSA, 50 mM Na<sub>2</sub>HPO<sub>4</sub>·Na<sub>3</sub>PO<sub>4</sub>, 2.5% herring sperm ssDNA, and 0.1% high grade SDS) without probes. The probes were generated by PCR with the use of GP5/6 primers on HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, and HPV-33 cloned DNA with the use of GP5/6 primers. Each PCR mixture contained 10 mM TRIS-HCl, pH 8.5; 50 mM KCl, 3.5 mM MgCl<sub>2</sub>, 200 µM nucleotides dATP, dGTP, dCTP, 134 µM dTTP, 66 µM Dig-11-dUTP (Boehringer Mannheim, Mannheim, Germany), 50 pmol of primers GP5 and GP6 and 2.5 U Taq polymerase (Promega). Hybridisation with 40 ng of each probe was done at 50°C overnight in the hybridisation solution. After hybridisation, the membranes were washed in 0.5X SSC, 0.1% SDS at 55°C three times for 30 minutes. Chemiluminescent detection was done using the DIG system (Boehringer Mannheim) according to the manufacturer's protocol. Briefly, the membranes were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) (Boehringer Mannheim) for 1 minute and incubated in a blocking solution (1% blocking reagent, 100 mM maleic acid, 150 mM NaCl, pH 7.5) (Boehringer Mannheim) for 45 minutes. Anti-Digoxigenin-AP (Boehringer Mannheim) was diluted 1:10 000 in the blocking solution and incubated with the membranes for 30 minutes. The membranes were then washed three times for 15 minutes in washing buffer and equilibrated in detection buffer (100 mM TRIS-HCl, pH 9.5, 100 mM NaCl). Each membrane was incubated with 750 µl of a CSPD ready to use solution (Boehringer Mannheim) for 5 minutes. The membranes were air dried and incubated at 37°C

**Table 1** PCR positivity with primers GP 5/6

Group	Total	PCR with GP 5/6 primers
Patients*	120	54 (45%)
Controls	208	11 (5.3%)
All	328	65 (19.8%)

\*Groups A, B, and C combined.

for 15 minutes. For detection of the chemiluminescent signal, the membranes were exposed to Lumi-Film (Boehringer Mannheim) for 30 minutes and 2 hours.

### Typing of HPV by PCR using type specific primers

Those samples whose GP 5/6 PCR products were positive after hybridisation with the mixture of long probes were submitted to nested PCR. The first step was their amplification with the MY09 and MY11 primers which amplify a 450 bp long fragment.<sup>27</sup> Fifty µl volumes of the reaction mixture contained 10 mM TRIS-HCl, pH 8.5, 50 mM KCl, 4.0 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.5 pmol of both the MY09/11 primer, and 2.5 U Taq polymerase. Initial denaturation at 94°C for 5 minutes was followed by 40 cycles, each consisting of 1 minute at 94°C, 1.5 minutes at 42°C, 1 minute at 72°C, and a final extension step at 72°C for 5 minutes. Volumes of 5 µl of the PCR product were used for the second, the nested PCR. This was performed with the use of primers specific for HPV-11, HPV-16, HPV-18, HPV-31, and HPV-51, which amplify an internal region to MY09/11 primers 410 bp long. The 50 µl volumes used in the second PCR reaction consisted of 10 mM TRIS-HCl, pH 8.5, 50 mM KCl, 4.0 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.5 pmol of each of the type specific primers (HPV-11: RDB 136 and RDB 137; HPV-16: MY74 and MY75; HPV-18: MY76 and MY77; HPV-31: MY49

and MY50; HPV-51: RDB 247 and RDB 248)<sup>28-29</sup> and 2.5 U Taq polymerase. The condition were the same as in the first PCR reaction. Aliquots of 10 µl from the second amplification reaction were separated electrophoretically on a 3% agarose gel (NuSieve 3:1, FMC BioProduct). The gels were stained with ethidium bromide, visualised, and photographed under ultra-violet transillumination.

### Serological tests

Sera from patients and controls taken at enrolment were tested for the presence of HPV specific antibodies with VLPs. The VLPs were prepared in a baculovirus recombinant system expressing L1 of HPV 16, L1 of HPV 18 and both L1 and L2 of HPV 33. The preparation of VLPs and serological detection of HPV specific antibodies were as described previously.<sup>17</sup>

### Statistical analysis

The Fisher exact test and the standard  $\chi^2$  test for trend were used. Odds ratios (OR) with 95% confidence intervals (CI) and two tailed p values were calculated using the GraphPad InStat (version 3.00) (GraphPad Software, San Diego, CA, USA). In all tests, the basic significance level was  $p = 0.05$ .

### RESULTS

In the present study, the total of 370 archival cervical smears were selected for HPV DNA presence testing by means of PCR. Forty two samples (11%)—namely, 31/239 (13%) controls and 11/131 (8%) cases were not amplified by the primers for internal control ( $\beta$  globin gene) and were therefore excluded from the study. Altogether, 328 samples were tested for the presence of HPV DNA; of these, 120 originated from patients and 208 from control women. With a single exception, all samples that were declared HPV DNA positive were only found to be so after hybridisation (see Materials and methods), which, in general,

**Table 2** HPV types detected in cervical smears

Group	Total	HPV					One type	Two types*	Three types†
		11	16	18	31	51			
Patients	28	0	21 (75%)	5 (17.9%)	13 (46.4%)	1 (3.6%)	18 (64.3%)	8 (28.6%)	2 (7.1%)
Controls	2	0	2 (100%)	0	1 (50%)	0	1 (50%)	1 (50%)	0
All	30	0	23 (76.7%)	5 (16.7%)	14 (46.7%)	1 (3.3%)	19 (63.3%)	9 (30%)	2 (6.7%)

\*HPV 16 + 18, once; HPV 16 + 31, 7 times; HPV 16 + 51, once.

†HPV 16 + 18 + 31, twice.

**Table 3** Presence of HPV DNA in women with cervical neoplasia at enrolment, in those who developed the disease in the course of subsequent follow up, and in matched control women

Group	Diagnosis	Total	HPV DNA positive	OR	CI	p Value
A	Cases	67	32 (47.8%)	18.3	7.4 to 44.9	<0.0001
	Controls	147	7 (4.8%)			
B*	Cases	26	12 (46.2%)	97.4	5.4 to 1745.3	<0.0001
	Controls	56	0 (0%)			
C†	Cases	27	10 (37.0%)	7.5	2.1 to 27.1	<0.01
	Controls	55	4 (7.3%)			
B+C‡	Cases	53	22 (41.5%)	18.5	5.9 to 57.6	<0.0001
	Controls	108§	4 (3.7%)			

OR = odds ratio; CI = confidence interval.

Group A = women with cervical neoplasia at enrolment (mean age 35 years); group B = women with slightly suspicious cytological and/or colposcopic findings at enrolment (mean age 34 years); group C = women with normal cytological and colposcopic findings at enrolment (mean age 34 years).

Control subjects were matched with the patients by age, age at first intercourse, number of sexual partners, and smoking habit. One to three controls were matched with each patient.

\*Relative risk (RR) = not done due to zero value of HPV DNA positive controls.

†RR = 5.0 (CI 1.8 to 14.8).

‡RR = 11.2 (CI 4.1 to 30.9).

§Those controls which were used with both groups B and C have been included only once in the row for groups B+C combined.

**Table 4** Relation between presence of HPV DNA and severity of disease in women with cervical neoplasia at enrolment and those who developed the disease in the course of subsequent follow up

Group	HPV DNA	Histological diagnosis			p for trend
		MSD	CIS	INCA	
A	Positive	17/45 (38%)	12/18 (67%)	3/4 (75%)	<0.05
B+C	Positive	16/35 (46%)	5/14 (36%)	1/4 (25%)	NS

MSD = moderate to severe dysplasia; CIS = carcinoma in situ; INCA = invasive carcinoma; for other abbreviations, see  
NS = not significant.

**Table 5** Relation between presence of antibody and HPV DNA

HPV DNA	Total	Antibody presence			
		HPV-16	HPV-18	HPV-33	Any
Positive	65	12 (18.5%)	13 (20%)	13 (20%)	21 (32.3%)
Negative	259	24 (9.3%)	25 (9.7%)	22 (8.5%)	42 (16.2%)
OR		2.2	2.3	2.7	2.5
CI		1.0 to 4.7	1.1 to 4.9	1.3 to 5.7	1.3 to 4.6
p Value		<0.05	<0.05	<0.05	<0.01

OR = odds ratio; CI = confidence interval.

Any = samples positive in ELISA test with any of the VLPs used (VLP-16, -18, -33).

There was no statistically significant difference in the presence of HPV DNA and HPV specific antibodies between the groups of cases and controls.

**Table 6** Relation between presence of antibody presence and HPV DNA in different groups of patients

Patient group	HPV DNA positive	Antibody presence			
		HPV-16	HPV-18	HPV-33	Any
A	32	8 (25%)	9 (28%)	10 (31%)	15 (47%)
B+C	22	3 (14%)	4 (14%)	1 (5%)	4 (18%)
OR		2.1	1.8	9.6	4.0
CI (95%)		0.5 to 9.1	0.5 to 6.7	1.1 to 81.2	1.1 to 14.4
p Value		0.4935	0.5234	<0.05	<0.05

For explanation of abbreviations see Table 5.

increases the sensitivity of HPV detection by a factor of 10.<sup>30</sup> At variance with these results, the  $\beta$  globin band, which is amplified from a gene present in all cells, was visible already on ethidium bromide stained gels in all instances. This suggests that the number of HPV infected cells in our samples was low.

A summary of the data is shown in table 1. In our cohort, 65 samples (19.8%) were HPV DNA positive. The positives were not equally distributed among the patients and control subjects: HPV DNA was detected in 54/120 (45%) of patients, while only 11/208 (5%) samples from control women were positive. This difference was statistically highly significant ( $p < 0.001$ ). In 30 of the 65 HPV DNA positive samples it was possible to amplify HPV DNA for the typing test (see Materials and methods) and reveal the type of HPV. As can be seen in table 2, 19/30 samples (63%) contained only one type of HPV, while in the remaining 11 the simultaneous presence of two or three HPV types was found. The most prevalent type was HPV 16, followed by HPV 31, HPV 18, and HPV 51. In the doubly infected, the combination of types 16 and 31 was the most frequent.

The prevalence of HPV DNA in the three groups of patients—that is, those with lesions at enrolment (group A) and those developing the disease in the course of the observation period (groups B and C), as well as in the corresponding controls, is shown in table 3. In all groups there was a statistically highly significant difference between the prevalence of HPV DNA in patients and matched control women, which

clearly indicated that the cervical HPV infection at enrolment was associated with a markedly increased risk of subsequent development of MSD or a more serious condition. When groups B and C were taken together, the relative risk of developing cervical neoplasia in HPV infected women was 11.2.

As indicated in table 4, in group A patients HPV DNA prevalence increased with severity of the disease. This was not observed in group B and C patients.

Serum samples from all 324 subjects studied were available for correlation of the presence of HPV DNA and HPV specific antibodies at enrolment. As table 5 shows, HPV DNA positive women possessed HPV antibodies against all three types tested significantly more frequently than did HPV DNA free subjects. There was no significant difference in the seroreactivity of HPV DNA positive patients and controls (results not shown).

Finally, an analysis was made to determine whether there was any difference in antibody prevalence between HPV DNA positive subjects found ill at enrolment (group A patients) and those who developed the disease later in the course of the observation period (group B and C patients). The results are presented in table 6. The data indicate that in all instances antibodies were encountered more frequently in HPV DNA positive group A patients than in groups B and C patients, although this difference was statistically significant only in the case of HPV 33 antibody or when antibodies against all three HPV types were taken together.

## DISCUSSION

In the present study, DNA was extracted from 370 Giemsa stained cervical smears that had been stored for nearly 25 years at room temperature. In spite of these materials not having originally been collected for future molecular assays, and despite their age and suboptimal conditions of their storage, the  $\beta$  globin gene used as an internal control was successfully amplified in 88% of the cervical smears. Only these smears were subsequently tested for HPV DNA presence.

The GP5/6 primers, which were repeatedly proved to be very sensitive and had provided highly reproducible results in many large scale epidemiological studies<sup>31–32</sup> were employed for HPV DNA detection in the present investigation. According to the results obtained, the prevalence of HPV DNA in women with CIS and INCA diagnosed at their enrolment into a large scale prospective research carried out in 1975–83 (see Introduction) was lower than in other recently reported studies.<sup>3</sup> Since we used primers for the *L1* region, we speculated that some false negative results might have been obtained owing to an interruption or rearrangement of the *L1* gene as a result of the integration of viral DNA into the host cell genome. In additional experiments, primers specific for the *E6* gene,<sup>33</sup> which is always preserved intact even when integration occurs, were accordingly used.<sup>34–36</sup> In general, this assay system proved less sensitive for detecting HPV DNA in the samples tested, and very few additional positive samples were detected (results not shown). Therefore we believe that the lower prevalence of HPV DNA detected in our cell smears that had been taken long ago from women with cervical neoplasia could largely be attributed to a limited number of cells recovered from the cervical smears, and, as mentioned above, to their long storage under suboptimal condition. This assumption is further supported by the fact that HPV DNA, if found to be present, was nearly always detected only by hybridisation tests with the PCR products, which is known to increase the sensitivity of the assay 10-fold.<sup>30</sup>

Overall, women with cervical neoplasias diagnosed at enrolment or subsequently developing the disease were more often HPV DNA positive than control women (45% versus 5.3%). More importantly, the differences were nearly as marked in those found ill at enrolment as in those who developed the disease in the course of the subsequent follow up. Thus, the present data indicate that the presence of HPV DNA in healthy women increased more than 11-fold the risk of subsequent development of cervical neoplasia. These results provide further support for the involvement of HPV in the pathogenesis of this disease. Recently, the first large scale prospective study has yielded similar results.<sup>8</sup> In that undertaking, however, control women were matched with patients by age and time of follow up, without taking into consideration sexual life associated attributes as in our study. In spite of this, the results of the two studies are not essentially different. Liaw *et al.*<sup>8</sup> have demonstrated that women with normal cytological findings who are HPV DNA positive were at 3.8-fold higher risk of developing low grade SIL and at 12.7-fold higher risk of developing high grade SIL. In the present study, HPV DNA positivity increased the risk of MSD development 12.3 times and the risk of CIS or INCA development 9.6 times. The somewhat lower risk associated with demonstrable HPV infection as determined in the present study might be due to the tighter matching, but could also have been influenced by the above mentioned shortcomings of the present material.

Another point of interest concerns the relation between HPV DNA positivity and severity of the disease. In our group A patients—that is, those found with neoplasia at enrolment, the prevalence of HPV DNA increased with severity of the disease, which is in agreement with earlier reports.<sup>13–37</sup> This was not observed in the group B and C patients, who were free of the disease at the enrolment but developed it in the course of the observation period. Assuming that the HPV DNA positiv-

ity in our assay reflected its original quantity, one could suggest that the risk of developing the more serious condition—that is, CIS and INCA, was not dependent on the virus load at the time of sampling. Still, because of the small number of patients and rather exclusive nature of the materials examined, the present observation should be interpreted with much caution.

There was a strong correlation between the presence of HPV DNA and the presence of antibodies reactive with HPV VLPs. This is not particularly surprising. It is noteworthy, however, that the occurrence of antibodies in HPV DNA positive patients was much more frequent in group A patients than in those who developed the disease in the course of the follow up period. This seems to be in line with a previous observation of ours that the development of the disease in originally healthy subjects is associated with HPV antibody increase,<sup>17</sup> as well as with earlier evidence of delayed antibody development in HPV infected subjects.<sup>17–38</sup>

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