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Polymerase chain reaction-assisted papillomavirus detection in cervicovaginal smears: stratification by clinical risk and cytology reports

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Abstract Seven hundred and twelve patients from cancer screening, pregnancy care, outpatient clinics for patients at risk for cervical dysplasia and human immunodeficiency virus (HIV) infection were tested simultaneously for cytological aberrations and human papillomavirus (HPV). Classification of these cases, and of all cytology records throughout 1991 and 1992 was performed according to the "Münchner Nomenklatur" and the Bethesda classification. HPV-directed polymerase chain reaction analysis was carried out with general primers, patients at risk for cervical dysplasia were tested by subsequent hybridization with HPV 16 and 18 probes. Patients from cancer screening and pregnancy care showed similar HPV prevalences ranging between 19.4%–24.6%. In contrast, patients from dysplasia and HIV units were infected in 56.2%–62.3% and 75.0%–76.9% respectively in centre of disease control stage III–IV. HPV detection rates in patients from dysplasia and HIV units increased gradually from 40.1%–52.9% in non-suspicious smears to 80.8%–100% in atypical smears. High risk HPV 16 and 18 infections were detected in 64% of smears with cytological evidence of HPV infection (koilocytosis) to 84.2% in severe dysplasia. Following the Bethesda guidelines, 2.9%–14.7% of all smears initially reported as Pap 2 K (suggestive of HPV infection) would be qualified as risk lesions (low grade squamous intraepithelial lesions), although they tested HPV negative in more than a third of cases. Thus, when using the Bethesda system, HPV analysis is needed to prevent overclassification and overtreatment. The "Münchner Nomenklatur" avoids this dilemma by not mixing morphological statements on infection, atypia and cancer risk.

Key words HPV · HIV · Dysplasia · Koilocytosis
Bethesda classification

Dedicated to Prof. Dr. H.-E. Stegner on behalf of his 65th birthday.

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Introduction

Experimental and clinical studies have shown a major role for human papillomavirus (HPV) infections of the genital mucosa in the aetiology of precancer and cancer of the cervix [24, 55]. High risk HPVs, especially HPV16 and 18 are much commoner than low risk HPVs in cancer and in high grade dysplasia [24, 37, 55]. Limited studies suggest that at least 10% of women with cervical HPV infection will develop dysplasia within 1–2 years [38]. In a screening program of college women 18–20 years of age at the University of Washington, 29–40% of HPV positive women initially recruited with normal cytological reports developed dysplasia within 1–2 years, relative risks for dysplasia evolution being calculated as 4.6 for HPVs 16 and 18 and 1.4 for HPVs 6 and 11 [27, 29]. In follow up studies of women with abnormal smears and/or colposcopy, the relative risk was 5.2 for the presence of high risk HPVs with subsequent progression from low grade to high grade dysplasia [15].

Unfortunately, the value of Pap tests in detecting HPV associated cytopathic effects and lesions, especially early lesions, is limited by several factors, among which the most important are sampling and screening errors [14, 17, 18, 28]. Over the last few years, a more clinically based cytological classification system of cervicovaginal smears has evolved, leading to the Bethesda proposal by the National Cancer Institute in 1988 [4, 39, 52, 54]. One of the most important and controversial aspects of this proposal was the introduction of two groups of cervical lesions instead of three or four subcategories. These groups were called squamous intraepithelial lesions (SIL) of low and high grade. Low grade SIL comprises HPV associated cytopathic effects (notably ko-

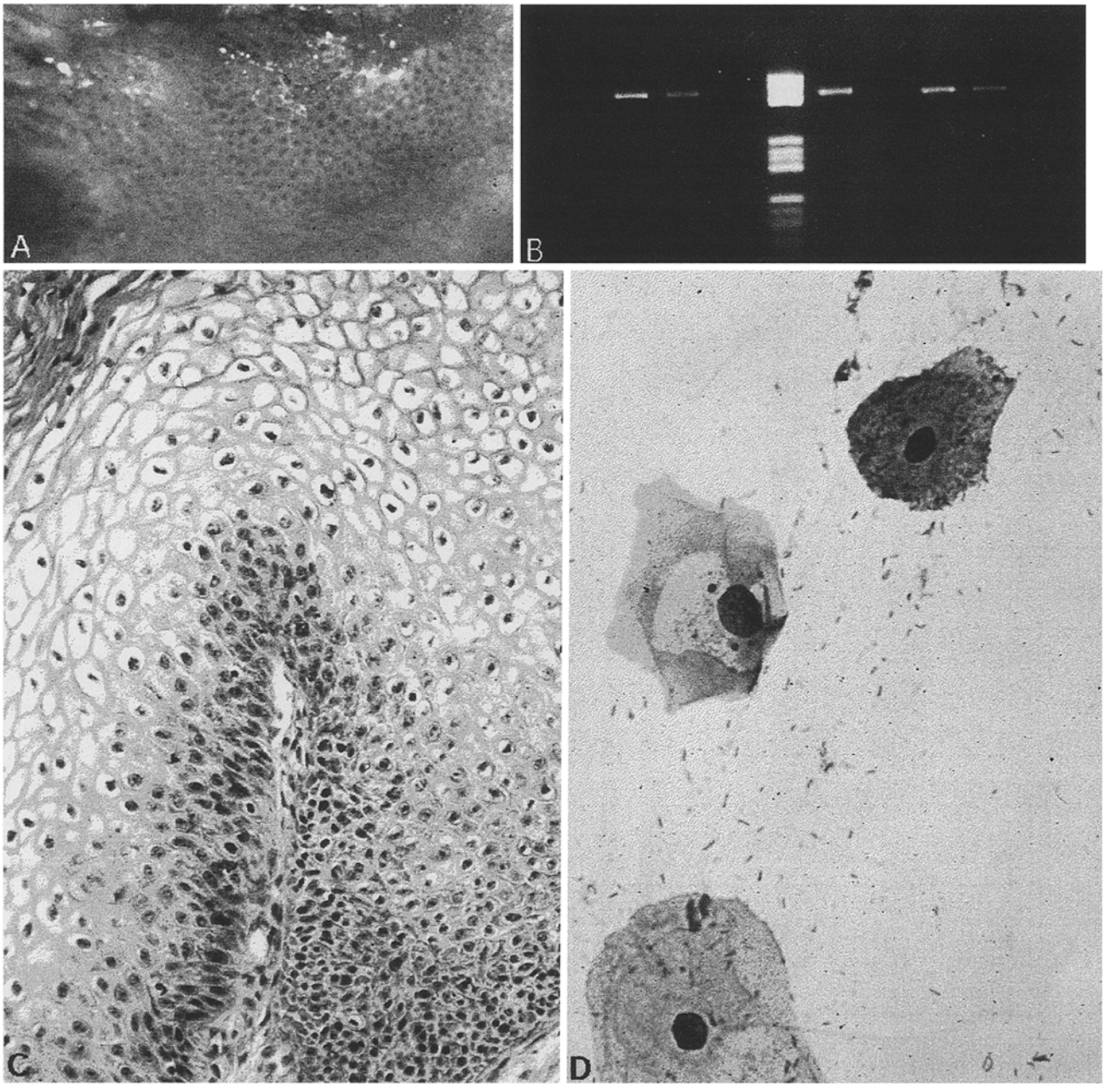

Fig. 1 **A** Colposcopic view of suspicious mucosa (mosaic pattern). **B** Agarose gel showing DNA bands amplified with general primers from different virus infected cell suspensions (centre of figure: molecular weight marker). **C** Koilocytic lesion. Haematoxylin and eosin, ×400. **D** Pap 2K: typical koilocytosis of intermediate keratinocyte. Papanicolaou stain, ×600

ilocytosis) without atypia, and mild dysplasia; high grade SIL includes moderate and severe dysplasia/carcinoma in situ.

HPV testing is increasingly carried out to identify latent and to recognize subclinical HPV associated lesions [23, 32, 37]. The most efficient and convenient method for detecting latent and subclinical HPV infection is the polymerase chain reaction (PCR) [5, 6, 7, 35, 36]. In order to assay HPV infection in patients at different clinical risk at our institution, detection was carried out by general primer (GP) targeting to recognize all relevant genital HPV infections (Fig. 1). At the same time Pap smears were obtained and screened according to the "Münchner Nomenklatur" [51] and the Bethesda system [39]. Patient groups included women under regular cancer screening, in ante-natal care, attending special outpatient units for diagnosis and treatment of dysplasia, and human immunodeficiency virus (HIV)-infected patients. This investigation was directed to assess HPV infection in patients at different clinical risk for dysplasia and HPV infection in patients with abnormal smears as classified by the "Münchner Nomenklatur" and the Bethesda classification.

Table 1 Human papillomavirus (HPV) infection in patients at different risk for dysplasia (n=712; *HIV* human immunodeficiency virus)

Group of patients	Mean age (years)	Number	HPV-positive (%)
A: routine cancer screening	39.5	65	16 (24.6)
B: pregnancy care unit	29.3	36	7 (19.4)
C: outpatients at risk for dysplasia	33.8	550	309 (56.2)
D: HIV-infected women	32.9	61	38 (62.3)

Table 2 Correlation of cytology reports and HPV analysis [*GP* general primers, *ND* not done, *Pap 1/2* non-suspicious for dysplasia, *Pap 2K* cytological signs of HPV infection=squamous intraepithelial lesions (SIL) low grade subgroup 1, *Pap 3D* indicative of mild dysplasia=SIL low grade subgroup 2 *Pap 4A* indicative of severe dysplasia and/or carcinoma in situ

Group		Pap	Number	(%)	HPV-GP positive	(%)	HPV 16/18	(%)[a]
A and B:	routine cancer screening (n=101)	1/2	101	100	23	22.8	ND	
		2K-4A	0					
C:	high-risk patients (n=550)	1/2	287	52.2	115	40.1	ND	
		2K	81	14.7	50	61.7	32	64.0
		3D	108	19.6	85	78.7	56	65.9
		4A[b]	74	13.5	59	79.7	48	81.4
		4A[c]	47	8.5	38	80.8	32	84.2
D:	HIV positive patients (n=61)	1/2	34	55.8	18	52.9	ND	
		2K	8	13.1	5	62.5	ND	
		3D	14	22.9	10	71.4	ND	
		4A	5	8.2	5	100.00	ND	

[a] HPV 16/18 positives out of HPV-GP positive cases
[b] SIL high grade subgroup 1=moderate dysplasia and subgroup 2=severe dysplasia/carcinoma in situ
[c] proportion of severe dysplasia/carcinoma in situ in[b]

Materials and methods

Patients were enrolled from routine cancer screening programs (patients from resident gynaecologists and outpatients of the university hospital admitted for various gynecological disorders), the hospital pregnancy care unit, and the Eppendorf outpatient clinic for women at risk for dysplasia (patients admitted for confirmation of diagnosis and treatment; women during follow-up after conization or laser therapy of cervical lesions; partners of male patients with HPV-associated genital lesions; women with recurrent vulvar condylomatous or bowenoid lesions). Others were seen at the Finkenau outpatient clinic for patients with HIV infection (see Table 1).

From January 1991 to December 1992, 10,227 cervical smears were evaluated according to the "Münchner Nomenklatur" and the Bethesda system by at least two observers. All cases with dysplasia/SIL were reevaluated by another cytopathologist at the end of the study period (for numbers see Tables 2 and 4). Only cells with one or more hyperchromatic and distorted nuclei and irregular perinuclear halos were regarded as koilocytes. Morphological effects suggestive of other infections (*Trichomonas, Gardnerella*) were distinguished from HPV associated cytopathic reactions as accurately as possible.

In 712 patients, material was analysed simultaneously for HPV infection. Cytological specimens were taken with two cotton swabs: one was used for Papanicolaou staining and cytological diagnosis, the other was transferred to 3 ml 0.9% sodium chloride and stored at −70° C before HPV detection.

For PCR cells were sedimented by centrifugation and proteins were digested in 100 µl PCR buffer containing 0.5% Tween 20 and proteinase K (200 µg/ml) for 1 h at 55° C. The PCR was performed as described previously [23], 5 µl of the mixture was used for priming with consensus oligonucleotides MY09 and MY11 (Perkin Elmer Cetus) [35], which amplify a 450 bp-segment of the *L1* gene of all relevant genital types. After amplification, 15 µl of the reaction mixture was analysed by electrophoresis in a 4% agarose gel containing 10 µg/ml ethidium bromide. Subsequently, hybridization with a HPV 16/18 probe mixture (WD 74 and My 14) [35] was carried out at 52° C in a cocktail containing 5×standard saline citrate, 0.1% sodium dodecyl sulphate, 5×Denhardt's solution, 0.1 mg/ml sheared denatured salmon sperm DNA and 1 pmol/ml of digoxigenin-labelled probe. Hybrid detection was performed using the Dig nucleic acid detection system (Boehringer, Mannheim). Presence and integrity of DNA was tested by amplification of a 268 bp human β-globin gene fragment (primers: GH20/PCO4; Perkin Elmer Cetus), HPV-negative cases without visible amplification products in agarose gels were excluded.

For assessing the significance of differences of HPV results, statistical analysis was carried out using the χ^2-test.

Results

A total of 712 women were screened simultaneously for cervical dysplasia and HPV infection. Outpatients from cancer screening programs and pregnancy care unit (mean age 39.5 and 29.3 years respectively) showed HPV prevalences ranging between 19.4%–24.6% (groups A and B, Table 1, non-significant difference). Patients from dysplasia and HIV units (groups C and D, mean age 32.9 and 33.8 years respectively) were infected in 56.2% and 62.3% of cases respectively (cumulative data, Table 1, difference between C and D not significant). Results from groups A and B together, however, were significantly different from either group C or D (P<0.001). HPV detection rates in group C and D increased gradually from 40.1%–52.9% in non-suspicious smears (Pap 1/2) to 80.8%–100% in highly atypical

smears (Pap 4A, Table 2, p<0.001). Most of the 40.1%–62.5% of HPV positive cases in groups C and D with smears without nuclear atypia (Pap 1/2 and Pap 2K) had a history of HPV infection or partner(s) with signs of HPV infections (data not shown). In group D, HPV infection rate increased strikingly from 44.4% in centre of disease control (CDC) stage II (asymptomatic HIV infection) to 75% in CDC III (persistent generalized lymphadenopathy) and 76.9% in CDC IV (AIDS with secondary disease; clinical data available from 38 patients, Table 3).

In correlating cytology and HPV infection 10,227 cervicovaginal smears were (re-) evaluated over 2 years (Table 4). Reports included the grading scheme of the "Münchner Nomenklatur" and the Bethesda classification (for numbers see Table 4). 9498 smears (92.9%) were classified as non-suspicious (Pap 1/2). When looking at selected groups and our cytological survey (Tables 2 and 4), high rates of dysplasia in groups C and D (19.6%–22.9% Pap 3D and 8.2%–8.5% Pap 4A) contrasted with general screening results (3.1% Pap 3D and 0.9% Pap 4A). Mild dysplasia/low grade SIL was diagnosed in 1.9% and high grade SIL (moderate and severe dysplasia) in 2.1% of cases in this 2 year survey. Within the Pap 3D group of the "Münchner Nomenklatur", approximately two-thirds of cases fell into the mild dysplasia/low grade SIL category (1.9%), the minor part into the moderate dysplasia group (1.2%). The latter were consequently reported as high grade SIL, together with Pap 4A cases. Cytological aberrations suggestive of HPV infection (Pap 2K) appeared in 13.1%–14.7% of patients in groups C and D compared with only 2.9% in the general cytological review (Tables 2 and 4).

Among 550 patients in group C (Tables 1 and 2), 81 women (14.7%) were cytologically suspicious of HPV infection, without evidence of atypia, corresponding to Pap IIK; low grade SIL of the Bethesda system. When analysed for HPV infection, only 61.7% were positive for HPV. Within the mild dysplasia/low grade SIL group (n=108; 19.6%), 78.7% were positive for HPV. In 74 women (13.5%), moderate or severe dysplasia was reported corresponding to high grade SIL, the HPV infection rate was 79.7%. When looking at Pap 4A alone within high grade SIL (n=47; 8.5%), the HPV infection rate was 80.8%. Differences in HPV infection rates between non-suspicious smears and either low grade SIL or high grade SIL were highly significant (P<0.001), those between subgroups were not. The rate of high risk HPV 16 and 18 infections increased from 64% in Pap 2K to 84.2% in Pap 4A cases (Table 2, P<0.01).

In group D, the HPV infection rate of non-suspicious smears was even more frequent than in group C (52.9%). As in group C, less than than two-thirds of cases reported as Pap 2K were positive for HPV (62.5%, Table 2). In dysplasia, a similar trend appeared with a gradual increase in HPV infection rate towards high grade SIL. All cases diagnosed as Pap 4A harboured HPV DNA.

Table 3 HPV in HIV-infected women: correlation to centre of disease control (CDC)-stage (n=38)

CDC-stage	Number	HPV positive (%)
I	0	0
II	9	4 (44.4)
III	16	12 (75.0)
IV	13	10 (76.9)

Discussion

Recognition and treatment of cervical dysplasia is mainly guided by the clinician's interpretation of colposcopic aberrations and the cytologists's report. Potential failures of cytology, however, are now increasingly attracting scientific discussion and public concern [28]. In the USA, the answer to this issue is rigorous quality control [8] and a new classification, the Bethesda proposal, which changes the Pap classification for dysplasias into a two-tier reporting system (low and high grade SIL), and includes HPV-associated cytopathic effects into the low grade SIL category [39]. We stratified our patients for clinical risk and degree of dysplasia, and for HPV. In risk groups, we asked for the predictive value of simply reporting cytomorphological signs of HPV infection by comparison with PCR-assisted HPV analysis. From this, answers on the value of the Bethesda system, and the need for additional HPV testing were expected.

Our investigation was done primarily by GP-primer targeting. Typing by PCR is laborious and expensive and virus heterogeneity is high in latent infections and low grade lesions [13, 21, 24, 25, 33]. Low risk HPV 6 and 11 infections account for less than 10% of infections [10, 37]. Although type-specific PCR was carried out by ourselves for HPVs 16/18 in group C, results do not influence clinical practice as yet. The widely accepted rationale is to follow up patients with any latent HPV infections at yearly intervals, as usual, and regardless of typ-

Table 4 Cervical cytology records 1991–1992 (n=10227)

"Münchner nomenklatur"	Number	(%)	Bethesda system
Pap 1/2	9498	92.9	Non-suspicious
Pap 2K	295	2.9	Suggestive of HPV low grade SIL
Pap 3D (mild dysplasia)	190	1.9	Low grade SIL
Pap 3D (moderate dysplasia)	125	1.2	High grade SIL
Pap 4A (severe dysplasia)	97	0.9	High grade SIL
Pap 5	22	0.2	Carcinoma

ing, to treat patients with subclinical/clinical infections according to the findings of colposcopy, cervical abrasion, and cytology.

Our observation of latent HPV in 22.8% of patients without clinical risk and with non-suspicious cytology was similar to the results of other authors [3: 16.8%; 26, 27: 20%; 30: 16%, 43: 18.1%; 49: 14.3–33%; 53: 14.2%]. However, HPV detection rates in screening may range from 6% (in older women 33–55 years of age) [5] to near 50% in young females (18–20 years) [31: 46%; 29: 32%]. The vast majority of our patients belonged to the first age group. Besides age, factors influencing HPV prevalences are sexual activity, and immunosuppression [2, 16, 20, 26, 31, 47]. Interestingly, our study did not show higher HPV infection rates in pregnant women as reported by other authors [42, 47, 48]. Increased HPV infection rates during pregnancy were not found in all studies [22, 41] and results may be biased by selection of the population under study. Submorphological HPV infection was very frequent in the presence of HIV infection (52.9%), and even more in diseased patients (CDC III-IV: 75%–76.9%). There is also a clear association of the frequency of cervical dysplasias with HIV infection, its clinical stage and with the impairment of lymphocyte function [9, 44]. HIV patients had a three-ten fold higher risk of developing dysplasia than HIV-negative controls with otherwise similar risk factors [1, 9, 12, 20, 34]. In our group D of HIV infected patients, 44.2% of the patients had abnormal smears [44: 41%]. Dependent on the degree of cytological aberration, 62.5%–100% were HPV-positive. Interestingly, however, invasive cancers are rarely seen in Western countries [1, 12, 44]; none of the HIV infected women at the HIV outpatient unit of the Finkenau Hospital has developed invasive cervical carcinoma. When comparing the risk of other virus associated tumours (Epstein-Barr virus-associated lymphomas), the influence of immunosuppression on the development of cervical neoplasia seems to be weak. Strategies on how HPV may escape immune surveillance were recently reviewed by Frazer and Tindle [11].

The most intensively studied patients derived from group C. Patients of this group were at risk of developing primary or recurrent HPV-associated disease. Correspondingly, the HPV detection rate was high, 40.1% of cases with non-suspicious cytology were HPV-positive reflecting a high proportion of latent infections.

In order to better understand the implications of the Bethesda System, we reviewed all our cytology reports over the last 2 years. This review revealed abnormal smears in 7.1%, in contrast to 44.2%–47.8% in patients at risk for dysplasia or with HIV infection. In spite of the selection of patients at our institution, numbers of Pap 4A reports are comparable to the recently presented Copenhagen population screening study [30], detecting abnormal smears in 3% of 11088 women, one third were classified as ASQUS (atypical squamous epithelial cells of undetermined significance), low grade, and high grade SIL. However, when including moderate and severe dysplasia (1.2% and 0.9%), our results out-numbered screening reports. The frequency of low grade SIL was particularly higher (4.8%), and of course exceedingly high when looking at groups C and D separately (low grade SIL; Pap 2K & Pap 3D-mild dysplasia: 34.3%–36%). While only 2.9% of all Pap records were morphologically suggestive of HPV infection (Pap 2K), 13.1%–14.7% of cytology reports in groups C and D were grouped into this category. These results are in line with other sexually transmitted disease clinic population studies [38]. Following the Bethesda guidelines, these 2.9%–14.7% of cytology reports with cytologic evidence of HPV infection had to be taken together with mild dysplasia as risk lesions.

In our groups C and D, most dysplasias were HPV positive (71.4%–100%), most of them hybridized with the HPV 16/18 cocktail (65.9%–84.2%; see also [30]: low grade SIL: 68% HPV 16; high grade SIL: 79% HPV16). However, 37.5%–38.3% of low grade SIL, subcategory HPV-associated cytopathic effects without atypia (Pap 2K), were HPV negative. Recent PCR investigations of cervical biopsies with morphological signs of HPV were negative in two-thirds of cases [50]. Hence, koilocytosis as a marker of HPV infection is questionable unless confirmed by molecular analysis (PCR, hybridization). This diagnostic window is also known from in situ and Southern blot investigations [19, 33, 45, 46] showing Pap 2K and/or "borderline atypia" to be positive in 24%–50% and low grade SIL in 60%–70%. Oncogenic HPV infections, however, may correlate with diagnostic Pap reports at higher rates [40].

While mild dysplasia still indicates a cancer risk independent of HPV analysis, the cytological report "cellular changes associated with HPV" – which tests negative for HPV – is simply a false positive and needs to be grouped into the Pap 1/2 category. The untested report of low grade SIL will inevitably produce a psychological trauma to the patient and influence the clinical management. In this regard, the Bethesda system encourages misclassification and overtreatment, which the "Münchner Nomenklatur" circumvents by not mixing up morphological statements about infection, atypia and cancer risk.

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