Pergamon

European Journal of Cancer, Vol. 34, No. 8, pp. 1230–1234, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00131-2

Original Paper

Risk Factors for HPV Detection in Archival Pap Smears. A Population-based Study from Greenland and Denmark

E.I. Svare,[1] S.K. Kjaer,[1] H.L. Smits,[3] P. Poll,[2] S.P. Tjong-A-Hung[3] and J. ter Schegget[3]

[1]Danish Cancer Society, Institute of Cancer Epidemiology, Strandboulevarden 49, Box 839, DK-2100, Copenhagen; [2]Department of Pathology, Nykøbing Falster Hospital, Denmark; and [3]Department of Virology, Academic Medical Centre, University of Amsterdam, The Netherlands

The most important risk factor for cervical cancer is genital infection with certain types of human papillomavirus (HPV). The presence of HPV was studied in archival smears from a random sample of women living in Greenland (GW) and Denmark (DW) having, respectively, a high risk and an intermediate risk for cervical cancer. Risk factors were also examined of the original 126 Danish and 129 Greenlandic archived smears collected during October and November 1988. 125 were located from each country including all abnormal smears. HPV DNA was isolated from the smears and detected by means of a consensus polymerase chain reaction (PCR) detecting a broad spectrum of genital HPV types. HPV was detected in all the abnormal smears and in 22 and 33% respectively of the cytological normal smears from DW and GW. Risk of HPV was significantly higher in smears from women who started sexual life relatively recently (respectively, ≤ 4 and ≤ 6 years ago in DW and GW) compared with ≥ 10 years ago (adjusted prevalence-OR: 9.3; 95% CI:2.2–39.2 in DW and 5.9; 95 % CI: 1.4–25.3 in GW). Among other important risk factors were age in both areas, lifetime number of sex partners and current smoking in DW and ever had gonorrhoea in GW. This study confirms the usefulness of the method as all abnormal smears were positive and, furthermore, the predictors for HPV presence in the normal smears corroborate with those found in recent studies of HPV in fresh cervical swabs. Thus, this method can be useful for large-scale epidemiological studies of HPV DNA in already sampled material. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: human papillomavirus (HPV), epidemiology, cervical cancer, population based study, random sample, general population, women, archival smears, polymerase chain reaction (PCR), risk factors
Eur J Cancer, Vol. 34, No. 8, pp. 1230–1234, 1998

INTRODUCTION

GENITAL INFECTION with certain types of human papillomavirus (HPV) is well recognised as the major risk factor for cervical neoplasia. Recently, it has become possible to detect the HPV genome by means of polymerase chain reaction (PCR) in DNA extracted from pap smears, which have been fixed, stained and archived for up to several years [1–3].

In fresh cervical swabs, an increased prevalence of HPV has been found with increasing severity of intra-epithelial lesions [4, 5]. Correspondingly, HPV has more frequently been found in archival smears with cytological changes than in archival smears with normal cytology [2]. Several investigations have studied risk factors for HPV DNA as detected with PCR in fresh cervical swabs from women with normal cytology [6–12]. Among the most consistent findings of these studies are an increased risk of HPV detection in women with multiple partners and a decreased risk with increasing age. Less consistently, an association has been found with other variables such as parity, oral contraceptive use and smoking.

Studies correlating presence of HPV in archival smears to relevant epidemiological data have not previously been reported. This study compares risk factors for HPV detection in archival smears from women randomly sampled from the general population in two countries, i.e. Greenland and Denmark. These two geographical areas were chosen because of a substantial difference in cervical cancer incidence which is, respectively, 45.8 per 100 000 in 1984–1988 in Greenland [13] and 16.4 per 100 000 in 1988 [14] in Denmark.

Correspondence to S.K. Kjaer.
Received 13 Oct. 1997; revised 9 Mar. 1998; accepted 20 Mar. 1998.

MATERIALS AND METHODS

Data collection

Recruitment procedure and examination of the participants has been described in detail elsewhere [15]. In brief, a random sample of 150 Greenlandic (GW) and 150 Danish women (DW) aged 20–39 years was drawn from the computerised Danish Central Person Register. In contrast to the Danish population which is essentially caucasian, the Greenlandic population is of Inuit origin with approximately 25–39% admixture of caucasian [16]. Of the eligible women, 126/144 (88%) DW and 129/133 (97%) GW were included from October to November 1988.

From each participant, material for HPV detection had been sampled using two cotton-tipped swabs and a routine pap smear had been taken by means of an Ayers spatula and a cytobrush from the cervical surface and the endocervical canal, respectively. The smears had been alcohol fixed and included in the normal routine procedures of the respective pathological departments. The archival smears for the study were located. We succeeded in collecting all abnormal smears (6 from Denmark and 7 from Greenland) and, respectively, 119 (99%) and 118 (97%) of the normal smears from Denmark and Greenland. In both areas, the smears had been stored 4 years before HPV DNA detection was done. Information on demographical and social background, reproductive history, contraception use, sexual habits, sexually transmitted diseases (STDs) and smoking was collected in a personal interview using a standardised questionnaire.

Preparation of DNA from archival cervical smears

All smears were marked with a random number leaving it impossible for the laboratory staff to distinguish between cytological diagnosis and geographical origin. DNA was prepared from the fixed and stained smears essentially as described by Smits and colleagues [1]. The slides were placed in xylene in individual containers to remove cover slips and left for 48 h at room temperature. After the cover slip was removed with a razor blade, the slide was submerged briefly in xylene to remove residual traces of glue and then in ethanol. Cells were collected from the slide using a fresh razor blade and transferred to an Eppendorf tube. To extract DNA, 0.9 ml cell-lysis buffer (5.25 M guanidinium isothiocyante, 50 mM Tris-HCI, Ph 6.4, 20 mM EDTA) and 40 µl activated silica (coarse) in 0.1 M HCL were added and incubated for 15 min at room temperature. After centrifugation, the silica and bound nucleic acids were washed twice with 5.24 M guandinium isothiocyante in 50 mM Tris-HCl, pH 6.4 twice with ethanol and once with acetone. Finally, nucleic acids were eluted from the dried silica in 140 µl TE buffer at 56°C. The quality of the DNA preparation was assessed by amplification of a 179 bp fragment of the human globin gene.

Detection and typing of HPV DNA

PCR amplification was done using the CPI/CPIIG consensus primer pair described by Tieben and coworkers [17]. This primer pair amplifies a 188 bp El fragment of a broad spectrum of genital HPV types. Amplification was done in a 100 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 3.6 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Perkin-Elmer, Emmersville, State, U.S.A.) and 150 ng of each primer. Forty cycles of amplification were performed (1 min at 55°C, 1 min at 55°C and 2 min at 72°C). The amplification product was analysed on an ethidium-bromide stained 2% agarose gel. For typing, the amplified 188 bp DNA fragment was excised from the gel and analysed by direct sequence analysis using the 3′PCR primer. The HPV type was assessed by a comparison with a panel of reference sequences.

Statistical methods

Categorial variables were analysed with Chi squared-test. Risk factors for HPV detection in the cytologically normal smears were analysed in the DW and the GW, separately. All variables were adjusted for potential confounders by means of a multiple logistic regression analysis which included all variables significantly related to risk of HPV detection ($P < 0.05$). In addition, lifetime number of sex partners was always included in the models. Prevalence odds ratios (PORs) as maximum likelihood estimates of the relative risk of HPV positivity were computed with 95% confidence interval (CI). The SAS software package was used for all analysis [18].

RESULTS

Some selected characteristics of the women with normal pap smears are listed in Table 1. In the DW 24, 20, 27 and 29% were aged 20–24, 25–29, 30–34 and 35–39 years, respectively. The GW were significantly younger with the corresponding age-distribution being, respectively, 33, 29, 24 and 14%. Judged by age at first intercourse, sexually active years, number of sexual partners and history of gonorrhoea, the GW were much more sexually active than the DW

Table 1. *Selected characteristics in women from Denmark and from Greenland with a normal pap smear*

	Denmark ($n = 119$)		Greenland ($n = 118$)		
Variable	*n*	(%)	*n*	(%)	*P*-value*
Age					
20–24	29	(24)	39	(33)	
25–29	24	(20)	34	(29)	
30–34	32	(27)	28	(24)	
35–39	34	(29)	17	(14)	0.03
Age at first sexual intercourse					
≥ 17	64	(54)	19	(16)	
14–16	52	(44)	83	(70)	
≤ 13	3	(3)	16	(14)	0.001
Sexually active years					
≤ 4	13	(11)	2	(2)	
5–9	29	(24)	32	(27)	
10–19	56	(47)	67	(57)	
≥ 20	21	(18)	17	(14)	0.02
Lifetime number of sexual partners					
0–1	28	(24)	0		
2–4	39	(33)	5	(4)	
5–9	38	(32)	18	(15)	
10–39	13	(11)	61	(52)	
≥ 40	1	(1)	34	(29)	0.001
Pregnancy					
Never	34	(29)	26	(22)	
Ever	85	(71)	92	(78)	0.2
Gonorrhoea					
Never	116	(97)	16	(14)	
Ever	3	(3)	102	(86)	0.001
Current smoking					
No	58	(49)	10	(8)	
Yes	61	(51)	108	(92)	0.001

*P-value for difference between the two populations.

Table 2. Prevalence of HPV DNA positivity in archival smears from Denmark and Greenland. Overall HPV DNA prevalence in cytological normal and abnormal smears

	HPV positivity			
	Denmark		Greenland	
Pap smear result	*n*/total	(% pos)	*n*/total	(% pos)
Normal*	26/119	(22)	39/118	(33)
Abnormal	6/6	(100)	7/7	(100)

**P*-value = 0.05 for difference between the two populations.

Table 3. Distribution of HPV types in archival smears with normal cytology from Denmark and Greenland

	Denmark ($n = 26$)	Greenland ($n = 39$)
HPV type *n* (% of all positive)		
HPV 16	10 (38)	25 (64)
HPV 18	3 (12)	3 (8)
HPV 31	4 (15)	2 (5)
HPV 33	0	1 (3)
HPV 58	2 (8)	0
Other types*	2 (8)	1 (3)
Unidentified	5 (19)	5 (13)
Typing not done	0	2 (5)

*HPV 1 or HPV 4.

(Table 1). For example, more GW than DW reported having had first coitus at age ≤ 16 years (84 versus 46%) and ≥ 40 sexual partners during lifetime (29 versus 1%). Also, current smoking was significantly more common in the GW than in the DW (92 versus 51%). By contrast, almost the same proportion of women from the two areas had ever been pregnant.

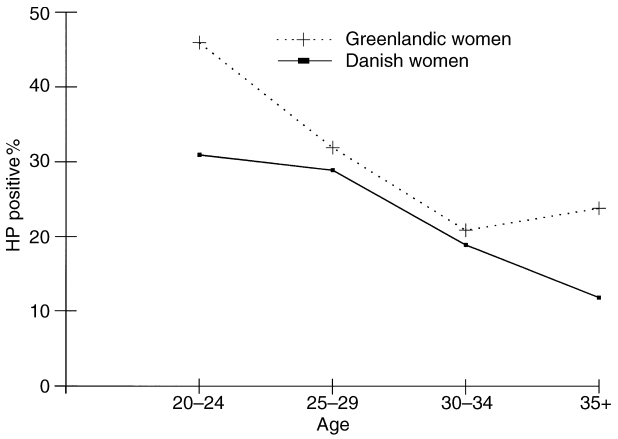

Figure 1. HPV DNA positivity in archival smears by age. Danish and Greenlandic women.

HPV DNA detection

HPV DNA was detected in 6/6 abnormal smears in DW and 7/7 abnormal smears in GW (Table 2). In the cytologically normal smears, HPV DNA was detected in 26 (22%) in DW and in 39 (33%) in GW. In both populations, HPV16 was by far the most commonly detected HPV type (Table 3).

Risk factors for HPV detection in cytologically normal smears

HPV was more frequently detected in smears from the youngest women than from the older women of each study group (Table 4; Figure 1). Among the DW, HPV was 2.5 times more frequently found in smears from women aged 20–24 years than in women aged 30–39 years. Correspondingly in the GW, HPV was three times more frequently detected in the smears from the youngest women compared to the smears from the oldest women. After taking potential confounders into account, the association remained marginally significant in both populations.

Table 4. Risk factors for HPV DNA positivity in cytologically normal archival smears from Denmark and Greenland

	Denmark						Greenland				
Variable	HPV DNA pos/total	(% pos)	Crude POR	POR*	(95% CI)	Variable†	HPV DNA pos/neg	(% pos)	Crude POR	POR‡	(95% CI)
Age§											
30–39	10/66	(15)	1.0	1.0			10/45	(22)	1.0	1.0	
25–29	7/24	(29)	2.3	1.7	(0.5–5.4)		11/34	(32)	1.7	1.5	(0.6–4.4)
20–24	9/29	(31)	2.5	2.5	(0.8–7.4)		18/39	(46)	3.0‖	2.4	(0.9–6.4)
Sexually active years											
≥ 10	11/77	(14)	1.0	1.0		≥ 10	22/84	(26)	1.0	1.0	
5–9	8/29	(28)	2.3	1.9	(0.6–5.5)	7–9	7/20	(35)	1.5	1.4	(0.5–4.2)
≤ 4	7/13	(54)	7.0‖	9.3	(2.2–39.2)	≤ 6	10/14	(71)	7.1‖	5.9	(1.4–25.3)
Lifetime no. of sex partners											
0–1	1/28	(4)	1.0	1.0		≤ 9	9/23	(39)	1.0	1.0	
2–4	11/39	(28)	10.6‖	7.9	(0.9–72.2)	10–29	17/48	(35)	0.9	2.2	(0.6–8.3)
≥ 5	14/52	(27)	10.0‖	9.0	(1.0–81.1)	≥ 30	13/47	(28)	0.6	2.2	(0.5–9.0)
Gonorrhoea											
Never	24/116	(21)	1.0	1.0			11/16	(69)	1.0	1.0	
Ever	2/3	(67)	7.7	6.5	(0.5–85.5)		28/102	(27)	0.2‖	0.2	(0.05–0.8)
Current smoking											
No	8/58	(14)	1.0	1.0			3/10	(30)	1.0	1.0	
Yes	18/61	(30)	2.6‖	2.9	(1.0–8.5)		36/108	(33)	1.2	1.4	(0.3–7.3)

*Prevalence odds ratio, adjusted when appropriate for sexually active years, lifetime number of partners and current smoking. †Same categorisation as in Denmark unless specified. ‡Adjusted when appropriate for sexually active years, lifetime number of partners and ever gonorrhoea. §Sexually active years not included in the model. ‖95% CI exclude 1.0.

Proximity to first intercourse was strongly related to risk in both populations. After adjustment, HPV was 9.3 times more likely (95% CI: 2.2–39.2) to be detected in the smears from DW, who had relatively few sexually active years (≤ 4 years versus ≥ 10 years). Likewise in GW, POR was 5.9 (95% CI: 1.4–25.3) for ≤ 6 sexually active years compared with ≥ 10 years.

In the DW, lifetime number of sexual partners was strongly related to risk of HPV presence in the archival smears, with a 10 times higher risk in women with ≥ 5 partners than in women with ≤ 1 partner (1 virgin included). Adjustment for potential confounders only changed the risk slightly (POR = 9.0; 95% CI: 1.0–81.1). A corresponding categorisation of lifetime number of partners was not possible in the GW (none had 0–1 partner and only 5 had 2–4 partners). Compared with smears from women with ≤ 9 partners during lifetime, increasing numbers of partners had no significant influence on the risk of HPV detection in the GW smears.

HPV was significantly less frequently found in the smears from GW who reported having ever had gonorrhoea than in the smears from women who had never had this disease. This association remained significant after adjustment for potential confounders. In the DW, the opposite trend was found, i.e. higher HPV risk in the smears from women who had ever had gonorrhoea. However, only 5 DW reported to have ever had this disease and the association was not significant.

Smears from DW who were current smokers were significantly more likely to contain HPV with adjusted POR being 2.9 (95% CI: 1.0–8.5). In the GW, where the majority were smokers (92%), there was no statistically significant association between smoking and risk of HPV in smears. Among variables not related to risk of HPV presence in any of the populations were age at first intercourse, parity, oral contraceptive use, marital status and educational level (data not shown).

DISCUSSION

Given the well-established strong relationship between cervical neoplasia and HPV, a high frequency of HPV DNA in cytologically abnormal smears should be expected. All abnormal archival smears in this study were HPV DNA positive confirming the usefulness of the assay.

The two groups of women participating in this study differed substantially with regard to several variables relevant for studies in cervical cancer epidemiology. Firstly, the cervical cancer incidence is 4 times higher in Greenland than in Denmark. Secondly, several behavioural risk factors were more commonly reported by the GW than by the DW, especially those related to sexual life. However, similarities were found in the predictors of HPV detection in normal smears in the two population, i.e. the association with sexually active years, age and sexual behaviour, e.g. lifetime number of partners.

The strong association with lifetime number of partners seen in the DW confirmed the sexually transmitted nature of the infection. The lack of correlation with this variable in the GW may seem contradictory but can most likely be explained by the few low-exposed women in this population. For instance, a possible change in risk from 0–1 to 2–4 partners, which represented a 10-fold increase in risk in the DW, was not computable in the GW. However, our results are in line with other studies of very sexually active women [8, 10]. In those populations, recent number of partners, i.e. within the last year was a better predictor of HPV infection than lifetime number. In the present study, however, we have no data on this variable.

Smears from women who had first intercourse relatively recently were at higher risk of being HPV positive than women who had been sexually active for a longer period. This finding is consistent with the hypothesis that some acquired immunological mechanism clears the infection or reduces the risk of reinfection [19].

The higher HPV detection rate in young women is consistent with several other studies of HPV in fresh cervical swabs [6–12]. As illustrated in Figure 1, the major decline in age-specific HPV detection rate occurred earlier in the GW than in the DW (at age 25 versus 30 years). This earlier 'peaking' of the age-specific prevalence of HPV in GW than in DW confirms previous studies of HPV in fresh cervical smears from women living in the same countries [10]. In penile swabs as well, a decrease with age was found which occurred earlier in Greenland than in Denmark [10]. Whereas the age curve for the DW continued to decline, the Greenlandic curve seemed to level off or even increase, leaving the HPV detection rate in age group ≥ 35 years higher in the GW (4/17 = 24%) than in the DW (4/34 = 12%), but, without the difference between the two populations being significant ($P = 0.2$). As HPV infection in women is considered more likely to be persistent with increasing age [20] and persistence to be a better marker for cervical cancer risk [21], the higher HPV risk in GW above the age of 35 years is in line with the higher cervical cancer risk in this area. A similar tendency was found in the fresh swabs from the same women [15] and in female attenders at venereal clinics in the same two countries [10]. However, only few women aged ≥ 35 years were included in these studies and our findings have to be reproduced in studies including a larger number of women before firm conclusions can be made.

The lower risk of HPV detection in smears from GW with a previous history of gonorrhoea was unexpected. However, we found the same results in fresh swabs from two other populations of very sexually active women [10].

It has been suggested that smoking, by means of impeding the local immunological response towards infection, increases the risk of HPV infection [23]. It has also been suggested that the association with smoking could be explained by sexual behaviour among smokers [8, 22]. However, among the DW in this study, the increased risk of HPV in current smokers could not be explained by the confounding effect of sexual variables. It is difficult to make firm conclusions regarding the smoking/HPV relationship in the Greenlandic population as there were very few GW who did not smoke.

In conclusion, our data confirm the usefulness of this method for detection of HPV in archival smears as all dysplastic smears were HPV positive *and* the predictors of HPV presence in the cytologically normal smears corroborate the risk factors found in recent studies of HPV in fresh cervical swabs. Thus, this method can be useful for large scale epidemiological studies of HPV DNA in already sampled material.

1. Smits HL, Tieben LM, Tjong AP, *et al.* Detection and typing of human papillomaviruses present in fixed and stained archival cervical smears by a consensus polymerase chain reaction and direct sequence analysis allow the identification of a broad spectrum of human papillomavirus types. *J Gen Virol* 1992, **73**, 3263–3268.

2. Chua KL, Hjerpe A. Polymerase chain reaction analysis of human papillomavirus in archival cervical cytologic smears. *Anal Quant Cytol Histol* 1995, **17**, 221–229.
3. de Roda Husman AM, Snijders PJ, Stel HV, van den Brule AJC, Meijer CJLM, Walboomers JMM. Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. *Br J Cancer* 1995, **72**, 412–417.
4. Schiffman MH, Bauer HM, Hoover RN, *et al.* Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. *J Natl Cancer Inst* 1993, **85**, 958–964.
5. Kjaer SK, van den Brule AJC, Bock JE, *et al.* Human papillomavirus—the most significant risk determinant of cervical intraepithelial neoplasia. *Int J Cancer* 1996, **65**, 601–606.
6. Wheeler CM, Parmenter CA, Hunt WC, *et al.* Determinants of genital human papillomavirus infection among cytologically normal women attending the University of New Mexico student health center. *Sex Transm Dis* 1993, **20**, 286–289.
7. Bauer HM, Hildesheim A, Schiffman MH, *et al.* Determinants of genital human papillomavirus infection in low-risk women in Portland, Oregon. *Sex Transm Dis* 1993, **20**, 274–278.
8. Hildesheim A, Gravitt P, Schiffman MH, *et al.* Determinants of genital human papillomavirus infection in low-income women in Washington, D.C. *Sex Transm Dis* 1993, **20**, 279–285.
9. Melkert PW, Hopman E, van den Brule AJC, *et al.* Prevalence of HPV in cytomorphologically normal cervical smears, as determined by the polymerase chain reaction, is age-dependent. *Int J Cancer* 1993, **53**, 919–923.
10. Svare EI, Kjaer SK, Worm AM, *et al.* Risk factors for HPV infection in women from sexually transmitted disease clinics: comparison between two areas with different cervical cancer incidence. *Int J Cancer* 1998, **75**, 1–8.
11. Burk RD, Kelly P, Feldman J, *et al.* Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. *Sex Transm Dis* 1996, **23**, 333–341.
12. Cuzick J, Szarewski A, Terry G, *et al.* Human papillomavirus testing in primary cervical screening. *Lancet* 1995, **345**, 1533–1536.
13. Kjaer SK, Nielsen NH. Cancer of the female genital tract in Circumpolar Inuit. *Acta Oncol* 1996, **35**, 581–587.
14. Storm HH, Manders T, Friis S, Bang S, eds. *Cancer Incidence in Denmark* 1988. Danish Cancer Society, Copenhagen 1991.
15. Kjaer SK, de Villiers EM, Caglayan H, *et al.* Human papillomavirus, herpes simplex virus and other potential risk factors for cervical cancer in a high-risk area (Greenland) and a low-risk area (Denmark)—a second look. *Br J Cancer* 1993, **67**, 830–837.
16. Kissmeyer-Nielsen F, Andersen H, Hauge M, Kjerbye KE, Mogensen B, Svejgaard A. HLA types in Danish Eskimos from Greenland. *Tiss Antigens* 1971, **1**, 74–80.
17. Tieben LM, Ter Schegget J, Minnaar RP, *et al.* Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. *J Virol Methods* 1993, **42**, 265–279.
18. SAS/STAT User's guide. Fourth Edition, Cary NC, ed. Version 6, Volume 1–2. SAS Institute Inc. 1989.
19. Schiffman MH. Epidemiology of cervical human papillomavirus infections. *Curr Top Microbiol Immunol* 1994, **186**, 55–81.
20. Hildesheim A, Schiffman MH, Gravitt PE, *et al.* Persistence of type-specific human papillomavirus infection among cytologically normal women. *J Infect Dis* 1994, **169**, 235–240.
21. Ho GY, Burk RD, Klein S, *et al.* Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. *J Natl Cancer Inst* 1995, **87**, 1365–1371.
22. Villa LL, Franco EL. Epidemiological correlates of cervical neoplasia and risk of human papillomavirus infection in asymptomatic women in Brazil. *J Natl Cancer Inst* 1989, **81**, 332–340.
23. Barton SE, Hollinworth A, Maddox PH, *et al.* Possible cofactors in the etiology of ucervical intraepithelial neoplasia. *J Reprod Med* 1989, **34**, 613–616.

Acknowledgements—We are grateful to A. Isobethsen, L.L. Petersen, A. Rønne and J. Jacobsen for assisting with the data collections. The study was supported through grants from the Danish Cancer Society, Copenhagen.