THE PREDICTABILITY OF INVASIVE CERVICAL NEOPLASIA BY THE PRESENCE OF SPECIFIC HUMAN PAPILLOMAVIRUS SEQUENCES IN PREINVASIVE CERVICAL NEOPLASIA

By

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ABSTRACT

Cancer of the cervix is the second most common form of cancer in women worldwide. The natural history of cervical cancer is thought to involve sequential changes from varying grades of precursor intra-epithelial lesions called CIN. However, little is known of the risk factors that can predict the oncogenic potential of a particular CIN lesion. Based on their preferential occurrence in cervical cancers and their potential oncogenic properties, the human papillomaviruses (HPV) particularly the genotypes 16, 18, 31, 33, 35 and 42 have been implicated in the etiology of invasive cervical cancer. However, these viruses could occur as either secondary pathogen of cancer or as predictor of those CIN lesions that are likely to progress to invasive disease. The hypothesis that was verified in this study was that HPV 16,33 and 18 are likely to be predictive of CIN lesions that progress to invasive cancer.

First of all, in order to characterize the prevalent type of HPV in the target study population of B.C., a cross-sectional study was conducted and the presence of specific HPV types ascertained by the tissue in situ hybridization. The frequency of HPV types 16, or 33, was found to vary with the severity of the CIN grades, in contrast to the frequency of HPV types 6/11 and 18 that segregated independently of the CIN grades.

Next, a case-control study was undertaken to verify the main hypothesis of HPV being predictive of CIN lesion progression to invasion. It was reasoned that the particular HPV would occur at higher

frequency in CIN biopsies of cervical cancer cases than in CIN biopsies of noncases (controls). A total of 47 cases and 94 controls were enrolled from patients registered by the Cancer Control Agency of the Province of British Columbia. A case was defined as a post-pubertal woman with invasive disease and who had a CIN diagnosis at least two years prior to the invasive disease. For each case an attempt was made to enrol two control matched on grade of CIN and year of diagnosis. On each subject attempt was made to gather demographic informations that are known to be associated with cervical cancer. The HPV probes that were used included HPV 16/33 and HPV 6/11. The relative frequency of occurrence of specific HPV in the preinvasive biopsies of cases and controls were as folow: HPV 16/33 occurred in 10.6% of controls and 12.8% of cases; HPV 18 was found in 3.2% of controls and 8.7% of cases and HPV 6/11 in 2.2% of controls and 8.7% of cases. Conditional Chi-square analysis showed that the difference in the proportions of HPV positivity between cases and controls was compatible with sampling variation. Hence, with a statistical power of approximately 60%, it was concluded that particular HPV could not be predictive of CIN lesions progression in the sample of population that was studied.

However an excess risk for incurring cervical cancer, by being exposed to particular HPV at the CIN stage, was noted. The relative risk for HPV 16/33 was 2.34, [95% CI 0.70 to 7.66]; for HPV 18 was 2.45, [95% CI 0.22 to 27.80]; for HPV 6/11 2.19, [95% CI 0.39 to 12.42] or for all HPV combined was 1.87, [95% CI 0.55 to 6.28].

Interestingly, a comparison of the frequency of HPV occurrences in the case-control study with that in the cross-sectional study revealed a lower rate of HPV positivity in the case-control component. This could possibly be due to a cohort effect.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

IST OF ABBREVIATIONS

BSA	Bovine serum albumin
CCABC	Cancer Control Agency of British Columbia
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
DNA	Deoxyribonucleic acid
dCTP	deoxy-cytosine triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
HPV	Human papillomaviruses
OR	Odds ratio
PBS	Phosphate buffered saline
PV	Papillomaviruses
RNA	Ribonucleic acid
RR	Relative risk
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TBE	Tris borate-EDTA
TE	Tris-EDTA

CHAPTER I

INTRODUCTION

I.0.0. Importance of cervical cancer.

Cancer of the cervix is the second most common cancer in women worldwide. When both males and female cancers are considered together, it ranks tenth among all cancer cases in developed countries and first in developing countries (Parkin et al., 1988). The impact of cancers of the genital tract on North American women has changed dramatically over the past 50 years. In 1930, uterine cancer was the leading cause of death among women. Today, only approximately 35,000 cancer deaths are annually due to cancer of the cervix (Pickle et al., 1987). This dramatic decrease is due, in part, to screening cytology which has also contributed to our knowledge on the pathogenesis and epidemiology of the disease. However, despite extensive studies, gaps still exist in our understanding of the natural history and causes of cervical cancer. The focus of this thesis will be on the possible role of certain human papillomaviruses (HPVs) which have been recently implicated in the etiology of cervical cancer (zur Hausen, 1985). Since the pathogenesis of cancer of the cervix takes into account function as well as structure, it is of interest to first look at some of the functional features of this organ.

I.1.0. The normal cervix.

The human cervix is a continuation of the lower end of the corpus uteri. It is cylindrical in shape and extends from the level of the internal os to the wall of the vagina. Histologically, it is

divided into three recognizable areas; the portio, the endocervical canal and the transformation zone (Stern, 1973). See Figure I.1. The portio of the cervix, also called the portio vaginalis or ectocervix, projects into the vagina and is covered by stratified squamous epithelium. The epithelium is composed of five distinct cell layers; the basal layer, the parabasal cell zone, the intermediate cell layer, intraepithelial zone and the superficial cell layer (see Figure I.2.). The endocervical canal, also known as the endocervix, extends from the internal os to the external os and is lined by tall, mucous-secreting columnar epithelium. The endocervical

cervical canal also contains numerous glands that are lined with mucus secreting columnar cells and located in the subepithelial layer. The transformation zone, also termed the squamo-columnar junction, is the junction of the two types of epithelium which generally coincides with the location of the external os in most women. It has been observed that the immature metaplastic cells of the transformation zone appear to have a unique sensitivity for neoplastic events (Fenoglio et al., 1982).

The normal cervix is histologically characterized by epithelial cells undergoing normal mitotic activity. These cells are of uniform sizes and shapes and are arranged in a definite architectural pattern. Variable areas of exposed cervical mucosa of the portio vaginalis are covered with glandular endocervical mucosa at birth or after parturition. These everted endocervical glandular surfaces are less resistant to infection or irritation and, consequently, the subepithelial layers are infiltrated by chronic inflammatory cells.

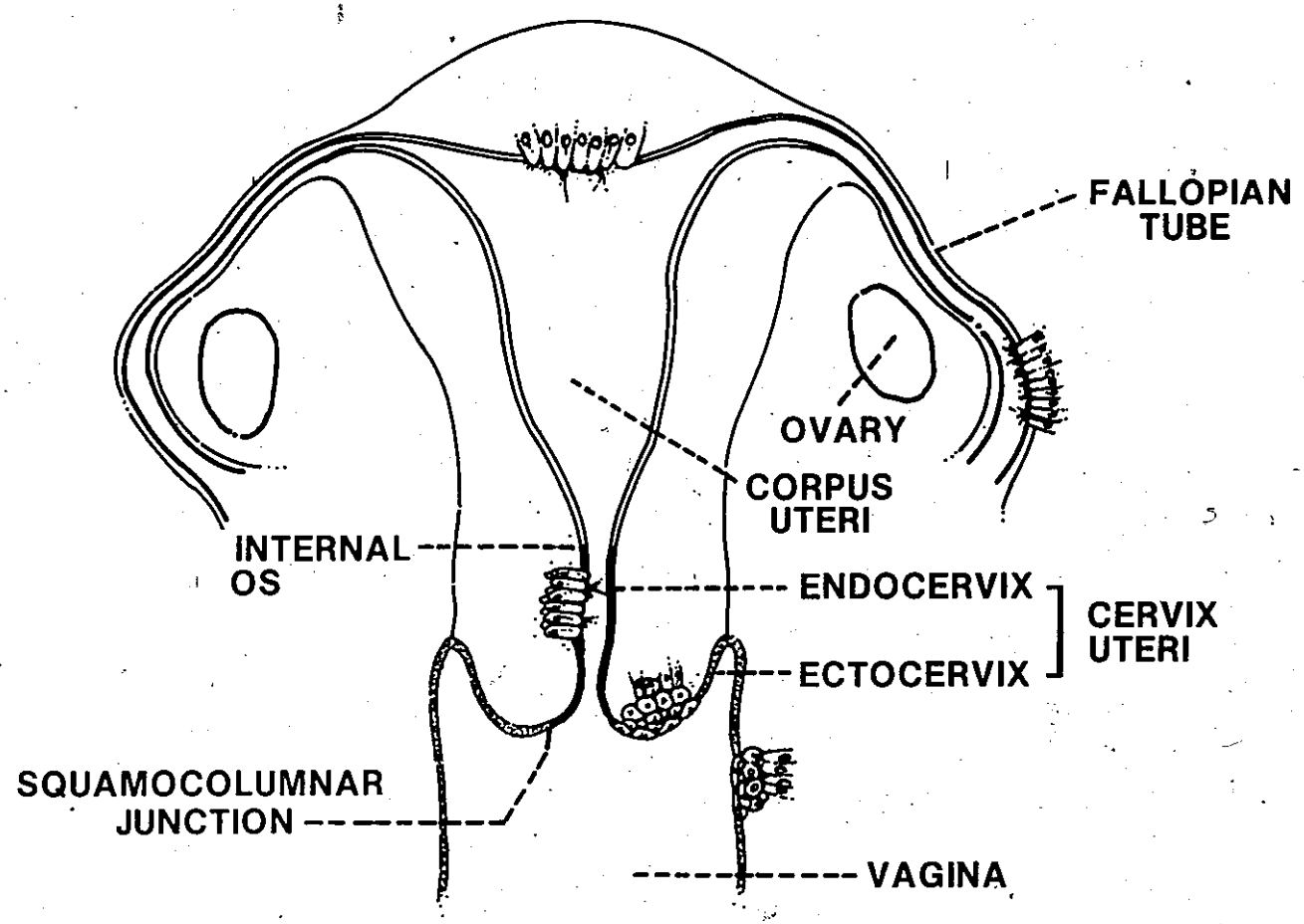


FIGURE I.1. DIAGRAMATIC REPRESENTATION OF INTERNAL FEMALE GENITALIA. (REF. STERN E,1973.)

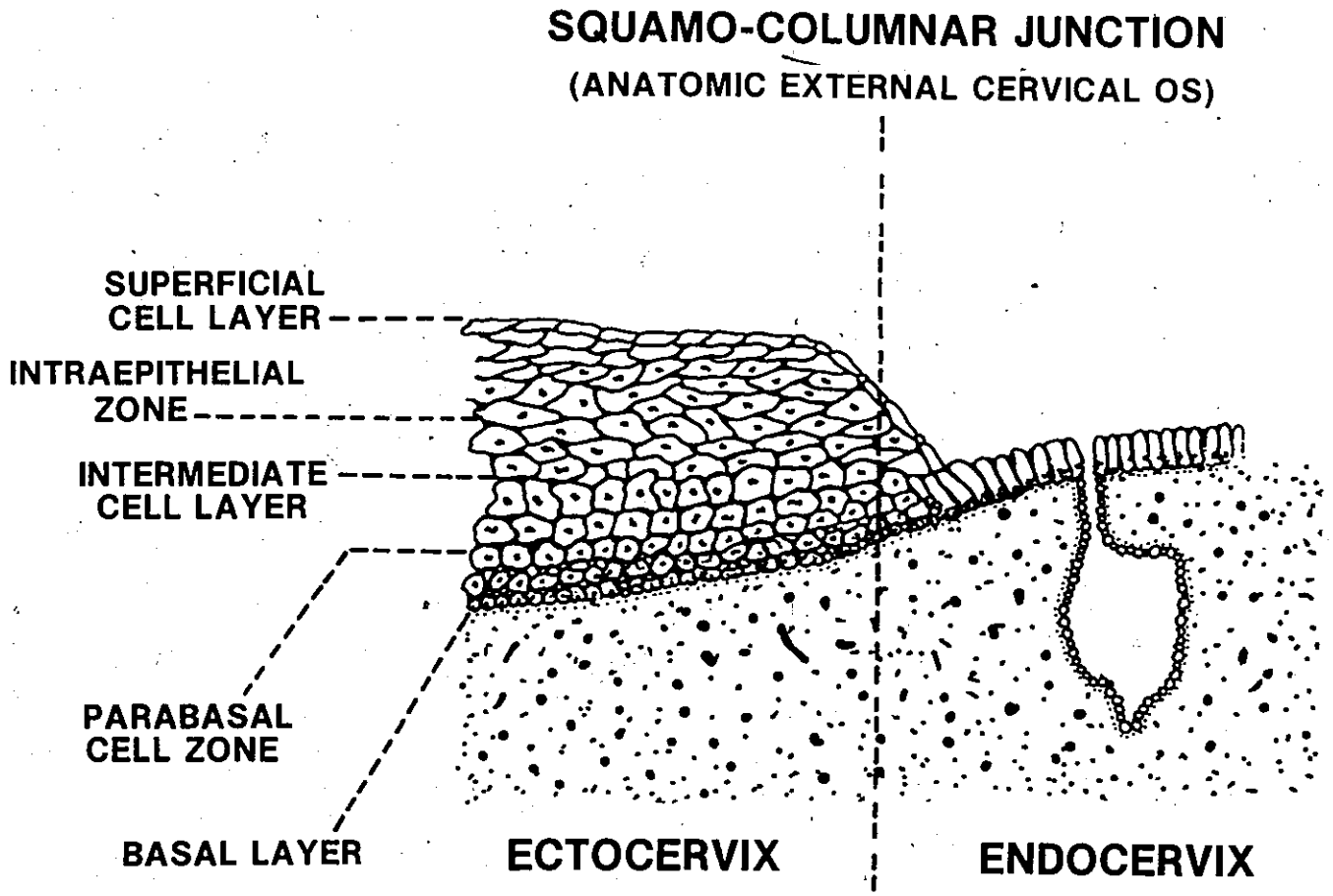


FIGURE I.2. HISTOLOGICAL FEATURES OF THE CERVIX (REF. KETCHING, 1986)

Under conditions of chronic irritation, the exposed glandular epithelium undergoes metaplasia: The columnar mucus secreting surfaces are replaced by stratified squamous epithelial surface. Metaplasia and chronic cervicitis are common morphological findings in women with normal reproduction cycles (Kraus, 1985).

I.1.2. The dysplastic cervix

Histologically, dysplasia of the cervix comprises a loss in the uniformity as well as a loss in the architectural orientation of the normally situated or metaplastic squamous epithelium (Robbins et al., 1981). It is characterized by three features: nuclear abnormalities, increased mitotic activity and a lack of order in the degree of maturation or differentiation. The grade of dysplasia can be ascertained by the amount of the epithelium involved in the process (Anderson, G.H., 1985). See Figure I.3.

In mild dysplasia, maturation of the squamous epithelial cells is confined to the superficial two thirds of the squamous mucosa while undifferentiated cells are present in the lower one third. The nuclei of the cells in the superficial two thirds of the mucosa show some enlargement and hyperchromasia.

Moderate dysplasia is marked by the superficial one third to two thirds of the squamous mucosa showing maturation of the cytoplasm. Undifferentiated cells occupy the lower one third to two thirds of the mucosa. Loss of polarity throughout the mucosa is more apparent than in the previous two categories.

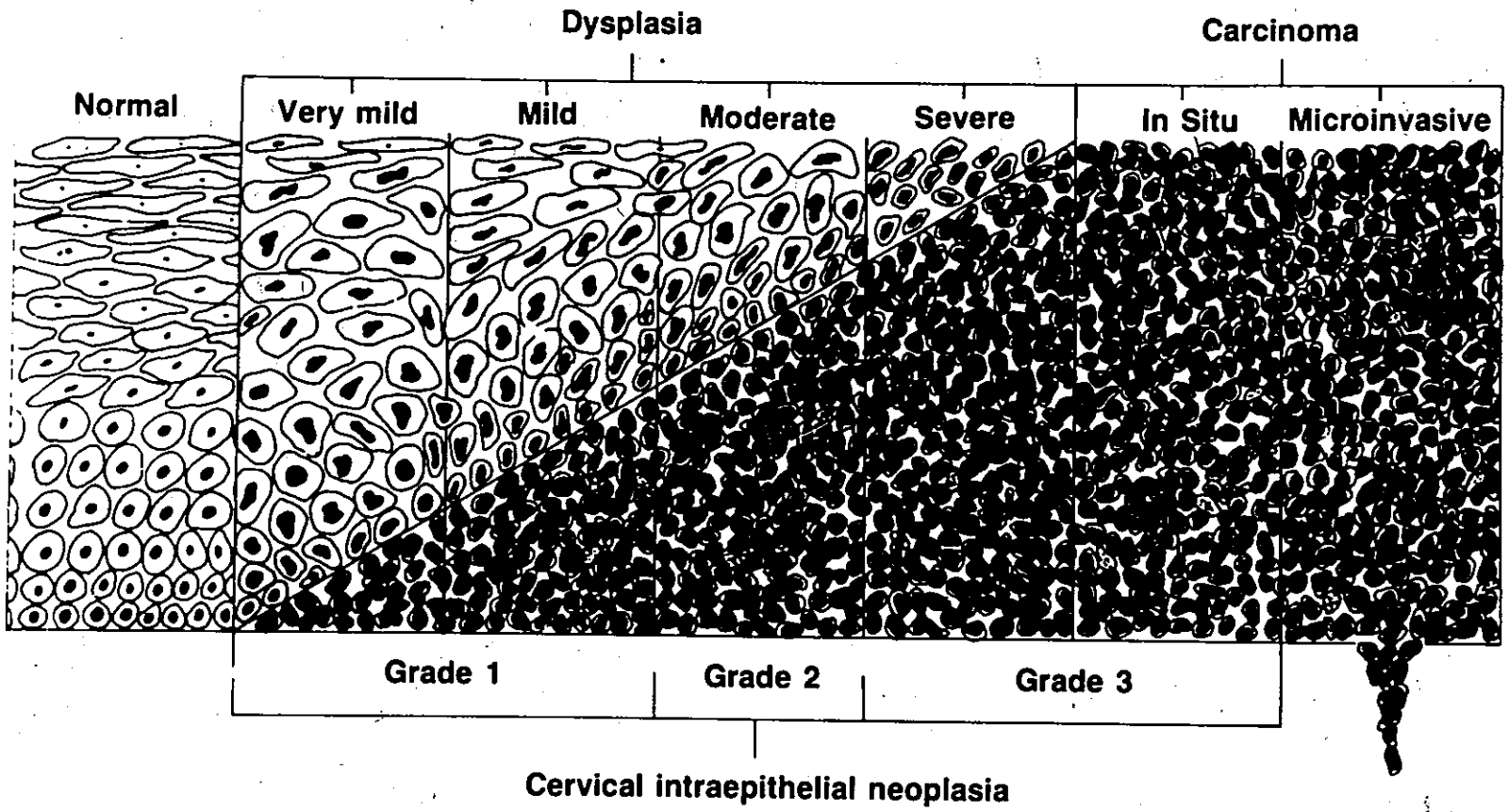


FIGURE I.3. RELATIONSHIP BETWEEN DYSPLASIA AND CERVICAL INTRAEPITHELIAL NEOPLASIA (REF. ROBBINS ET AL., 1981)

In severe dysplasia more than two thirds of the thickness of the squamous mucosa is occupied by undifferentiated cells which show nuclear hyperchromasia and loss of polarity. Mitotic figures may be found in the lower two thirds of the mucosa.

Carcinoma in situ (CIS) is characterized by the entire thickness of the epithelium being occupied by neoplastic cells. These cells show evidence of differentiation mostly in the superficial layers. Mitotic figures may be found at all levels of the mucosa.

Based on the observation that only the epithelium layer is involved in dysplasia, Richart (1987) has coined the term cervical intraepithelial neoplasia or CIN to describe the whole spectrum of dysplastic changes including CIS. The corresponding CIN classification for mild dysplasia is CIN 1; for moderate dysplasia, CIN 2 and for severe dysplasia including CIS, CIN 3. See Figure I.3. The chief objection to the CIN classification is that it does not distinguish between severe dysplasia and CIS (Anderson, G.H., 1985). However, since there is no consensus for distinguishing between CIS and severe dysplasia, such distinction has been rather arbitrary and the CIN classification is widely used.

I.1.3. The neoplastic cervix

Tumours of the cervix may be classified as benign or malignant. By far the most common tumour of the cervix is the squamous cell carcinoma which accounts for 80-95% of the cases. Adenocarcinomas make up 4% to 8% of the total and the remaining few include sarcomas,

lymphomas and melanomas (Anderson, M.C., 1985). Adenocarcinomas originate in the glandular epithelium while squamous cell carcinomas, the topic of this study, have their origin at or near the squamo-columnar junction of the ectocervix.

A staging system has been established to indicate the degree of invasion of the squamous cell carcinoma which may spread through the adjacent tissues, the lymphatics, veins, perineural space or nerves. Stage 0 is characterized by the carcinoma being strictly confined to the cervix. It is divided into a clinically unrecognizable stage IA and clinically recognizable stage IB. The stage IA is further subdivided into microinvasive carcinoma and occult carcinoma (Van Nagell et al, 1983). Microinvasive carcinoma is characterized by a small discrete foci of invasion extending to a depth of no greater than 5 mm through the basement membrane. Histologically, occult carcinomas show a greater depth of stromal invasion (Coppleson, 1985). Stage II involves carcinoma that has progressed beyond the cervix but not extended into the pelvic wall. When the carcinoma has extended into the pelvic wall and the lower third of the vagina, it is classed as stage III. Finally, stage IV is marked by extension of the carcinoma beyond the pelvis and may include involvement of the mucosa of the bladder or rectum. However, as has been discussed by Ferenczy (1977), there are some controversies in the present classification systems.

I.2.0. Natural history of cervical cancer.

The natural history of cervical cancer can be traced as the

evolution of the disease from a preclinical phase to the invasive stage. The widely cited working model of cervical cancer is that it evolves as a sequential process from varying grades of preinvasive dysplasias to invasive cancer (Richart, 1984). However, the study of natural history of cervical neoplasia is constrained by the nature of the end-point of the disease. If invasive cervical cancer is taken as the end-point, it is unethical not to treat women before the end-point is reached. By necessity then, knowledge of the natural history of cervical cancer has been largely derived from a highly selected sub-group of patients who have escaped treatment through refusal, missed diagnosis or loss to contact. The basis for the model of cervical cancer as obtained from a number of observational studies on different populations is reviewed below.

Richart and Baron (1969) followed 462 patients with a cytological diagnosis of severe dysplasia for 1 to 3 years. To follow the natural course of the disease, no biopsies were taken until the patients developed CIS. They observed that the mean period of progression from severe dysplasia to CIS was 12 months. The average rate of regression to normality was only 1.3%; of progression to CIS was 20.3%, and persistence was 78.3%. By contrast, Johnson et al. (1968) observed a regression rate of 50.4% in dysplastic patients who were followed by cytology and biopsy over periods of 10 years. In this interval only 1.4% progressed to CIS. Unfortunately their results were not analyzed according to severity of dysplasia. When Galvin et al. (1955) looked at the grades of dysplasia, they found that of 93 cases of the mild dysplasia 53.9% regressed to normality; 44.7%

persisted and 1.4% progressed to CIS. In the group of 63 patients with moderate dysplasias, 44% regressed to normality; 32% persisted and 24% progressed to CIS. In contrast, from the category of severe dysplasia, only 17.1% of the subjects regressed to normality; however, 14.6% persisted and 67.7% progressed to CIS. This tendency for milder forms of dysplasia to regress spontaneously, and for severe ones to progress to a more advanced state, has also been noted by other investigators. Thus, Stern and Nelly (1963) found that among 94 women with dysplasia, 48% were still dysplastic 1 to 7 years later; 40% regressed to normality, and 12% progressed to CIS. Hall and Walton (1968) reported a 29% progression rate of severe dysplasia to CIS over a 1-14 year period. The natural history of cervical dysplasia is summarized in Table I.1. Based on these and other similar data, it has been estimated that dysplasia is most commonly diagnosed among women in their 20s and CIS, in women between 30-39 years (Task Force, 1976).

The mode of evolution of invasive cancer from CIS is of prognostic significance for intervention of invasive cancer. The best evidence for a relationship between CIS and invasive squamous cell carcinomas was obtained by Petersen (1955) who, over a period of 9 years, followed 127 untreated Danish patients with CIS lesions. The following progression rates to cervical cancer were observed: 4% after one year, 11% after 3 years, 22% after 5 years and 33% after 9 years. Koss et al. (1962) followed 67 patients with initial diagnoses of CIS or "borderline" cytological abnormality over a period of 6 months to 10 years. They observed that among those with an initial CIN diagnosis, 25.4% regressed to normality; 61.2% persisted and 5.9%

Table I.1.
Summary of main studies showing the natural history of dysplasias

Sample	Diagnosis of dysplasia: Starting Point	Diagnosis of dysplasia: End Point	Percent at end point	Period of follow-up	Reference
93	mild	normal	53.9	4	Galvin et al. 1955
		mild	44.7		
		CIS	1.4		
63	moderate	normal	44		
		moderate	32		
		CIS	24		
41	severe	normal	17.1		
		severe	14.6		
		CIS	67.7		
97	slight	normal	62.0	1-14	Hall & Walton, 1968
		slight	35.6		
		CIS	13.4		
85	moderate	normal	32.9		
		moderate	48.7		
		CIS	18.4		
24	severe	normal	19.1		
		severe	33.3		
		CIS	47.6		
119	unspecified	normal	50.4	10	Johnson et al. 1968
		CIS	1.4		
		persisted	48.2		
462	severe	normal	1.3	1-3	Richart et al. 1969
		CIS	20.3		
		severe	78.3		
94	unspecified	unspecified	48	1-7	Stern & Nelly, 1962
		normal	40		
		CIS	12		

CIS, carcinoma in situ.

progressed to invasive cervical carcinomas. When the assessment of follow up was confined to lesions with "borderline" abnormalities, it was observed that 38.2% regressed to normal cytology; 15.4% persisted and 3.8% progressed to invasive carcinomas. Kottmeir (1961) reported that of 30 women who had been followed for at least 12 years, 22 (17%) developed invasive disease. Green and Donovan (1970) reported on a large series of women who had diagnoses of CIS made by cone biopsy. In this study, only 0.17% developed invasive carcinoma after 11 years of follow up. The last observation apparently indicates that there is little or no relationship between CIN and invasive carcinoma; however, these figures may have been biased downwards because of the curative effect of the biopsy. To overcome this difficulty, Kinlen and Spriggs (1978) traced 52 women who had abnormal pap smears diagnosed at least two years previously but who had not had any biopsies or treatment. Nineteen percent developed invasive cancer and 36.6% showed no apparent abnormality. These data on the evolution of invasive cancer from presumed CIS precursor are summarized in Table I.2.

The figures quoted for the progression of CIN lesions to invasive carcinoma thus vary between 0.17% and 70%. At least three possible explanations may be advanced for this variation: (a) an inception cohort was not assembled, (b) the variation in the sensitivity and specificity of the diagnostic assays was not defined and (c) cervical cancer may exist in more than one form.

If an inception cohort is not assembled to study the natural history of the disease, then the various subjects under observation will be at different stages of the disease and, consequently, will

Table I.2

Summary of Main Studies Showing Evolution of Invasive Clinical Cervical Carcinoma from Presumed Precursor Lesions.

Sample Size	Starting points	Percent showing end point (clinical carcinoma)	Length of follow-up	Reference
31	CIS	71	7 years	Kottmeir et al. 1961
94	Unspecified dysplasia	11	1–7 years	Stern & Nelly, 1962
127	Unspecified precancerous lesions	4 11 22 33	1 year 3 years 5 years 9 years	Petersen, 1955
180	CIS	0.55 1.1 1.6	1 year 2 years 3 years	Jordan et al. 1964
67 26	CIS "borderline"	5.9 3.8	1/2–10 years 1/2–10 years	Koss et al. 1963
576	CIS	0.17	2 years	Green, 1969
53	Pap smear IV/V	13	2–8 1/2 years	Kinlen and Sprigg, 1978

CIS, carcinoma in situ.

reach the end point at varying times. The mean duration of CIS stage is particularly relevant in this context. Cytologic (Richart and Barron, 1981) or epidemiologic (Fidler et al., 1968; Green and Donovan, 1970) observations indicate that CIS has a mean duration of 10 years. Statistical modelling puts forward a range of 3-10 years (Baron et al., 1978). Thus, the mean progression from CIS to invasive cancer for most women will be between 3-10 years but a few will invariably fall at the two extreme ranges of the distribution curve of the length of the natural history for CIS. Given that CIS is generally diagnosed in women between 30-39 years of age, it seems reasonable to speculate that some may never progress in thei lifetime while others may have a very rapid onset. Inclusion of these patients in a study may thus obscure the true rate of progression of CIS to invasive cancer.

The sensitivity and specificity of the diagnostic tests, if not accurately defined, will equivocally affect any studies relying on such methods for defining the starting point and the end point of a given disease. Cervical cytology and histology show considerable variations and there seems to be a lack of objective criteria for scoring such assays in the past (Miller, 1986). Use of these assays could account for false positive and false negative results in the follow-up studies and distort the true rate of evolution of cervical neoplasia.

It is conceivable that not all cases of invasive cervical cancer have a CIN precursor. Based on the observation that the age-specific incidence curve of cervical carcinoma shows two poorly

defined peaks at about age 50 and age 70, some investigators have even postulated two variants of squamous cell carcinoma (Ashley, 1966; Hakama et al., 1981). The commoner form is thought to occur in younger women and has a recognizable preinvasive phase; the other is found in older women and does not appear to be preceded by a CIN precursor. Recently, a converse pattern has been observed, the rapidly progressing invasive cancer is seen in younger patients and the slower evolving one is confined to the older patients (Berkely et al., 1980). While the existence of two forms of squamous carcinomas of the cervix remains a possibility, it must be pointed out that this is not a unique explanation as the age-related peaks in the incidence curve could be due to a cohort effect. Different birth cohorts may have different expectations of acquiring this disease if environmental factors are involved in the genesis of the disease (Editorial 1981). Since most of the studies were conducted on older cohorts in the 1950s and 1960s, when the disease was more prevalent, it is unclear whether the same natural history would be expected in younger cohorts today.

I.3.0. The epidemiology of cervical cancer

I.3.1. Methodological issues

Generally, a risk factor for cervical cancer indicates a variable that is believed to be related to the probability of an individual developing cervical cancer. A number of risk factors have been identified for cervical cancer and their magnitude assessed by epidemiological studies. However, the strengths of the conclusion

depend on the type of study designs that were used to investigate these relationships. Hence, it is relevant to briefly review the main types of epidemiologic designs before examining the risk factors.

Basic epidemiologic designs can be either observational or analytic in nature. The case-series study is an example of an observational design while the case-control and the cohort studies are two examples of the most common analytic designs used in studying the etiology of cancer (Kleinbaum et al., 1982). The case series or cross-sectional design consists of a one-time examination of the exposure and outcome variables. Although widely used in prevalence studies it is of limited value in establishing temporality as both the outcome and the exposure variables are concomittantly examined. Therefore, it cannot be used to distinguish between risk factors which increase the risk of getting a disease and prognostic ones which increase the probability of a case developing a certain outcome. The case control study design depends on the assembly of a group of patients with cervical neoplasia (cases) and a matched non-disease group (controls). Cases and controls are then compared with respect to previous exposure to the suspected etiologic agents and the risk can be assessed as odds ratio. Case-control studies generally cannot establish temporality as information on the risk factors is obtained after the diagnosis of cancer. An alternative design is the prospective cohort in which information of the exposure variable is known for all subjects at the beginning of the study period. The risk of developing cancer is then measured as relative risk at the end of the study period. The cohort study permits the demonstration that the hypothe-

sized cause preceded the diagnosis of cancer.

The strength of the conclusions that can be drawn from these designs varies. A number of inaccuracies or biases can distort the results of all these study designs and show either an indirect or spurious association between a given risk factor and cervical cancer. One principal source of inaccuracy is a confounding factor, an extraneous variable affecting the causal relationship being studied. Factors such as age, sex or race can all act as confounders and may totally or partially account for the apparent effect of the observed exposure factor. Alternatively, they may mask the association between cervical cancer and the exposure variable.

I.3.2. Key risk factors for cervical cancer

Because of the lack of certainty concerning the probability of an individual developing cervical cancer, epidemiologic studies have initially measured risk factors as opposed to causal factor (Kleinbaum et al., 1982). The key risk factors which have been identified for cervical cancer include socioeconomic ones, marital and sexual factors, smoking, use of oral contraceptive and infectious agents.

Several observational and analytic studies have demonstrated that cervical cancer is more prevalent in women in lower social classes. Generally, the risk for cervical cancer is about five times greater for women in the lowest social class than those in the highest classes (Stocks, 1955; Fasal et al., 1981).

One of the first risk factors identified for cervical cancer

was age at which a woman was married. The odds ratio reported in several case-control studies indicated that a woman who marries before age 20 has twice the risk of developing cervical cancer when compared to a woman who marries after age 20 (Martin, 1967; Terris et al., 1967). As more investigations were conducted, it was found that age at first coitus was a stronger correlate of risk than age at first marriage (Rotkin, 1967).

A related risk factor for cervical cancer is the number of sexual partners for a woman. The risk of developing the disease is greater for those with higher numbers of sexual partners than those with fewer numbers of sexual partners. Women with two or more sexual partners, often indexed by multiple marriages, separations or divorces, have a twofold to threefold increase in risks when compared to women with only one or no sexual partners (Rotkin, 1967). Recently, the sexual behaviour of the male partner has also been recognized to influence a woman's risk of cervical cancer. In many studies it has been demonstrated that the husbands of cases have significantly more sexual partners than those of controls (Buckley et al., 1981; Zunzunegi et al., 1986).

The role of smoking as an etiological factor in the development of cancer of the uterine cervix has been controversial. Epidemiologic studies using either the case-control or the prospective cohort approaches have demonstrated that current or long term smokers are at increased risk for both preinvasive and invasive cervical disease (Harris et al., 1980; Clarke et al., 1982; Greenberg et al., 1985). Unfortunately, these results might have been confounded by sexual

behaviour producing an artefactual association in older women. However, recently a multi-centre case-control study from the U.S. indicates that smoking may be an independent variable. A relative risk of 1.5 was found after adjusting for age at first intercourse and the number of sexual partners (Brinton et al., 1986b).

The relationship between use of oral contraceptives and cervical neoplasia, like that of smoking, has been a controversial one. However, the results of two case-control studies have been particularly informative. The degree of confounding produced by sexual life-style or screening history was appropriately controlled in a large multi-centre case-control study conducted by the World Health Organization (WHO collaborative study of neoplasia and oral contraceptive, 1985). In this study, an adjusted relative risk of 1.2 was observed for any pill user and this risk increased to 1.5 for users of 5 of more years. Similar trends have also been noted by a multi-centre case-control study done in the U.S. (Brinton et al., 1986c).

The epidemiologic obervations on the key risk factors indicate that cervical neoplasia has many of the attributes of a venereal disease. This has prompted a search for sexually transmitted infectious agents capable of causing cervical cancer (Rotkin, 1973; Kessler, 1981). Numerous viruses, bacteria, chlamydiae, parasites and fungi are known to be sexually transmitted. However, viruses have remained the principal candidate since they are capable of inducing cellular transformation. This phenomenon appears roughly analogous to naturally occurring tumours since the virally transformed cells share many traits in common with the tumour cells. Furthermore, viruses can

transmit new genetic information that may be required for the maintenance of the neoplastic phenotypes of the transformed cell (Fenoglio, 1982a). The plausibility for the role of viruses in the etiology of cervical cancer is thus a biologic one, however, a number of pitfalls exists in epidemiologically linking a suspected virus in the etiology of a chronic disease such as cervical cancer. Consequently, in this thesis, the model for causality in such an instance will be considered next, before the epidemiologic evidence for the particular viruses will be presented.

I.3.3. Models of causality

Classically the definition of causality may be derived from the formal logic theory of pure determinism. It formulates a constant, unique and perfectly predictable connection between two factors; X the causative one, and Y the effect (Kleinbaum et al., 1982). Two important corollaries of this logic are the specificity of cause and the specificity of effect. The specificity of cause implies that the cause must be a necessary and sufficient element in bringing about the effect. The specificity of effect in turn implies that only one effect is ascribed to the cause.

The logic of pure determinism has been successfully applied to acute infectious diseases with short latency period (Rothman, 1981). However, the rigorous application of these concepts to a disease of long latency such as cervical cancer is limited. Many of the risk factors, including the viruses, have a multiplicity of effects thus

invalidating the specificity of effect. Finally our knowledge of the natural history of the disease is incomplete as very little is known about the initiation process or the oncogenic potential of a precursor lesion. In view of these limitations, epidemiologists working on the etiology of chronic diseases rely on alternative strategies that incorporate some of the concept of pure determinism as well as concepts derived from empirical observations (Lillienfeld, 1959; Lilienfeld et al., 1979; Evans, 1978; Susser, 1986 and Yerushalmy et al., 1959 and Sartwell, 1960). Before etiologically linking a virus to a chronic disease the evidence is graded by a set of seven diagnostic rules (Trout, 1981). These are, strength of association, dose-response effect, lack of temporal ambiguity, consistency of findings, biologic plausibility of the hypothesis, epidemiologic coherence of the evidence, and specificity of association. These rules will be applied to review the evidence implicating the candidate human papillomaviruses in the genesis of cervical cancer.

I.3.4.0. The candidate human papillomaviruses

A role for HPV in the genesis of cervical cancer had been earlier postulated by zur Hausen (1974) who based his argument on the presence of these viruses in genital warts. This argumen has been strengthened by the finding that significantly more patients with genital warts show dyskaryotic changes of CIN than those without warts (Francheschi et al., 1983). Recently, with the advent of molecular cloning and the availability of viral probes this interest has been

revived (zur Hausen, 1985).

The papillomaviruses (PV) belong to a sub-group of papovaviridae (Tooze, 1980). All of the viral genetic information is contained within a small, circular, double-stranded DNA genome of about 8000 nucleotide base pairs (Danos and Yaniv, 1983). These viruses are normally species specific, producing hyperplasia and neoplasia in their natural hosts. In humans, over 45 different types of HPVs have been identified and isolated from such diverse clinical conditions as cutaneous warts, laryngeal papillomas, cervical intraepithelial lesions, anogenital warts, squamous carcinomas and epidermodysplasia verruciformis (Pfister, 1984; zur Hausen, 1977; Jenson et al., 1984).

Specific HPVs have been implicated in the genesis of cervical neoplasia (Richart, 1984 and 1987; Fletcher, 1983; Fuji et al., 1984; Koss, 1987). Infection due to HPVs is thought to result in three growth patterns on the cervix; the exophytic condyloma, inverted papilloma and the so-called flat condyloma. The most common form appears to be flat lesions that originate within the transformation zone and display the clinical and colposcopic appearance of the classical CIN spectrum of disease (Richart, 1984).

I.3.5.0. Epidemiologic evidence linking HPV with cervical cancer

I.3.5.1. Consistency of the observation between the association of particular HPVs and cervical cancer

The evidence for a causal relationship is strengthened when

several studies, conducted at different times, with different settings and with different patients all come to the same conclusion (Trout, 1981).

The possible role of HPVs in genital neoplasias were first derived from cytologic studies. Numerous case-series and prospective cohort studies showed that between 50 to 70% of condylomatous lesions were associated with CIN lesions (Meisel et al., 1981a and 1981b; Syrjanen 1980; Syrjanen et al., 1981, Reid et al., 1982a, 1982b and 1983; de Brux et al., 1981). In most of these studies approximately 10% of lesions, diagnosed by demonstrating HPV koilocytes or HPV antigen, progressed to more severe CIN. Unfortunately, due to the limitation of the cytologic diagnosis it is not possible to incriminate any particular types of HPV in the process.

The application of cloned viral DNA for type specific identification of HPV revealed that subtypes of HPVs are associated with specific disease (zur Hausen, 1985; Pfister, 1984; Koss, 1987). The genotypes of HPV predominantly infecting the genital tract include HPV types 6, 11, 18, 31, 33 and 35. Among these, HPV types 6 and 11 generally segregate in preinvasive lesions and the remaining genotypes are confined to advanced CIN lesions and or invasive lesions. Interestingly, the relative frequency of these viruses vary in different reports.

Gissman et al. (1982) first observed that HPV 6, and its homologously related HPV 11, viral DNA sequences were present in about 95% of genital wart samples and about 18% invasive carcinoma samples from German patients. Various other subsequent reports have confirmed

Table I.3 Summary of Occurrence of HPV 16 in Squamous Cervical Carcinomas

Assay	Hybridization Stringency	Sample Size	Percent Positive	Confidence Interval	Reference
DB	S	30	53	34,71	Choo et al. 1986
SB	S	13	46	19,75	diLuca et al. 1986
SB	NS	18* 23**	61 35	35,82 16,57	Durst et al. 1983
SB	S	7	14	0,57	Fukushima et al. 1985
SB	NS	11	73	39,93	Lancaster et al. 1986
SB	NS	13	92	63,99	McCance et al. 1985
SB	NS	9	89	51,99	McNab et al. 1986
SB	S	47	66	50,79	Meanwell et al. 1986
SB	NS	20	60	36,80	Prakash et al. 1985
SB	NS	11	45	16,66	Reid et al. 1987
SB	S	11	45	16,76	Scholl et al. 1985
DB	S	6	50	11,88	Shirasawa et al. 1986
SB	S	9	33	7,70	Tsunokawa et al. 1986
SB	S	50	36	22,50	Yoshikawa et al. 1985

SB: Southern blot; DB: Dot blot; NS: Non-stringent; S: Stringent

* German; ** Kenyan/Brazilian

the presence of these HPV in condyloma acuminata (Yoshikawa et al., 1985; Beckman et al., 1985; Stoler et al., 1986; Tomita et al., 1986; Reid et al., 1987). The occurrence of HPV type 6 and 11 DNA sequences in CIN lesions appears to be inversely related to the severity of the lesions. Thus, Wagner et al. (1984) found HPV 6 and 11 sequences to be present in 30% of CIN I/II lesions and in only 18% of CIN III lesions. Most other studies have confirmed this trend (Schneider et al., 1985, Kadish et al., 1986). Similarly, Reid et al. (1987) found HPV 6 and 11 sequences in 33% of CIN I lesions; 15% of CIN II lesions and only 4.5% of CIN III lesions.

The occurrence of HPV 16 DNA sequences was first reported by Durst et al. (1983). They found that 61.1% of cervical cancer samples from German and 34.8% from Kenyan and Brazilian patients contained HPV 16 sequences. Most other studies have found the positivity rates to vary in these ranges. Thus, the observed positivity rates are: 92% (McCance et al., 1985a); 60-65% (Prakash et al., 1985; Meanwell et al., 1987); 45% (Scholl et al., 1985, di Luca et al., 1986); 50-55% (Shirasawa et al., 1986; Choo et al., 1987); and about 35% (Yoshikawa et al., 1985, Tsunokawa et al., 1986). Some other studies have found the positivity rate to be as low as 14% (Fukushima et al., 1985). These rates, together with the appropriate confidence intervals, have been summarized in Table I-3.

The occurrence of HPV 16 in dysplasias seems to vary directly with the severity of the disease and a wide range has been reported so far. The relative frequency of HPV 16 sequences in CIN I lesions has been observed to be 0% by Pater et al. (1986); 16.6% by Reid et

Table I.4 Summary of Occurrence of HPV 16 in Cervical Intraepithelial Neoplasias (CIN)

Assay	Hybridization Stringency	Sample Size	CIN grade	Percent Positive	95% CI	Reference
TISH	S	18	unspecified	55.5	30.6,78.4	Crum et al. 1986
SB	S	4	II/III	75.0	19.4,99.3	diLuca et al. 1982
SB	NS	20	unspecified	10.0	1.2,31.7	Gismann et al. 1983
		9	III	22.2	2.8,60.6	
SB	NS	4	I	50	6.2,93.2	Kadish et al. 1986
		12	II	15	2.0,48.4	
		6	III	26	4.3,77.7	
SB	NS	20	I	55.0	22.2,77.8	McCance et al. 1985
		30	II	66	40.6,77.3	
		28	III	71	51.3,86.3	
SB	S	6	I	66.6	22.2,95.6	Murdoch et al. 1988
		10	II	60.0	26.2,87.8	
		5	III	80.0	28.3,99.5	
SB	S	6	I	0.0		Pater et al. 1986
		30	II	23.0	9.9,42.3	
		54	III	50.0	36.0,63.9	
SB	S	18	I	16.6	3.6,41.4	Reid et al. 1987
		15	II	39.4	24.0,56.5	
		22	III	72.7	49.8,89.3	

TISH: Tissue in situ hybridization; SB: Southern blot;
S: Stringent; NS: Non-stringent

al. (1987); 50% by Kadish et al. (1986); 55% by McCance et al. (1985a, 1985b and 1985c); and 66% by Murdoch et al. (1988). The occurrences in CIN II lesions have similarly been found to have a wide range of 15% (Kadish et al., 1986); 23% (Pater et al., 1986; 39.4% (Reid et al. (1987); 60% (Murdoch et al., 1988) and 66% (McCance et al. (1985). In CIN III lesions the rates have been estimated to be 22% (Gissmann et al., 1983); 26% (Kadish et al., 1986); 50% (Pater et al., 1986); 71% (McCance et al., 1985); 72.7% (Reid et al., 1987); and 80% (Murdoch et al., 1988). These rates, together with the appropriate confidence intervals, have been summarized in Table I.4.

Boshart et al. (1984) first observed that HPV 18 DNA sequences were present in 25% of cervical carcinoma samples from African and Brazilian patients and in 15% of cervical carcinoma samples from German patients. Subsequent reports have found that approximately 5% (Yoshikawa et al., 1985) and 22% (Tsunokawa et al., 1986) of invasive cancer samples contain HPV 18 sequences.

The occurrence of HPV 18 sequences in various grades of dysplasias seems to be independent of the severity of the lesions. However, the respective rates vary in different reports. Reid et al. (1987) observed 5% of CIN I, 2% of CIN II and 4% of CIN III to contain HPV 18 sequences while Pater et al. (1986) found these HPV sequences to occur in 23% of CIN I, 20% of CIN II and 26% of CIN III lesions. An extreme observation is that of Kadish et al. (1986) who found approximately 25% of mild and moderate dysplasias (CIN I/II)and no CIN III lesions to contain HPV 18 sequences. Other investigators have studied the joint occurrence of HPV 16 and 18 sequences by the use of a mixed

probe (Wagner et al., 1984; Schneider et al., 1985). In such studies it has been observed that HPV 16/18 sequences seem to preferentially segregate in higher grades of dysplasias than in lower grades. However, it is difficult to distinguish if the observed effect is due to the HPV 16 probe in the mixture.

The occurrence of the newly discovered genital HPV 31, 33 and 35 has not been as extensively studied. HPV 31 has been originally reported to be present in approximately 20% of mild and moderate dysplasias and 6% of invasive cancers (Lorincz et al., 1986, 1987a and 1987b). Reid et al. (1987) found that HPV 31 sequences occurred in 11% of CIN I lesions, 13% of CIN II lesions, 13% of CIN III lesions and 9% of invasive cancers. Beaudenon et al. (1986) have detected HPV 33 sequences in 4-8% of CIN lesions and cervical invasive carcinomas. The joint prevalence of HPV 16/33 sequences has been found to be 83% in penile intraepithelial lesions (Barosso et al., 1987). Finally, HPV 35 sequences have been found in 1% of anogenital intraepithelial neoplasias and 4% of anogenital cancer (Lorincz et al., 1987b).

The prevalence data thus indicate a definite association between certain HPV and cervical cancer. In particular the association between HPV 16 and cervical cancer is fairly consistent as summarized in Table I.3. Nonetheless, considerable variation in these rates has been observed; the reasons are not known but a number of explanations are possible. First, these variations could reflect one in diagnostic assays. As will be discussed in Chapter II, a number of different assays are available for the detection of HPV sequences and variation in the performance of these assays will inherently lead to different

results. It is evident from Table I.3 that different types of assays and hybridization conditions have been used for the detection of HPV sequences. Secondly, different selection biases may accordingly distort the observation. It is difficult to compare the prevalence data from the various studies as the referral pattern and demographic characterization of the study subjects have been insufficiently defined. Thirdly, the number of study subjects has generally ranged from 7 to 50 and the confidence intervals on the HPV 16 positive proportions are very large. With such overlapping and wide confidence intervals derived from small sample size, it is precarious to comment on the significance of the apparent geographic variations (Munoz et al., 1988). Nonetheless, the possibility of a true geographic difference cannot be presently excluded.

I.3.5.2. Specificity of association between particular HPV and cervical cancer

If specific types of HPVs are causally related to cervical cancer, then the occurrences of these viruses might exhibit some degree of specificity. Consequently, they should be present at a higher frequency in cervical cancer cases than in non-cervical cancers or normal tissues. It has been observed, from virtually all prevalence studies, that HPV 16 is preferentially associated with invasive cancers. However, between 60 to 80% of samples from precancerous squamous cell conditions such as Mobus Bowen and Bowenoid papulosis (Ikenberg et al., 1983), or vulvar intraepithelial lesions (Reid et

al., 1987) as well as 33% of adenocarcinomas (Yoshikawa et al., 1985b) are found to contain HPV 16 or 18 sequences. Other non-cervical squamous cancers have also been shown to contain HPV sequences. These include 80% of samples from vulvar carcinomas (Reid et al., 1987); 49% of penile cancer (McCance et al., 1986b); 60% of anal squamous carcinomas (Palmer et al.,1987); 4.1% of anaplastic lung carcinomas (Stremlau et al., 1984); 46% (Maitland et al., 1986) and 1.6% (Lonig et al., 1985) of oral carcinomas; 28% of human tongue carcinomas (de Villiers et al., 1985) and 2% of carcinomas of head and neck (Ostrow et al., 1987). In addition, about 25% of samples from squamous cell carcinomas of the esophagus have been found to react to mixed probes containing HPV 16, 18, 11 and 13 (Kulski et al., 1986) and about 30% of samples from bronchial squamous cell carcinomas have been found to react to mixed probes containing HPV 16, 18, 11 and 30 (Syrjanen et al., 1987).

The occurrence of HPV 16 and 18 in normal cervical tissues is not accurately known. Estimates of these sequences vary from 0% to 48%. Thus, 0% has been reported by Wagner et al. (1984) and Scholl et al. (1985); 1.75% by Schneider et al., (1985); 3.2% by Fuchs et al. (1988); 11% by McNab et al. (1986) and Toon et al. (1986); 20% by Burk et al. (1985) and 48% by Murdoch et al. (1988). When age matched controls have been used, the estimate of HPV 16 sequences has been found to be 28% (Meanwell et al., 1987) and that of HPV 16 and 18 sequences between 18-64% depending on the age and geographic location of the patients (Reeves et al., 1987). One possible explanation for this discrepancy is that most studies have used statistically small

numbers of unmatched controls. Furthermore, the sampling method for the cases and controls have varied; Meanwell et al. (1987) and Murdoch et al. (1988) have collected full depth biopsies including the basal layers from paired matched internal control (i.e. histologically normal cervices). It is not always possible to ascertain which sampling methods were used by other investigators.

Taken together, these data seem to indicate an apparent lack of specificity for HPV 16/18 sequences in cervical cancer. While the specificity argument enhances the plausibility of causal inference, lack of it should not negate it (Susser, 1986). Departure from the ideal will occur if other factors including HPVs are posed as causes of cervical cancer or, alternatively, if HPV 16 and 18 are considered as the causes of many cancers. Indeed, zur Hausen (1982) has argued that HPVs act in synergism with other co-factors in the genesis of cervical cancer. Based on the prevalence data, it is plausible that HPVs could also be involved in the genesis of noncervical squamous carcinomas.

I.3.5.3. Dose response relationship of the association between particular HPV and cervical cancer

A dose response relationship is present when varying amounts of the putative causal factor are directly related to varying amounts of the effects (Weiss, 1981). This effect can be assessed either as a function of intensity of exposure or duration of exposure. Both types of exposure can be measured at the individual or the population

levels. Therefore, an individual who is exposed to the particular HPV by either type of exposure should have an increased risk of developing cervical cancer. Such exposure could occur by venereal means as there is compelling evidence to believe that the HPVs found in the genital tract are venereally transmitted. Prevalence studies point to the ubiquity of these viruses in both the male and the female genital tracts (Reid et al.,-1987); McCance et al., 1985c); de Villiers et al., 1987; Grussendorf-Conen et al., 1986a and 1986b). The presence of HPV has also been reported in semen (Ostrow et al., 1986) and the presence of HPVs also correlates with that of other venereally transmitted agents, thus suggesting that the HPV share a common mode of transmission with other venereally transmitted agents (Syrjanen et al., 1984). Consort studies have also demonstrated that these viruses are transmitted from female to male (Stein, 1980; Levine et al., 1984; Barosso et al., 1987 Schneider et al., 1987b). Based on the fact that a woman's sexual lifestyle is a risk factor for cervical cancer, it is plausible to postulate that the more a woman is exposed to venereally transmitted HPV the higher is her probability of developing cervical cancer. By virtue of its venereal etiology, cervical cancer is bound to be associated with sexually transmitted agents. However, if a sexually transmitted HPV is etiologically related to cervical cancer, then these HPV must show an association which is much stronger than the other sexually transmitted agents. Unfortunately, the present state of knowledge does not enable one to distinguish if HPV and cervical cancer are simply covariables of sexual behaviour.

The application of the dose-response concept for viral infec-

tion at the population level has been reviewed by Rawls (1982). The relationship between the incidence of virus induced cancers in a population, in which the cancers are occurring at a spontaneous rate, can be mathematically formulated. Under these circumstances, the incidence of cervical cancer attributable to HPV should be linearly related to the fraction of the population exposed to the HPV. In two studies that have addressed this issue, the evidence has been conflicting. A study by Kjaier et al. (1988) compared the prevalence of HPV 16/18 in Denmark to that in Greenland, a region where the incidence of cervical cancer is 5.7 times higher than that in Denmark. Surprisingly, the age adjusted prevalence rate of HPV 16/18 in Greenland was only 67% that of Denmark, thus not supporting a role for these viruses in the incidence of cervical cancer. By contrast, some support for the dose-response concept is derived from the observation that Latin American populations with a high incidence of cervical cancers (Reeves et al., 1982) have a high proportion of the population exposed to HPV (Reeves et al., 1987). Since both studies used the same assay, the most likely explanation for the observed difference is one of different HPV types being involved in the two different populations.

I.3.5.4. Biologic plausibility of the association between particular HPV and cervical cancer

Biologic plausibility rests on the assertion that cause and effect are consistent with the current pathogenic mechanism of the disease, be it at a cellular level or from an animal model (Fletcher

et al., 1982). Currently postulated experimental models of viral carcinogenesis assert that the initiating virus persists in the induced tumours either as DNA, RNA or transforming proteins (Branton et al., 1985). In addition the virus must be able to induce cellular transformation in vitro and such cells should induce tumours when inoculated in experimental animals.

This paradigm has been fulfilled to a large extent in the case of HPV 16 and, to a lesser extent, in the case of HPV 18. Most studies show that over 50% of invasive cervical tumours harbor HPV 16 and in many instances the particular HPVs appear to be integrated in the tumour cell (McNab et al., 1986; Choo et al., 1987; Shirasawa et al., 1986; Meanwell et al., 1987; McCance et al., 1985b; Yoshikawa et al., 1985b; Tsunokawa et al., 1986; and diLuca et al., 1986). In only one instance, however has it been demonstrated that the HPV 16 DNA is unambiguously integrated in the host cell genome (Durst et al., 1985). In the other instances the possibility of experimental variation accounting for the apparent integration cannot be excluded (Maitland et al., 1987). Studies mapping the integration sites of HPV 16/18 on specific chromosomes have shown that the viral sequences are integrated on different chromosomes. For the sample studied, the integration of HPV 18 occurs in the 5'-end of the c-myc proto-oncogenes (Popescu et al., 1987a and 1987b; Durst et al., 1987). In the case of the established cervical epithelial cells the integration patterns show remarkable specificity in opening the circular viral DNA within the early genes 1 and 2 (E1-E2) open reading frames (Schwarz et al., 1985). In addition, the E6-E7 open reading frames of HPV 16/18 are

specifically transcribed or expressed as protein in cervical cancer cell lines and in most of the primary tumour biopsy specimens tested so far (Schwarz et al., 1985; Pater et al., 1985, Smotkin et al., 1986; Smotkin et al., 1987, Schneider-Gadicker et al., 1986; Seedorf et al., 1987, Androphy et al., 1987, Alwardy et al., 1987; Choo et al., 1987; Spence et al., 1988; Banks et al., 1987). The expression of the E6/E7 proteins suggests that they may function as transforming proteins in the maintenance of the cancer phenotypes.

The phenomenon of in vitro cellular transformation by HPV 16 has been studied in some detail. HPV 16 has been shown to induce malignant transformation of NIH 3T3 cell lines (Yasumoto et al., 1986; Tsunokawa et al., 1986); to immortalize primary human keratinocytes (Pirisi et al., 1987) or to induce oncogenic transformation of primary rat cells in cooperation with ras oncogenes (Matlashewski et al., 1987).

The concept of oncogenic potential of HPV is further strengthened by parallel studies from animal papillomaviruses or other malignant human disease. Both the Shope papillomavirus in cotton tailed rabbit and bovine papillomavirus (BPV) in cattle have been shown to have oncogenic potential under natural and experimental conditions (Lancaster and Olson, 1982). In the case of BPV, an early gene product E6, similar to that of HPV 16, has been found to be expressed in the transformed cell lines (Yang et al., 1985). Shope papillomavirus normally gives rise to self-limiting benign papillomas that generally regress spontaneously except in about 25% of cases. Under these circumstances, transformation to squamous cell carcinoma

is observed and a certain proportion of the episomal PV genomes is found to be integrated in the cellular DNA (Wettstein and Steven, 1982). In the human, epidermodysplasia verruciformis (EV), a skin condition predisposing to malignancy, has been linked to certain HPVs. It is currently believed that HPV type 5, 17 and 37 may act as a co-factor in malignantly converting sun-exposed areas of EV patients (Orth, 1977).

I.3.5.5. Strength of association between particular HPV and cervical cancer

A strong association between a cause and an effect is generally accepted as better evidence for a causal relationship than a weak one (Evans, 1978). Estimate of the strength of the association between HPV 16 and 18 and cervical cancer is fraught with many problems and inaccuracies. Uncontrolled case-series observation has found a relative risk of 13.9 for having invasive cervical cancer and HPV 16 (Prakash et al., 1985). A case control study of Australian women reported a relative risk of 16 for cervical cancer and HPV koilocytic cells (Mitchell et al., 1986). Unfortunately, no specific HPV can be incriminated in this study as koilocytes are a relatively insensitive indicator of specific HPV infections. Odds ratio estimate from a case-control study of Latin American women found that the risk for HPV varied. The odds ratio for HPV 16 and 18 was 5.8 for women below 30 years and 1.6 for women between 30 to 49 years (Reeves et al., 1987). This trend is also supported by the observation of Meanwell et al

(1987) who found no significant difference between cases and controls when the age of the subjects was controlled.

I.3.5.6. Temporal relationship of the association between particular HPVs and cervical cancer

The correct sequence of events for a causal relationship necessitates that cause should precede effect. Therefore, if HPV 16 and 18 are related to cervical cancer, infections by these particular viruses should precede the outcome of cancer. Various prospective cohort studies have shown that untreated cervical HPV lesions evolve like CIN lesions (Syrjanen et al., 1985a). Epidemiologic observations show that HPV 16, and to a lesser extent HPV 18 sequences, preferentially segregate in invasive cervical cancer than in CIN lesions. However, this observation is open to two equally plausible interpretations. First, it is possible that the particular subset of patients who are infected with these HPV at the CIN stage will eventually progress to cervical cancer. Alternatively, it is equally possible that the invasive cancer patients have an increased susceptibility to superinfection by these viruses. To date there is only one documented case-report of an HPV antigen positive patient progressing from CIN stage to cervical cancer (Syrjanen et al .,1985b). By virtue of the inherent limitations of the case-series designs, it is not possible to distinguish if these viruses causally precede the cancer or are secondary, opportunistic pathogens of the cancer state.

I.3.5.7. Epidemioligic coherence of the association between particular HPVs and cervical cancer

The assertion that these particular viruses are etiologically related to cervical cancer is compatible with both the biologic properties of these viruses and the natural history of cervical cancer. Furthermore, an association between HPV 16 and cervical cancer has been consistently demonstrated in all the studies. However, the temporal relationship of these viruses to disease has not been demonstrated since no study to date has shown that the presence of these viruses predicts the development of cancer.

Epidemiologically, the concept of specificity of cause implies that the cause must be a necessary and sufficient element in bringing about the effect. If HPVs are necessary cause of cervical cancer, then all cases of cervical cancers should have the viruses. But the prevalence data so far do not support this contention. It has been argued that the HPV negative cervical cancer cases are due to hitherto undiscovered HPVs (zur Hausen, 1985). If HPVs are sufficient in causing cervical cancer, then noncases of the disease should not have the virus. This contention is again not supported by the prevalence data so far. It has been argued that HPV per se may be insufficient in causing cervical cancer and that cofactors such as smoking or Herpes simplex virus type II may be synergistically required in the genesis of cervical cancer (zur Hausen, 1982). However, this contention, probable as it may be, is not supported by either epidemiologic (Brinton et al., 1986b) or molecular evidence (Prakash

et al., 1985; McNab et al., 1986; Kjaier et al., 1988).

Taken together, the epidemiologic and molecular data are too sparse for one to unequivocally conclude whether the association between HPVs and cervical cancer represents one of major causality, risk factor or covariability of sexual behaviour. Clearly more defined epidemiologic studies are required to delineate the exact role of particular HPVs in the genesis of cervical cancer.

I.4.0. Hypothesis

The natural history of invasive squamous cell carcinoma of the cervix is thought to involve a gradual progression from an initiation event through preinvasive stage to invasive cancer. Based on the body of evidence implicating particular HPV in the genesis of cervical cancer, together with the potential oncogenic properties of these viruses, it is hypothesized that certain types of HPV initiate neoplastic changes which progress to invasive cancer. Available evidence suggests that a number of types of HPV may induce changes of the cervix called CIN, but only those associated with HPV 16, 18, 31, 33 or 35 are likely to progress to invasive cancer.

I.4.1 Aims of the study

It is proposed to verify this hypothesis by means of a retrospective, matched case-control study design. By comparing the relative frequency of these particular HPV in the preinvasive biopsies of cases

and appropriately matched controls, it could be possible to establish whether the presence of HPVs predicts the developent of cervical cancer. If particular HPVs are predictive of cervical cancer, then the occurrence rates of these viruses should be higher in the pre-invasive biopsies of cases than in those of controls. By contrast, if the HPVs are secondary to the cervical cancer then the relative frequency of these viruses would be equal in the preinvasive biopsies of cases and controls. However, the feasibility of this study heavily depends on the ability to detect viral markers in retrospective specimens. This issue is addressed in chapter II, and the study design is elaborated in chapter III. The results and discussion of the case-controls study are presented in chapter IV.

The uniqueness of this study is the examination of preinvasive biopsies of cervical cancer cases and controls for exposure to specific HPVs. To date no published studies have been so designed. Thus, the conclusion that can be derived from this study would also contribute to new knowledge on the possible association between HPV and cervical cancer. It will distinguish whether or not these viruses are predictive of the disease or are secondary pathogens of the diseased state.

CHAPTER II

LABORATORY DETECTION OF VIRAL MARKERS

II.1.0. Biology of human papillomaviruses.

The papillomaviruses (PV) belong to genus A of the family papovaviridae while the polyomaviruses constitute genus B (Tooze, 1980). These two genera differ in important genome composition and biological properties. Characterization of the PV virions has been hampered by the lack of suitable in vitro systems for culturing these viruses or the availability of sufficient virions from natural hosts. The notable exceptions are the Bovine papilloma viruses (BPV) and the HPVs inducing warts in human. Consequently, most of the knowledge of the genetic organization and expression of the PVs have been derived from BPV in productively infected fibropapilloma and in the abortively transformed mouse, C127 cell line. In humans, the HPV causing deep palmo-plantar wart has been a ready source of virions for characterization of the HPV.

The basic organization of all the PVs so far examined seems to be similar. The naked virions are composed of a 55 nm icosahedral capsid that encloses a double stranded, circular DNA genome spanning approximately 8000 nucleotide pairs in length and having a molecular weight of $5X10^6$. In virions or infected cells, the PV genome normally exists in three forms called form I, II and III (Jenson et al., 1984). Form I is a covalently closed, circular, supercoiled molecule with a sedimentation coefficient of 23S; form II is an open circular molecule with a sedimentation coefficient of 17S and form III is a linear molecule with a sedimentation coefficient of 16S. All three forms of DNAs appear to exist as episomal, non-integrated, replicating plasmids

in both benign and malignant lesions (Jenson et al., 1984).

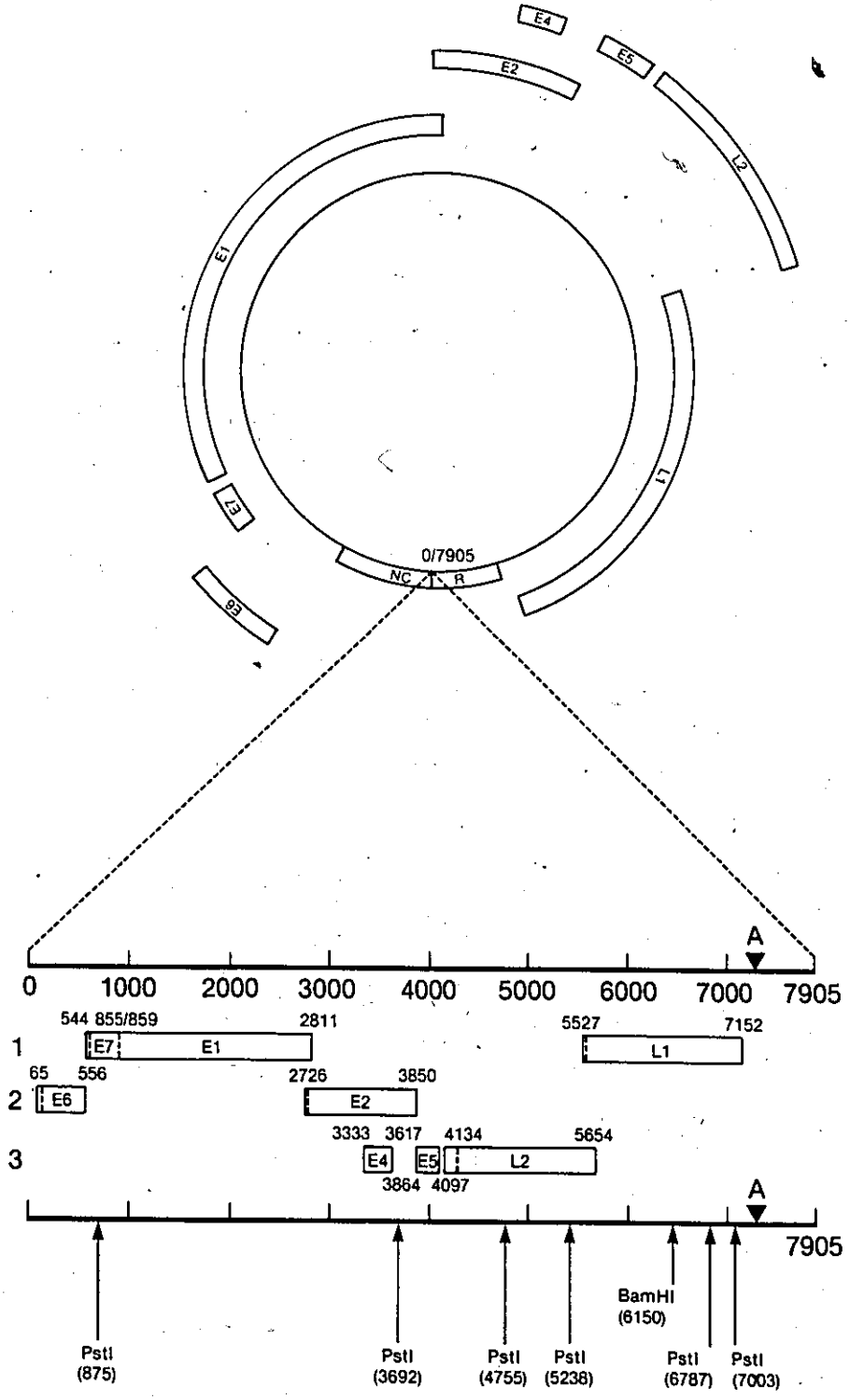
Molecular cloning and nucleotide sequence determination have shown that all the genetic information of PVs, in contrast to the polyoma virus, is encoded in only one strand of the genome. This is consistent with the unique polarity of the viral mRNAs detected in many systems (Stenlund et al., 1985). By analogy to the polyoma viruses, the coding strand has been functionally divided into early (E) and late (L) domains. The early domain, encoding functions required prior to DNA relication, is transcribed in BPV transformed cells and consists of 8 open reading frames, designated E1 to E8. The late domain, encoding functions required for viral capsid synthesis, is transcribed in productively infected bovine fibropapilloma and consists of 2 open reading frames, L1 and L2 (Howley, 1983, Baker, 1987). By convention, the corresponding domains of all PVs are called E and L region in reference to the BPV genome (Danos et al., 1982, 1983). In addition, a non-coding region (nrc) is located in each papillomavirus genome between the stop codon of L1 and the first start codon of the E6. For the BPV, functions implicated in genome replication and gene expression are controlled by the non-coding region. The 3' end of this domain contains a polyadenylation signal for the transcript of L region, while the 5' end contains typical promoter elements for polymerase II transcription. The early region domain is required to establish and maintain viral control over the infected cell (Baker et al., 1987.) and probably contains information for the virus replication (Lusky et al., 1983, 1984, and 1985) and transformation functions (Dimao et al., 1986a and 1986b, Yang et al.,

1985). The late domain is involved in capsid protein synthesis and virion assembly. The L1 ORF, encoding major capsid component, is highly conserved among the PVs and this is consistent with the observed cross-reactivity of the genus specific antisera directed against disrupted virions (Jenson et al., 1980, Schiller, 1987, Nakai et al., 1986). The L2 ORF is less conserved among PVs and probably encodes a type specific, minor capsid component (Komly et al., 1986). The HPVs are believed to encode similar functions, although the evidence is indirect (Giri and Danos,1986). The genetic organization of HPV type 16 genome, based on the BPV genome organizaton, is shown in Figure II.1. The polyadenylation signal for the L1 transcript is indicated by the letter A.

PVs are classified into types and subtypes based on species specificity and polynucleotide sequence homology. Virus isolates are considered new types if there is less than 50% nucleic acid homology with existing known types; PV with greater than 50% but less than 100% nucleic acid homology are classified as subtypes (Coggin and zur Hausen, 1979). Thus, reassociation kinetics have shown that HPV 11 and 6, which were originally defined as different subtypes, share 82% of homology (Giri and Danos,1986). Similarly, heteroduplex analysis combined with genome sequencing have shown that the E1, E2, E6, E7, L1 and L2 ORFs of HPV 16 and 33 share between 65 and 75% homology and hence could be regarded as subtype of the same virus (Cole and Streeck,1986).

PVs are host specific and show a high degree of epitheliotropism (Jenson et al., 1984, Orth et al., 1978). They are unique in

Fig. II. 1. Genome organization of HPV 16 based on Seedorf et al., 1985



that virion assembly is linked to the cellular events of squamous differentiation and keratinization. All three types of epithelia namely; cutaneous, mucosal and metaplastic ones are susceptible to infection by PVs. In productive wart infection, vegetative viral DNA synthesis can be detected in differentiating cells of the epithelium but not in the basal layer or the underlying fibroblasts. Viral capsid production and virion assembly occur in the upper, terminally differentiated layer (Orth et al., 1971). The phenotypic expression of most cutaneous warts and mucosal papillomas are believed to be similar. Productive HPV infection is characterized by proliferation of the prickle cells (acanthosis), cytoplasmic vacuolization (koilocytosis), nuclear alteration (atypia or pyknosis) and the production of excess keratin. In flat condyloma of the cervix, principally associated with HPV16, hyperplasia, koilocytosis and cytological atypia are commonly observed (Richart and Crum, 1984).

II.1.1. The detection of HPV markers.

The accurate diagnosis of HPVs depends on the presence of suitable viral markers. The most common viral markers are one of the following: antibodies to the particular HPV, viral proteins (antigen), whole virions or viral nucleic acid sequences. In addition, the presence of HPV koilocytes has also been used as a means of diagnosing HPV. The relative sensitivity and specificity of these markers will vary according to the clinical condition because the expression of these viral markers are intimately linked to the state of the cellular

differentiation.

Little is known of HPV serotypes to enable serodiagnosis of these viruses. Therefore, the detection of antibodies to specific HPV is not feasible; a notable exception being HPV 1 which replicates to high copy numbers in cutaneous warts. Virions for immunological studies can be readily purified from these warts (Komly et al., 1986). However, detection of viral antigen may be possible under certain circumstances. The L1 open reading frame of all PVs appears to encode a major group specific antigen that can be detected by appropriate immunochemical means (Nakai et al., 1986). By contrast, the assay for detecting L1 antigen has two serious limitations. First of all it is an insensitive indicator of specific HPV infection by virtue of being shared by all PVs. Secondly, the expression of these late proteins is intimately tied with the state of differentiation of the infected cell. Thus, poorly differentiated neoplastic cells of CIN 3 or invasive lesions will not permit completion of the viral life cycle, and hence will be negative for viral L1 proteins. Current estimates of the rate of detecting HPV L1 antigen vary between 50-70% in plantar wart (Jenson et al., 1980), 40-60% in verrucae (Jenson et al., 1982) and 40-70% in mild dysplasia (Jenson et al., 1984). Diagnostic antisera to the putative protein of L2 open reading believed to contain type specific epitotype is not currently available.

The detection of complete virion particles can be done by electron microscopy (Almeida et al., 1962). This assay, however, will again be seriously limited as in the case of the viral antigen. First

of all, all PVs are morphologically indistinguishable, hence no specific types of HPV can be identified by this technique. Secondly, the assembly of complete virions will be absent in CIN 3 or invasive cervical cancer cells. The sensitivity of electron microscopy for the detection of HPV virions varies between 20-45% (Pilotti et al., 1984, Laverty, 1979, Meisel et al, 1981 and 1985).

The presence of koilocytes is considered to be pathognomonic for HPV infection (Koss, 1987). Although, the evidence is indirect, studies using electron microscopy (Laverty, 1979), immunochemistry (Jensen et al. ,1980) and in situ hybridization (Syrajen et al., 1986), have shown that about 50% of samples with koilocytes contain virions, viral antigen or viral DNA. Gross et al., (1982) have suggested that the morphology of the koilocytes may predict the particular type of HPV involved. However, like the detection of virions or viral antigen, there are inherent problems in using koilocytes as a viral marker (Boon et al., 1985). Since virally induced changes require a certain degree of cellular maturity for expression, the expression of koilocytes will decrease as the severity of the CIN increases (Winkler et al., 1984). Hence, the absence of koilocytes may not necessarily reflect the absence of virus. This has been observed with HPV types 6,11 and 42 that have been isolated from 44% of intraepithelial lesions without clear koilocytosis (Barrosso et al., 1987). Furthermore, koilocytosis may be the pathologic manifestation of some HPV infection and not of all HPVs. Finally, koilocytes may be an insensitive marker of specific viral markers and the histologic diagnosis of koilocytes have been shown to

be inherently prone to a high degree of inter- and intra-observer variations (Horn et al., 1984).

The most accurate and sensitive way of detecting specific HPV, to date, is by the technique of molecular hybridization. This assay depends on the use of single stranded (denatured) known molecularly cloned HPV DNA as probe to anneal to identically denatured target HPV sequences in a suitable hybridization reaction. The conditions of hybridization can be varied so as to detect only homologous HPVs (stringent) or partially homologous HPVs (non-stringent). The stringency of the hybridization reaction is largely controlled by how much below the theoretical melting temperature (T_m) is the hybridization reaction performed. Generally, the lower the reaction temperature from the T_m, the lower is the stringency (Heilman et al., 1980). The lowering of the T_m can be achieved by altering the incubation temperature of the reaction and the use of chemicals such as formamide or salt in the hybridization reaction. Three hybridization methods are currently used, these are; Southern blot, filter in situ and tissue in situ.

Before the analytic, case-control study could be carried out, it was necessary to establish the validity of the assay to be used for measuring the presence of HPV sequences. The validation of an assay requires extensive laboratory characterization prior to use. The chief features of validation studies include determination of sensitivity, specificity, and the range of normal values for that test. The sensitivity of an assay is defined as the proportion of subjects with the disease and positive test result, while the

specificity of an assay is defined as the proportion of subjects without a disease and a negative test result (Fleiss, 1981). Thus, these two test parameters will define the true positive and the true negative and delineate the range of normal. Other important parameters of such studies are: adequate sample size, control for important confounders such as age or severity of cervical neoplasia and the reliability of the assay. The reliability of an assay i.e. the degree of constancy when the assay is repeatedly applied to the same individuals can also potentially affect selection in a case-control study (Fleiss,1986).

The choice of an assay for conducting the case-control study was dictated by the retrospective nature of the study. It was necessary to use the tissue in situ hybridizaion method to detect the presence of HPV sequences in formalin fixed cervical biopsies that had been preserved over the years. Although the tissue in situ hybridization method has been used for the detection of HPV sequences, it has not been properly validated. The first aim, therefore was to characterize the tissue in situ hybridization in relation to two most commonly used hybridization methods viz the Southern blot method and the filter in situ hybridization. For this purpose, a pilot study, consisting of a mixed population with various disease categories, was used. Once the main laboratory parameters of the tissue in situ hybridization were determined, a cross-sectional study of the target population in British Columbia was undertaken to determine the main parameters of this assay at a population level.

II.2.0.0. Materials and methods.

II.2.1.0. Pilot study population for comparison of assays.

The choice of study subjects for the pilot study was dependent on the availability of appropriate patients who could be sampled for all three assays. Three categories of patients from two different populations were sampled. The first category consisted of 26 Canadian patients having genital condylomas, and seen at University affiliated clinics in Hamilton. Clinical specimens from 6 penile lesions, 6 vulvar lesions, 2 groin lesions and 12 cervico-vaginal lesions were collected. The second category consisted of 23 Panamanian women with newly diagnosed cervical cancers. The third category consisted of 21 Canadian and 12 Panamanian patients who had undergone hysterectomy for diseases other than neoplasia. Histologic examination confirmed the diagnoses of condyloma or invasive cervical cancer in the first and second categories and the absence of these lesions in the third category. Although the third category was considered "normal" for the purpose of this study, histologic examination revealed 9 patients with cervicitis, 11 patients with cervical metaplasia and 13 patients with unremarkable cervices.

II.2.1.1 Target study population

The characterization of the tissue *in situ* hybridization assay at a population level was done in the target population of patients

who reside in the province of British Columbia. These women had been evaluated for cervical disease, at some point of their lives, by the gynecological cytology screening laboratory of the Cancer Control Agency of the Province of British Columbia (CCABC).

The CCABC offers free Province wide cytology service to hospitals, clinics and private practitioners. Out of the 600,000 smears that are annually examined, 90% of the materials are referred by the general practitioners. A complete follow-up is available on virtually all patients including those having suspicious or positive cytology. Follow-up on the latter cases is achieved by writing letters to the doctors with a request for repeat smears at annual intervals. If the smears are not received on the date requested two subsequent letters are sent at two monthly intervals and if there is still no response the Public Health Nurses contact the patients directly asking them to go to their physicians (Fidler et al., 1957).

The composition of the population served by this facility, based on information provided by the Provincial Division of Vital Statistics, has been detailed elsewhere (Boyes et al., 1970; Boyes et al., 1981). The proportion of women ever screened by the Agency was assessed in 1969. It was found that approximately 78% of all women in the Province and over the age of 20 were screened by the Agency. In 1983 there were 1,023,400 women over age 20 years in British Columbia and 490,407 smears were evaluated, indicating that approximately 48% of the women are screened annually. Even though the patients served by the facility vary from 14 to 75 years in age, the bulk of the patients fall within the 25 to 29 year age group. The

mortality rates and the incidence of cervical neoplasia over the past three decades are shown in Table II.1 & II.2, respectively. From this population, approximately 40 patients from each of the following histologically diagnosed disease category were sampled. These included CIN I, II, III, microinvasive, occult invasive and clinically invasive. Biopsies from the CIN group were obtained from patients attending the colposcopy clinic for the investigation of abnormal Pap smear or colposcopic examination. The collection of biopsies from the CIN categories were continuously carried out over a three week period until the required number was reached. No attempt was made to select samples on the basis of koilocytosis or any other selection criteria. Biopsies from the invasive groups, i.e. microinvasive, occult invasive and clinically invasive categories, were obtained by doing a computer search for all such patients treated at the CCABC and then locating the available pathology specimens from the various hospitals. In addition, approximately 40 biopsies were obtained from women undergoing hysterectomies for reasons other than cervical neoplasia. The histologically normal cervices were derived from one of the following sources; cone biopsies, routine histologic biopsies or hysterectomy biopsies. Histological examination for the latter category revealed that 20 patients had normal squamo-columnar junctions or unremarkable cervices; 14 had cervicitis; 3 had atypia and 5 had metaplasia. Based on the published prevalence of HPV sequences in invasive cervical cancer and CIN lesions, a sample size of 40 patients per histologic diagnosis was chosen in order to establish whether or not the observed differences in the various disease categories are due to sampling variation (Fleiss,1981).

Table II.1. Summary of Mortality Rates and Incidence Rates of Cervical Carcinoma in British Columbia

Refined Mortality Rates for Squamous Carcinoma of the Cervix in British Columbia

Year	Population in thousands over age 20	No. of Deaths	Rate per 100,000
1958	473.0	54	11.4
1962	503.4	65	12.9
1967	592.4	38	6.4
1972	713.1	43	6.0
1977	846.4	40	4.7
1983	1023.4	34	3.3

Incidence of Clinical Invasive Squamous Carcinoma of the Cervix Uteri in Women Over 20 years of Age in British Columbia

Year	Population in Thousands	Total Cases	Incidence per 100,000
1955	422.9	120	28.4
1962	503.0	78	15.5
1967	592.4	85	14.3
1972	713.1	66	9.2
1977	845.5	64	7.6
1983	1023.4	71	6.9

Table II.2 Cases of In Situ Carcinoma and Preclinical Invasive Carcinoma Detected in B.C.

Year	Cases Screened	In Situ Carcinoma	In Situ With Micro-Invasion	Occult Invasive Carcinoma	% Age Micro and Occult
1949-54	21,593	111	6	9	13.5
1955-59	114,250	506	33	21	10.7
1960-64	500,626	1,506	109	105	13.9
1965-69	1,055,626	3,158	140	160	9.5
1970-74	1,718,894	3,926	96	142	6.1
1975-79	2,462,733	7,510	103	112	2.9
1980-83	1,854,620	6,893	103	71	2.5
Total	7,728,620	23,645	590	620	5.5

II.2.1.2. Study designs and specimens collection.

An unmatched case-series study design was used for both parts of the investigation. From each patient whose samples were used for the pilot comparative studies, an attempt was made to collect cells from the lesions for filter *in situ* hybridization and then to obtain two biopsies of the lesions. This was not achieved in all cases. For the filter *in situ* hybridization method, cells from the cervical os or cervical lesions were collected by means of a cotton tipped swab and resuspended in 4 ml of phosphate buffered saline (PBS). The number of cells was counted in a hemocytometer and the cells were stored at -20°C until tested. The cell counts ranged from $1x10^4$ to $1x10^7$ epithelial cells per sample. Biopsies intended for the Southern blot analysis were snap-frozen in liquid nitrogen and transported to the laboratory where they were subsequently stored at -70°C until tested. Biopsies for histopathological examination and tissue *in situ* hybridization methods were fixed in 10% buffered, neutral formalin and processed by routine histologic methods.

From each patient whose biopsies were to be used in the tissue *in situ* hybridization method, sections were cut at 5 μm thickness from paraffin-embedded blocks. The sections were placed on poly-D-lysine (Sigma Chemical Co., St. Louis, MO) coated glass slides, fixed by baking at 60°C overnight, and shipped to McMaster University as such.

II.2.2.1. Preparation of Probe DNA.

Recombinant plasmid DNA containing HPV type 6C (de Villiers et al., 1981); HPV type 11 (Gissmann et al., 1982); HPV type 16 (Durst et al., 1983) and HPV type 18 (Boshart et al., 1984) were kindly provided by Dr. L. Gissmann and Dr. E. deVilliers (Deutsches Krebsforschung Zentrum, Heidelberg); those containing HPV 33 (Beaudenon et al., 1986) were obtained from Dr. G. Orth (Institut Pasteur, Paris). The HPV DNAs were used to transform E. coli for producing sufficient quantities for probes.

II.2.2.2. Transformation of E. coli.

The strain LE392 of E. coli, Ampicillin and Tetracycline Sensitive, was transformed by the Calcium Chloride procedure (Maniatis et al., 1982) as follows: A culture of E. coli in the logarithmic phase (0.4 to 0.6 Optical Density at 260 nm, approximately equal to 6 x 10^7 cells/ml) grown in Luria broth (10 g/L Bactotryptone, 5 g/L Bacto-yeast extract and 10 g/L NaCl) was chilled on ice for 10 minutes then pelleted at 4,000 g for 5 minutes. The pellet was re-suspended in 1/2 the original volume in transformation buffer (50 mM $CaCl_2$ and 10 mM Tris-Cl, pH 8.0), held at 4°C for 15 minutes then the cells were pelleted and re-suspended in 1/15 the original volume in transformation buffer and held at 4°C overnight. Approximately 100 ng of the recombinant HPV plasmid was added to the $CaCl_2$-treated cultures which were heat-shocked at 42°C for 2 minutes to maximize the efficiency of

DNA uptake. The cultures containing the appropriate antibiotics were then heated for 45 minutes at 37°C to allow the bacteria to recover and tenfold dilutions of the bacterial culture were plated on Luria agar containing Ampicillin at a concentration of 15 μg/ml.

Insertion of viral DNA into the Bam H1 site of the pBR322 inactivates the Tetracycline resistance gene. Several picked colonies growing on the Ampicillin agar were replica-plated on separate agar plates containing Tetracycline and Ampicillin respectively. Only those colonies showing Ampicillin resistance and Tetracycline sensitivity were selected for further analysis.

II.2.2.3. Small scale analytical purification of plasmid DNA from E. Coli.

The method of Birnboim and Doly (1979) was used for rapid isolation of plasmid DNA from E. coli. Two ml of an overnight culture, grown from a single colony in Luria broth containing 25 μg/ml of chloramphenicol, was centrifuged for one minute in an Eppendorf tube and the pellet re-suspended then digested for 10 minutes with 5 μg/ml of lysozyme in 100 μl of an ice-cold lysozyme buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0). The bacterial spheroplasts so formed were lyzed and the DNA concomitantly denatured with a 200 μl solution of 0.2 N NaOH and 1% SDS. The bacterial DNA was then selectively precipitated, with 150 μl of 3 M potassium acetate at pH 4.8 and was removed by centrifugation for 5 minutes. The plasmid DNA was precipitated from the supernatant with 2 volumes

of absolute ethanol at room temperature and pelleted by centrifugation as before. The pellet was washed once with 70% alcohol and dissolved in 50 µl of Tris-EDTA (TE) buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0).

II.2.2.4. Large scale isolation of plasmid DNA for radiolabelling.

The HPV DNAs were purified by the cesium chloride banding technique for the purpose of unambiguous restriction analysis and labelling (Maniatis et al., 1982). A scaled-up version of the Birnboim and Doly procedure was used and the DNA was extracted from the plasmids as in the previous Section with the exception that 500 ml of bacterial culture was initially used. After dissolution in TE buffer the DNA was further purified by cesium chloride-ethidium bromide density gradient. One gram of cesium chloride per ml of DNA solution was used and 0.8 ml of ethidium bromide (10 µg/ml) was added per 10 ml of DNA solution. This solution was centrifuged in a Beckman type-50 rotor at 45,000 rpm for 36 hours at 20°C. The band containing plasmid DNA was collected and the Ethidium bromide removed by 5 extraction cycles with a solution of equal volume of 1-butanol saturated with iso-amyl alcohol (Maniatis et al., 1982). The aqueous phase was extensively dialysed against several changes of TE buffer and the amount of DNA estimated by absorbance value at 260 nm (1.0 D unit 260=50 µg/ml DNA, (Davis et al., 1986).

II.2.2.5. Restriction enzyme analysis of HPV clones.

All HPV clones were characterized by digestion with the restriction enzyme PstI according to standard procedure (Maniatis et al., 1982). The digested DNA was then analyzed by two kinds of gel electrophoresis-the minigel and vertical gel (Maniatis et al., 1982). In both types, 1 to 1.2% agarose gel was used; in the minigel, electrophoresis was carried out at 60V for 2 hours while in the vertical gel, elctrophoresis was carried out for 10 hours at 25V. In both cases, the electrophoresis buffer was TBE buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.01 M EDTA). The DNA was stained with ethidium bromide, at a concentration of 0.5 μg/ml, and photographed using UV illumination.

II.2.2.6. Radiolabelling of Probe viral DNA.

For use as probes in the filter in situ and Southern blot hybridization method ,the HPV DNAs were excised from the plasmids to avoid possible false positive reactions due to the presence of pBR322 sequences in some clinical specimens (Ambinder et al., 1986, Barbacid, 1984). These DNAs were radiolabelled by the "oligo" reaction procedure. The HPV DNAs were not excised from plasmids when used as probes in the tissue in situ hybridization in order to enhance the signal associated with the probe (Singer et al., 1986). These DNAs were radiolabelled by the nick translation procedure.

A commerical nick translation kit (Bethesda Research

Laboratories, Gaithersburg, MD), based on the method of Rigby et al., (1977) was used to label the DNA by nick translation. One μg of DNA was incubated for one hour at 13°C in a 50 μl reaction mixture containing Nick translation buffer (50 mM Tris-Cl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 μg/ml bovine serum albumin (BSA), 0.25 ng of DNase I in 0.15 M NaCl, 10 mM of each of the following nucleotides dATP, dTTP and dGTP, 6 units of E. coli DNA polymerase I and 100 μCi of either α-[^{32}P]dCTP, or α-[^{35}S]dCTP (New England Nuclear, Lachine, Quebec, Specific activity 300 - 600 Ci/mMol). The reaction was stopped with 25 μl of 250 mM EDTA pH 7.9 and the final volume brought to 100 μl with TE buffer. The labelled DNA was separated from the unincorporated nucleotides by spinning the mixture (1600 g, 4 minutes at 4°C) in a Sephadex G-50 column (bed volume 0.9 ml) that has previously been equilibriated in TE buffer (Maniatis et al., 1982).

Probe DNA was also labelled by the "oligo" reaction using the method of Feinsberg and Vogelstein (1983). Approximately 50 ng of restriction endonuclease treated, gel purified HPV fragment DNA was denatured by boiling in 5 ul of distilled water. After cooling for 15 minutes, a solution containing 25 mM of Tris-HCl, 2.5 mM MgCl, 5 mM of 2-mercaptoethanol (pH 8.0), and 100 uM of each of the following nucleotides dATP, dGTP, dTTP in 1 mM Tris-EDTA (pH 7.5) containing 90 optical density unit/ml of a random oligonucleotide was added to the reaction and mixed by vortexing gently. The reaction was started by adding 1 ug of nucleic acid grade BSA, 100 uCi of α-[^{35}S]dCTP and 2.5 units of large fragment DNA polymerase I (Kornberg polymerase). The

tube was gently mixed and incubated at room temperature overnight. The reaction was then stopped and the labelled DNA was separated as for the nick-translation method.

A specific activity of greater than 10^8 cpm/μg DNA was achieved for each probe. Two types of probes were used in all the hybridization methods used in the laboratory characterization of the assays; (a) a mixed probe composed of HPV 16 and 18 DNA and, (b) a mixed probe composed of HPV 6C and 11 DNA. In order to determine the prevalent type of HPVs as detected by the tissue in situ hybridization in the target population, individual probe for HPV 16, 18, and 33 and a mixed probe for HPV 11 and 6C were used. The choice of mixed HPV 11 and 6C probe in this instance was justified by the fact that these two viruses share extensive homologies (Wagner et al., 1985).

II.2.2.7. Filter in situ hybridization method.

For the filter in situ method, the clinical samples were thawed, the cells pelleted at 2,000g for 10 minutes and resuspended in 400 μl of a DNA diluent buffer consisting of 5 μg/ml of denatured salmon sperm DNA in 6X SSC (1X SSC = 0.15M sodium chloride and 0.015M sodium citrate, pH 7.0).

Filter in situ hybridization was performed by a modification of the methods of Wagner et al., (1984) and that of Grunstein and Hogness (1975). Samples consisting of 100 μl were applied under vacuum to a nitrocellulose filter by means of a 96-well manifold apparatus (Schleicher and Schuell, Keene, N.H.). Cell lysis and DNA

denaturation were effected by placing the filter for 5 minutes onto 3 MM Whatman paper which had been previously soaked in 1M NaCl/0.5M NaOH. The procedure was repeated once. The filters were then neutralized by overlaying them twice for 5 minutes onto 3 MM Whatman filter paper previously soaked in 1.5M NaCl/0.5M Tris buffer (pH 7.4). In order to digest cellular debris and mucus, the filters were then air-dried for approximately 15 minutes, placed into a plastic bag, and treated with 2 mg/ml of proteinase K in 10 mM Tris (pH 7.8), 0.5% sodium dodecyl sulfate (SDS) and 0.5 mM EDTA. The filters were washed three times in chloroform (5 minutes each) and given one final wash in 2X SSC to remove loose cellular debris. The DNA was fixed onto the filter by baking at 80°C for 2 hours. The HPV DNA sequences were detected by a modificiation of the method described by Thomas (1980). The filters were prehybridized for 2-3 hours at 42°C in a prehybridization buffer consisting of 50% formamide, 5X SSC, 50 mM sodium phosphate, pH 6.5, 250 μg/ml of sonicated, denatured salmon sperm DNA and 5X Denhardt's solution, containing 0.02% each of BSA, Ficoll and polyvinylpyrrolidone. The hybridization buffer consisted of 4 parts of prehybridization buffer, 1 part of 50% (w/v) dextran sulphate and $5X10^5$ cpm/ml of ^{32}P-labelled specific DNA probes. Prior to use, the hybridization cocktail was boiled for 5 minutes at 100°C and then cooled on ice to achieve denaturation of the probe DNA. Following previously published reports (20,28), hybridization was carried out under stringent conditions at 42°C (Tm-17°C) overnight and the filters were washed four times under non-stringent conditions (Tm-42°C) at 42°C in 2X SSC containing 0.1% SDS, each wash lasting for

1 hour. The filters were enclosed in Saran wrap and exposed for 1-3 days to x-ray film at -70°C using a Kodak intensifying screen. The filters were subsequently washed under stringent conditions (Tm-17°C) using the conditions of non-stringent wash except that the procedure was carried at 68°C. A final wash in 0.1X SSC and 0.1% SDS was carried out at room temperature for 15 minutes and autoradiography was done as before. All autoradiograms were independently scored by three observers and those specimens recorded as positive by two or more observers were considered as positive.

The specificity of the hybridization reaction was controlled by testing serial dilutions of Caski cells which contain 500 copies of HPV-16 per cell (Yee et al., 1986) and similar dilutions of human embryonic fibroblast cells which do not contain HPV DNA. The limit of detection of this hybridization method was determined by a reconstruction experiment in which tenfold dilutions of HPV 16 DNA ranging from 100 pg to 10 ng were added to 10^6 human embryonic fibroblast cells.

II.2.2.8. Southern blot hybridization method.

The frozen biopsies were thawed, washed in phosphate-buffered saline (PBS) and resuspended in a small volume of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM Na_2 EDTA. The specimens were minced thoroughly with scissors and digested for 24 hr at 37°C with Proteinase K at a concentration of 500 μg/ml, in the presence of 1% (w/v) sodium dodecyl sulphate (SDS). After 2 extractions with

re-distilled phenol saturated with 100 mM Tris-HCl pH 8.0, 10 mM Na_2 EDTA, the aqueous phases were pooled and extracted twice with water saturated anhydrous ethyl ether. Nucleic acids were then precipitated from solution at -20°C by addition of two volumes of 95% ethanol in the presence of 0.3M sodium acetate, pelleted (15,000 g for 15 min at 4°C), rinsed briefly with 95% ethanol, drained thoroughly and re-dissolved in 50 mM Tris-HCl pH 7.5, 100 mM NaCl and 2 mM Na_2 EDTA. This solution was incubated first for 2 hr at 37°C in the presence of 50 μg/ml RNase A (from bovine pancreas) and then for 24 hr with 100 μg/ml proteinase K. Phenol extraction, ether extraction and ethanol precipitation were repeated and the DNA pellet was dissolved in 10 mM Tris-HCl pH 7.5, 1 mM Na_2 EDTA (TE buffer).

The concentration of DNA was determined by optical density (OD) at 260 nm. Complete digestion of each sample, as estimated by staining with Ethidium Bromide, was obtained with 10 units of restriction endonuclease Bam Hl per μg of DNA in a reaction mixture containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol (Cleland's Reagent; DTT), 1 mM spermidine and 100 μl/ml nuclease-free BSA, for at least 5 hr at 37°C. Phenol extraction, ether extraction and ethanol precipitation were again repeated and the resulting DNA pellet was dissolved in TE buffer at a concentration of 0.5 mg/ml. Gel electrophoresis on 1% (w/v) agarose gels was performed using 20 μg aliquots of the digested DNA sample in a volume of 40 ul. Following electrophoresis, the gels were stained with 1 μg/ml ethidium bromide for ultraviolet visualization. The transfer of DNA from the gels to nitrocellulose membranes was accomplished by the Southern

blotting method (Southern, 1975). The DNA containing gel was soaked in an excess of 50 mM NaOH for 40 minutes then neutralized with a solution containing 3 M NaCl, 0.5 M Tris-Cl, pH 7.4 for one hour and transferred to nitrocellulose filter as follows. The gel was placed on 2 sheets of Whatmann 3 MM chromatography paper saturated with 20X SSC (Standard Saline Citrate) and the borders sealed with plastic Saran wrap. The nitrocellulose filter paper (Sartorius) was placed on the gel which was overlaid with Whatmann 3 MM paper, followed by a stack of paper towels and a light weight. Transfer was done overnight then the filter was fixed by baking for 3 hours at 80°C and stored in sealed plastic bags until used for hybridization. Prehybridization and hybridization were then done as for the filter *in situ* method. However, in keeping with standard practice to initially identify partially homologous HPV (Burk *et al.*, 1985, Crum *et al.*, 1985, Durst *et al.*, 1983, Gissmann *et al.*, 1983), prehybridization and hybridization were carried out in 20% formamide at 42°C under non-stringent conditions (Tm-42°C). In order to identify any homologous HPV, the filters were first washed at non-stringent conditions, then washed at stringent condition to identify only specific types of HPV. For the purpose of comparison, the results obtained under stringent wash for the Southern blot were regarded as being comparable to those obtained under stringent hybridization and wash for the remaining two assays (Gissmann *et al.*, 1983). The autoradiograms were scored by three observers as for the filter *in situ* hybridization method.

The limit of detection of the Southern blot method was

determined by a reconstruction experiment containing 100 to 1 genome equivalent of HPV 16 DNA per cell.

II.2.2.9. Tissue in situ hybridization.

A modification of the method of Haase et al.,(1985) was used for the tissue in situ hybridization. Tissue sections were deparaffinized in xylene and sequentially hydrated in descending grades of alcohol ranging from 30% to 100%. Each hydration step lasted 5 minutes. The DNA was denatured in situ by treating the section in 0.2N HCl for 10 minutes. In order to facilitate diffusion of the probe, the sections were treated with a 25 μg/ml of proteinase K in 0.05M Tris-HCl, pH 7.4, for 10 minutes at 37°C. The sections were then fixed in a 4% paraformaldehyde solution in PBS and washed in PBS containing 0.2% (w/v) glycine for 15 minutes to remove the paraformaldehyde. This was followed by a final wash in PBS. The sections were then dehydrated in ascending grades of alcohol and air-dried for at least 30 minutes. Hybridization cocktail contained 2X SSC, 400 μg/ml of denatured salmon sperm DNA and varying concentrations of formamide. Formamide concentration of 10% (TM-42°C) and 50% (TM-17°C) were used for non-stringent and stringent conditions, respectively. To the hybridization solution was added $1x10^5$ cpm/ml of [^{35}S]-labelled specific HPV DNA. The whole hybridization cocktail was denatured by boiling for 5 minutes for 100°C, chilled on ice and adjusted to a concentration of 10 mM in dithiothreitol. Hybridization was carried out at 37°C for 18 hours and the sections were washed as follows: one

hour wash at room temperature in 2X SSC containing 1mM DTT followed by one 30 minute wash in 2X SSC. The slides were then washed for 10 minutes in two changes of a high wash buffer containing 50% formamide, 2X SSC, 0.01M Tris-HCl, pH 7.4, and 0.001 M EDTA. Finally, the slides were rinsed two times for 15 minutes in 2X SSC and dehydrated in ascending grades of alcohol. Autoradiography was carried out by dipping the slides in melted Kodak NTB2 nuclear track emulsion which was diluted in distilled water as directed by the manufacturer (Kodak Canada Ltd., Toronto). The slides were dried in the dark for 1/2 hour and exposed at 4°C for 4 weeks in a light-proof plastic box containing calcium sulfate as dessicant. Before developing, the slides were equilibrated to room temperature and immersed for 3 minutes in Kodak D-19 developer, followed by 1 minute in 1% acetic acid and 3 minutes in Kodak fixer. After 10 minutes washing in distilled water, the sections were lightly stained by hematoxylin then eosin and independently scored by three readers as for the two previous hybridization methods. Cytopreparation of Caski cells and Siha cells respectively harboring 500 and 10 copies of HPV-16 (Yee et al., 1986) were included to determine the lowest copy number of HPV genomes detectable by this method. The specificity of the hybridization signals was controlled by the following procedures. The specificity of the DNA signal was demonstrated by digesting the proteinase K treated sections with 200 μg/ml of DNase (Sigma) in 20 mM Tris (pH 7.4) for 60 minutes at 37°C. The specificity of the RNA signal was demonstrated by treating the proteinase K digested sections with a solution of 100 μg/ml of DNase-free ribonuclease A and 10 units of

ribonuclease T1 (Promega) in X2SSC for 30 minutes at 37°C. In both cases the slides were then washed in X2 SSC and post-fixed in paraformaldehyde and hybridized as before. The sequence specificity of the HPV probes was determined by performing the hybridization with the heterologous plasmid vector (pBR-322) probe or an adenovirus type 12 probe which shows no known specificity for cervical tissues.

II.2.2.10. Statistical analysis.

The reliability of the hybridization methods in the diagnosis of HPV was examined by the percent agreement and by using an unweighted Cohen's kappa statistic (Fleiss, 1981). The percent agreement is the number of samples for which two methods yielded identical results relative to the total number of samples examined. The kappa statistic is an attempt to correct the percent agreement for agreement occurring by chance alone. The association of HPV with cervical neoplasia was characterized by contingency table type analysis and the Chi Square test for independence of the row and column variables (Everritt, 1977). In addition, the log-linear model was used to test whether the association between HPV and cervical neoplasia depended on a third variable such as the age of the patient. Chi Square analysis and the kappa statistic were done on the HP3000 mainframe computer at the health science computational centre, McMaster University, using in-house fortran algorithms. The remaining data handling and statistical analysis were done by means of the SAS commercial statistical package (Carey, North Carolina) on the VAX and the IBM mainframe computers.

II.2.2.11. Scoring criteria.

Each of the assays considered here is subject to interpretive procedure and thus prone to inter-and intra-observer variation bias. To adjust for such potential biases the consenus readings of three independent raters were used for each of the assay. In addition, for the tissue in situ assay, the following set of objective criteria was developed and independently used by each of the three readers. A scale of 0 to 8 was used whereby 0 denoted negative; 1, doubtful; 2 denoted 1 positive cell in the entire section; 3 denoted between 2-3 cells per section; 4 denoted a weak positive; 5 denoted between 6-10 positive cells per section; 6 denoted between 11-25 positive cells per section; 7 denoted a classic positive usually consisting of more than 25 cells per section, and 8 denoted an unsatisfactory specimen. These criteria were applied irrespective of whether the signals were in the epithelium or in the stroma, however, the histogical location of the reactions were carefully noted in each case.

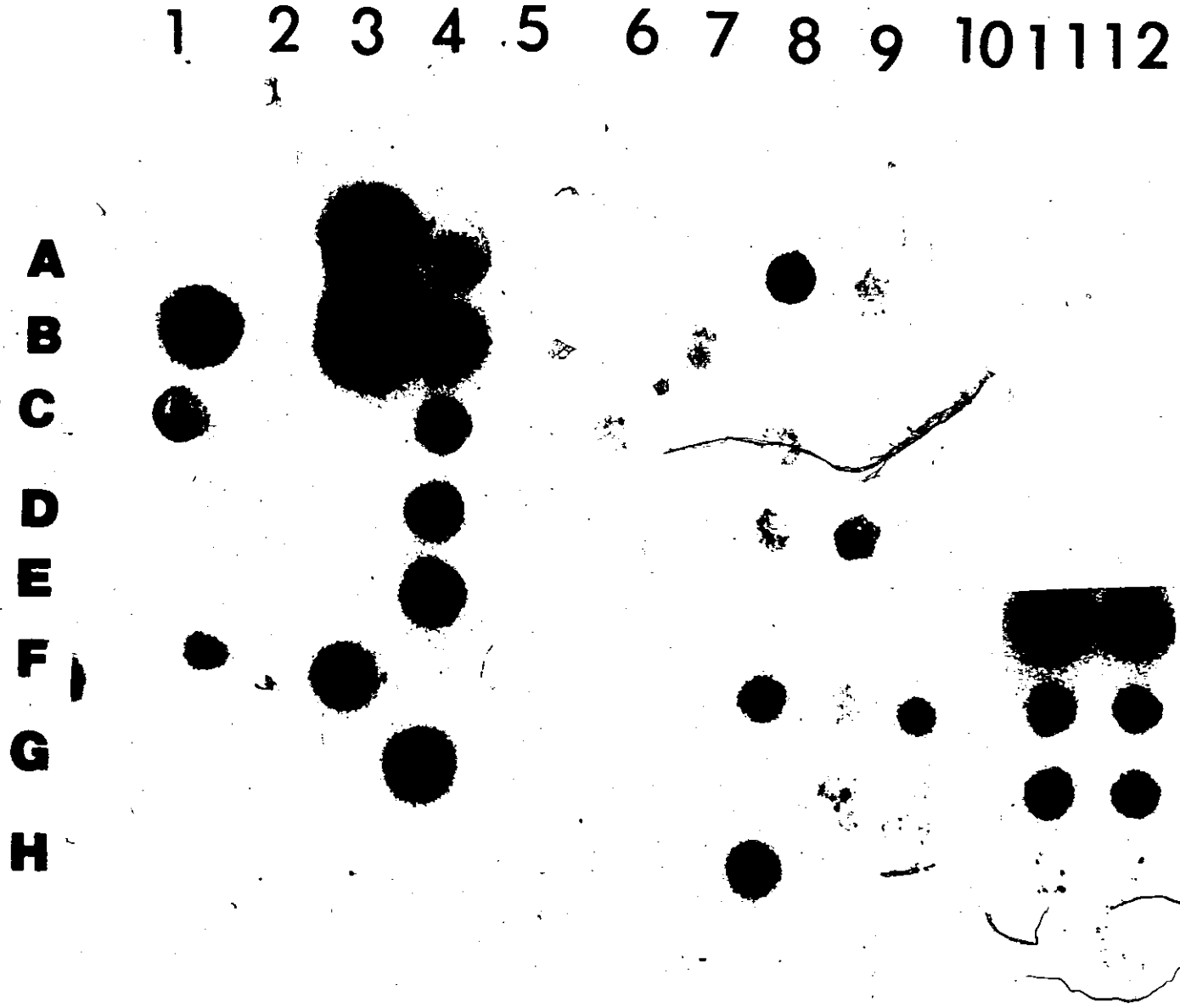
II.2.3.0. Results of the hybridization methods.

II.2.3.1. Filter in situ hybridization method.

The type of hybridization signals observed in clinical samples by the filter in situ hybridization method is shown in figure II. 2. This method appears capable of detecting HPV sequences within aggregates of cells (Wagner et al., 1985), giving rise to a discrete

Figure II.2. **Autoradiogram obtained by the filter in situ hybridization method.**

Exfoliated epithelial cells from the cervix were trapped on nitrocellusose paper, lysed with proteinase K and hybridized to HPV 16/18 probes under stringent conditions as described in the text. Rows A to H, columnns 1 to 9 contained clinical samples. The inset (rows A to D, columns 11 and 12) shows that there was no hybridization signal when a ten-fold dilution of human cells ranging from 10,000 to 10 cells per well was included as negative control. A positive control consisted of similar diiutuion of Caski cells which harbour HPV 16 sequences (rows E to H, columns 11 and 12). Any samples showing signals comparable to or weaker than those in rows H (Columns 11 and 12 were scored negative; any signals stronger than those were recorded as positive. A **clearly positive** signal is exemplified by the signal in row A column 3; a **clearly negative**, by that in row A,coumn 1, and **boderline** (+/-) signals in row A column 6.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

dot filling the whole well, as exemplified by samples in rows D and G, column 9 of figure II.2 or samples in rows F and G, columns 11 and 12. However, no correlation was observed between the number of cells initially present in the samples and the intensity of the signal, suggesting that the number of infected cells was probably more important than the absolute number of cells. When varying dilutions of HPV-16 DNA containing Caski cells were used (rows E-H, columns 11 and 12, Figure II.2), hybridization signals were clearly identifiable in wells receiving as few as 100 cells (row G, columns 11 and 12). However, when similar experiments were done with an HPV negative human fibroblast cell line, no detectable hybridization signals were observed even at the highest concentration of cells that was tested (rows A-D, columns 11 and 12, figure II. 2).

As shown in Table II.3., the filter in situ hybridization method detected HPV sequences in 62% of samples from condylomas. Of these, 35% reacted with HPV types 11/6; 12% with HPV types 16/18; 4% with all HPV probes and 12% were untypable. Among the 89% of HPV detected in invasive cancer cases, 22% comprised of HPV6/11; 44% of HPV 16/18 and 22% reacted with both types of probes. The specimens from the histologically normal cervices were found to contain HPV to a lesser extent; only 9.5% were found to contain HPV of type 6/11. Interestingly, all were from cervices with histological evidence of metaplasia.

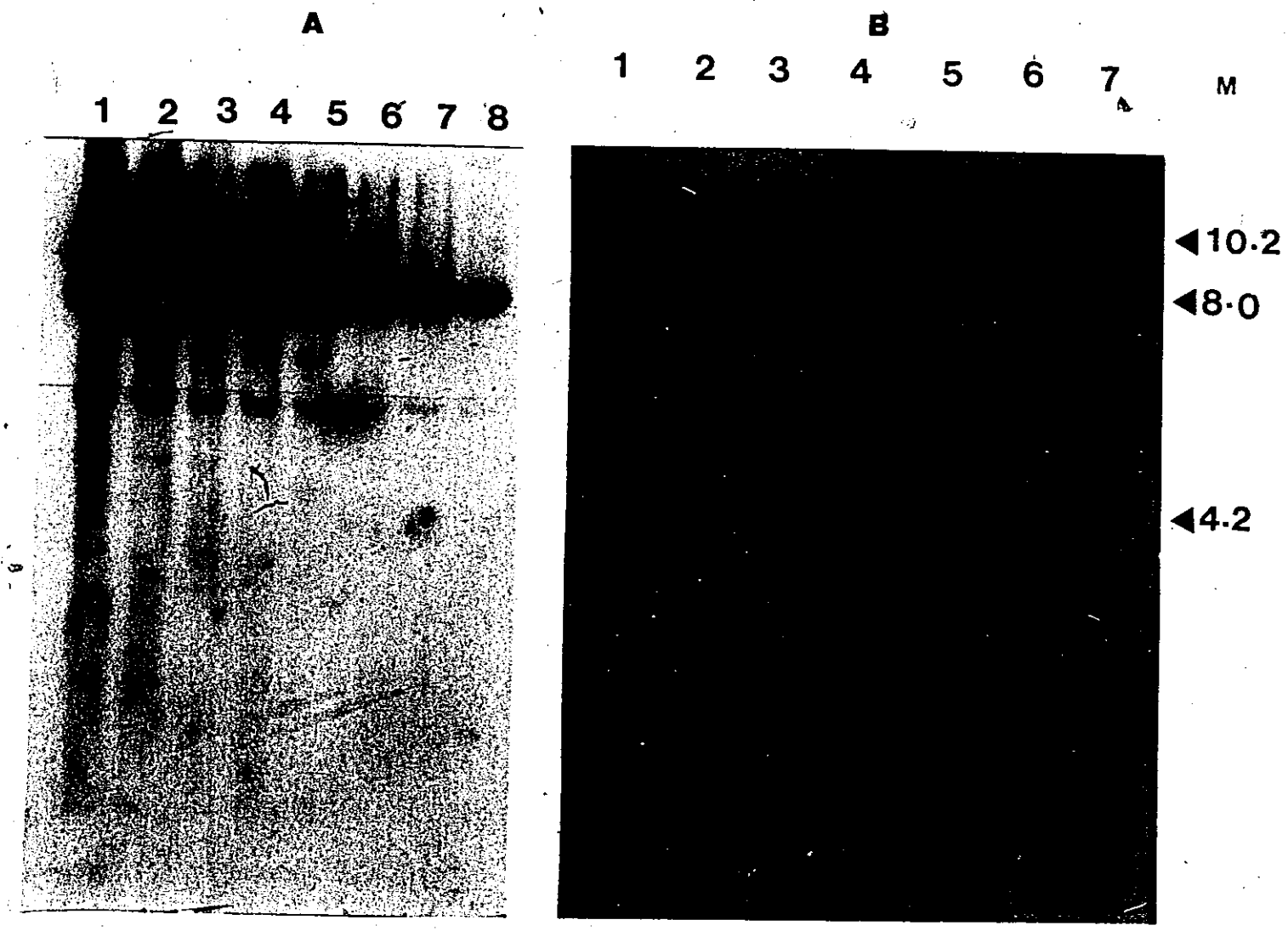
II.2.3.2. The Southern Blot Method

The Southern blot method could detect as low as 0.2 pg of HPV

Table II.3. Occurrence of HPV type by clinical conditions

Hybridization Assay	Histologic Diagnosis	No. positive/No. tested (%) for the following HPV types:			
		6 and 11	16 and 18	Other	Total
In situ filter	Condyloma	9/26 (35)	3/26 (12)	3/26 (12)	16/26 (62)
	Invasive cancer	4/18 (22)	8/18 (44)	0/18 (0)	16/18 (89)
	Normal	2/21 (9.5)	0/21 (0)	0/21 (0)	2/21 (9.5)
Southern blot	Condyloma	8/17 (47)	1/17 (6)	3/17 (18)	14/17 (82)
	Invasive cancer	0/20 (0)	13/20 (65)	1/20 (5)	14/20 (70)
	Normal	1/31 (3)	1/31 (3)	2/31 (6)	4/31 (13)
In situ tissue	Condyloma	13/25 (52)	3/25 (12)	(0)	18/25 (72)
	Invasive cancer	2/23 (7)	3/23 (13)	(0)	7/23 (30)
	Normal	2/33 (6)	0/33 (0)	(0)	2/33 (6)

Figure II.3. **Autoradiogram of Southern blot hybridization analysis.** High molecular weight DNA was extracted from cervical biopsies, cleaved with the restriction enzyme, Bam H1, and fractionated by gel electrophoresis then transferred to nitrocellulose paper by the method of Southern. The target DNA on the nitrocellulose paper was hybridized under non-stringent conditions then washed under non-sringent conditions. **Panel A** illustrates a reconstruction experiment to determine the lower limit of detection by this method. Varying concentrations of HPV 16 DNA were added to 5 ug of salmon sperm DNA. Lane 1 to 7 respectively contained 500 pg, 250 pg, 125 pg, 50 pg, 25 pg, 20 pg, and 10 pg of HPV 16 DNA. Lane 8 contained 0.2 pg of HPV DNA corresponding to 0.2 to 0.3 HPV genome per cell and is still positive. **Panel B** illustrates the types of hybridization signals seen in clinical samples. Lanes 1 to 4 contained DNA from condylomatous lesions and lanes 5 and 7, DNA from invasive cancer cells. The typical episomal HPV molecule is exemplified by the sample in lane 1 while the signals in lanes 5 and 7 could be consistent with either the HPV DNA being integrated in the cellular genomes or the existence of oligomeric forms of DNA.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 M
10·2
8·0
4·2

DNA, corresponding to 0.2 to 0.3 HPV genome per cell(Figure II.3.a). When the Southern blot method was applied to clinical samples a variation was the pattern of HPV DNA was observed. The HPV DNA from condylomatous lesions existed as episomal molecules. This was exemplified by the appearance of an 8 kb fragment after digestion with the single cut enzyme, Bam HI (Figure II.3.b., lane 1). Similar analysis of HPV DNA extracted from invasive cervical cancers sometimes yielded a series of bands (Figure II.3.b., lanes 5 and 7). This feature could be consistent with either the HPV DNA being integrated into the cellular DNA or the existence of oligomeric forms of HPV DNAs.

The Southern blot assay showed that 82% of samples from condylomas were positive for HPV sequences. Interestingly, 47% were HPV 6/11; 6% were HPV 16/18; 12% reacted with both probes and 18% were untypable. It was observed that 70% of samples from invasive disease contained HPV sequences comprised of 65% type 16/18 and 5% untypable. The percentage of samples from normal cervices reacting with HPV was found to be 13% which comprised of 3% type 6/11; 3% type 16/18 and 6% were untypable.

II.2.3.3. The tissue in situ hybridization method

For the tissue in situ hybridization assay the following parameters were investigated: method of labelling the probe DNA, stringency of the hybridization reaction and wash, length of exposure of the autoradiographic slides, sequence specificity of the HPV

Figure II.4. **Parameters of the tissue in situ hybridization method.** Tissue sections from paraffin-embedded biopsy samples were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K, and hybridized with ^{35}S-labelled HPV probes. **Panel a** illustrates the stronger signals observed when using probe labelled by the nick-translation procedure as compared to **panel b** which illustrates the weaker signals obtained with probe labelled by the "oligo" reaction method. **Panel c** illustrates the stronger signals observed by performing the reactions at non-stringent condition (Tm-42 as) compared to **Panel d** which illustrates the weaker signals obtained by performing the reaction at stringent conditions (Tm-17). The final magnification for all photomicrographs was X250. The final magnification for all photomicrographs was X250.

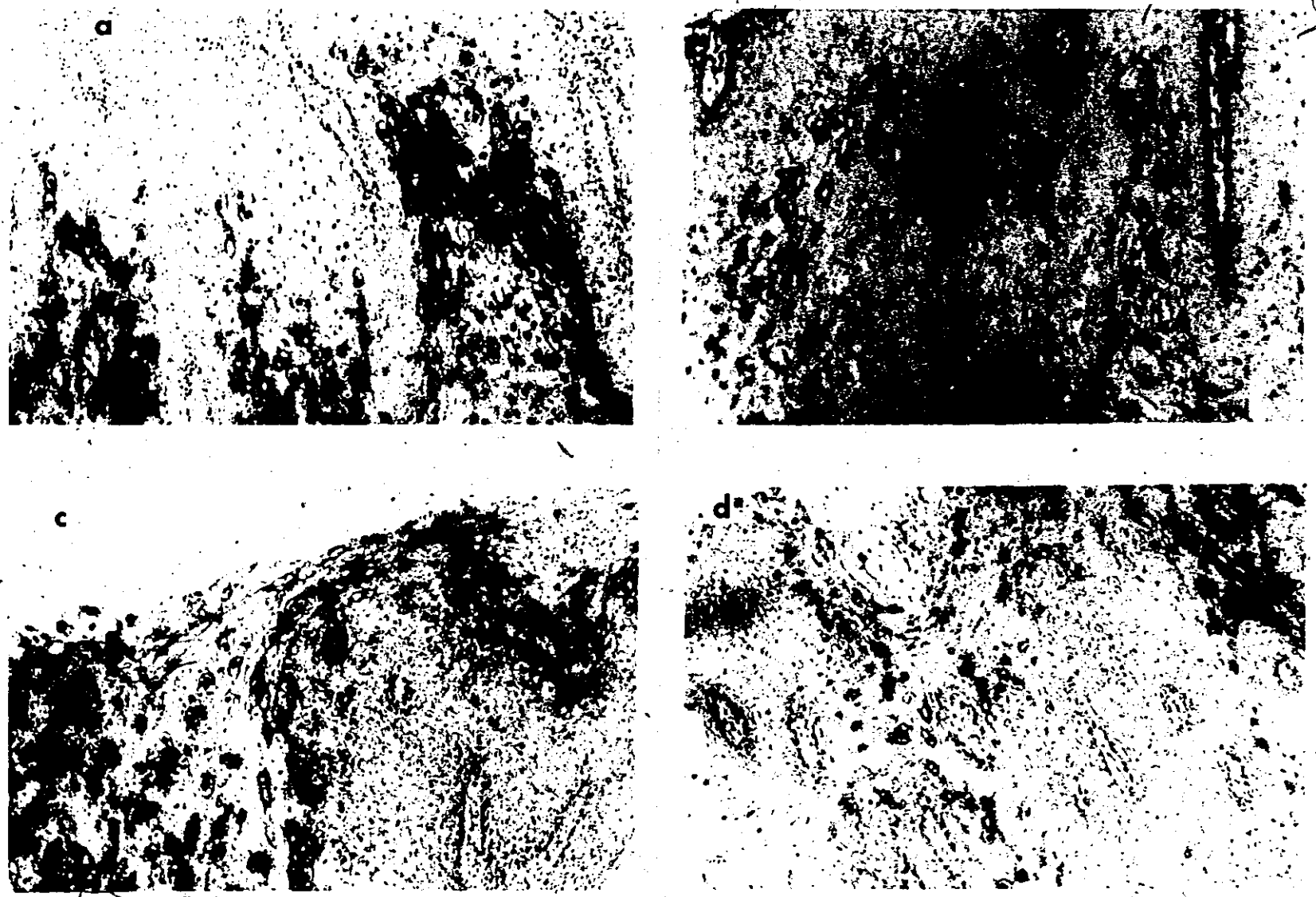
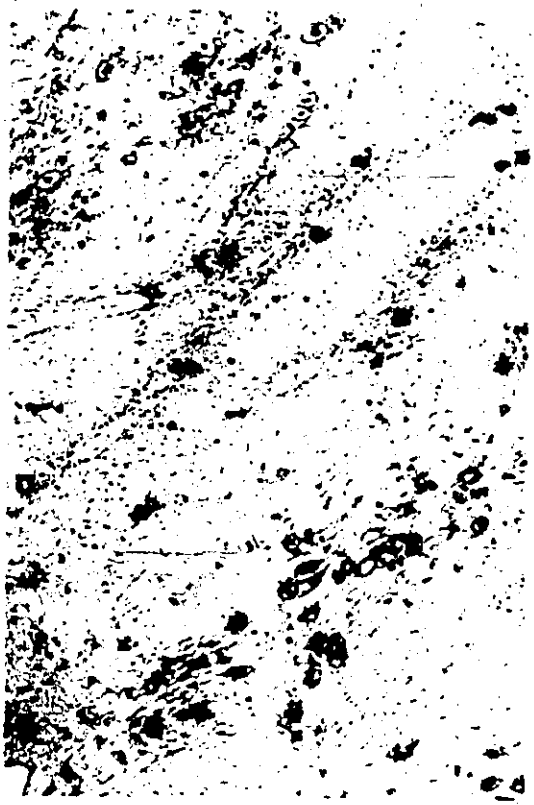

a
c
d

Figure II.5. Parameters of the tissue in situ hybridization method. Tissue sections from paraffin-embedded biopsy samples were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K, and hybridized with ^{35}S-labelled HPV probes. Panel a illustrates the non-specific signal observed when 10 mM DTT was omitted from the hybridization reaction and panel b illustrates the loss of signals when the hybrids were washed in high wash buffer at 50 ^{0}C. Panel c illustrates the stronger signals observed after 3 to 4 weeks of exposure as compared to Panel d which illustrates the weaker signals obtained after one week of exposure. The final magnification for all photomicrographs was X250.


b


d




c

probes and the limit of detection of this hybridization method. The most easily scored hybridization signal was observed when the probe DNA was labelled by the nick-translation technique using the whole HPV genome linked to the vector, pBR-322 DNA (Fig II.4.a). By contrast, a weaker signal was observed when the probe DNA consisted of only HPV fragment labelled by the "oligo" reaction method (Fig II.4.b). It was observed that hybridization reaction performed in 20% formamide resulted in stronger signal (Fig II.4.c) than that in 50% formamide (Fig II.4.d). When 10 mM DDT was omitted from the hybridization reaction, a fair degree of non-specific reactions was observed (Fig II.5.a). Furthermore, washing in high wash buffer at 50°C, led to essentially all of the autoradiographic signals being lost (Fig II.5.b). The best exposure time for the detection of the autoradiographic signal was found to be between 3-4 weeks (Fig II.5.c) and the weakest signals were seen after one week (Fig II.5.d). The sequence specificity of the particular HPV probe was demonstrated by the lack of significant hybridization when an HPV 6/11 containing condyloma specimen was hybridized to the heterologous plasmid vector pBR-322 (Fig II.6.a) or to an adenovirus probe with no known sequence specificity for cervical tissue (Fig II.6.b). The specificity of the hybridization signal for nucleic acid sequences was demonstrated by nuclease digestion. The bulk of the sequences appeared to be DNA as noticed by its RNase resistance (Figure II.6.c) and by its sensitivity to DNase (Figure II.6.d). The limit of detection of the tissue in situ hybridization method was estimated by using Caski and Siha cells which contain approximately 500 and 10

Figure II.6. **Characteristics of the tissue in situ hybridization method.**

Tissue sections from paraffin-embedded biopsy samples were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K and hybridized with ^{35}S-labelled HPV probes. **Panel a** illustrates the lack of hybridization signals observed when an HPV 6/11 positive specimen was hybridized to the heterologous pBR-322 probe. **Panel b** illustrates the absence of hybridization signals when the specimen was hybridizd to an unrelated adenovirus probe. **Panel c** illustrates that the signals obtained with HPV probe was resistant to RNase digestion . **Panel d** illustrates that the bulk of the hybridization signals was sensitive to DNase digestion. The final magnification for all photomicrographs was X250.


b

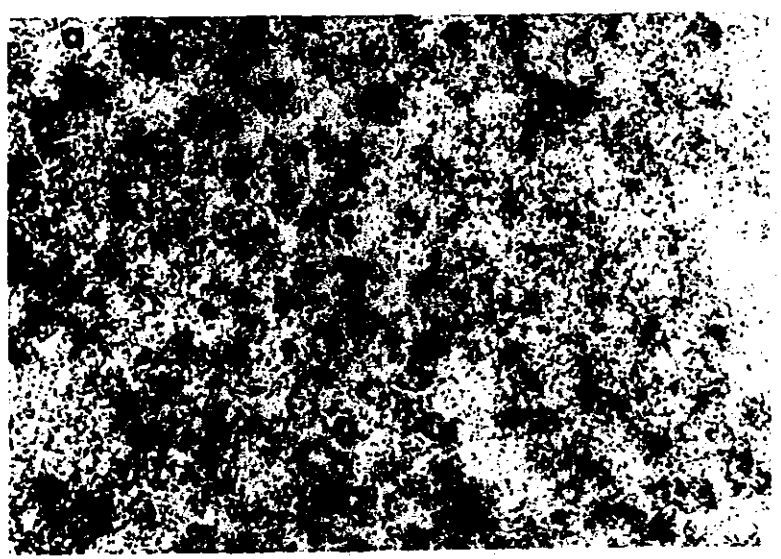

P

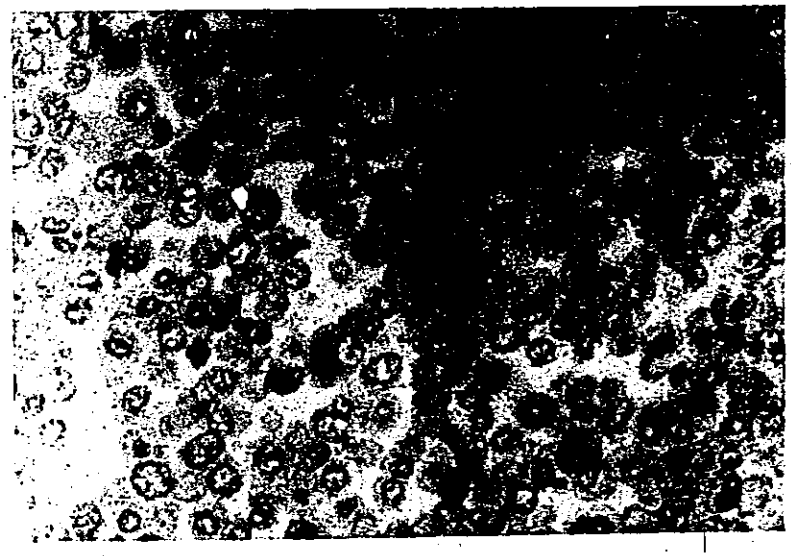

a


c

Figure II.7. **Parameters of the tissue in situ hybridization method and illustration of signals seen in condylomatous tissue.**

Tissue sections from paraffin-embedded biopsy samples and sections of cell cultures embedded in paraffin block were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K, and hybridized with ^{35}S-labelled HPV probes. **Panel a** illustrates that Caski cells containing 500 copies of HPV 16 genomes per cell were positive by the assay. **Panel b** illustrates the absence of hybridization signals in Siha cells containing 10 copies of HPV 16 genomes per cell. **Panel c** illustrates that the strongest signals were seen at the terminally differentiated epithelial layer of a condylomatous tissue. **Panel d** illustrates that the hybridization signals were located in the koilocytic cells of the condyloma. The final magnifications for the photomicrographs were X400 for panels a and b; X80 for panel c and X250 for panel d.





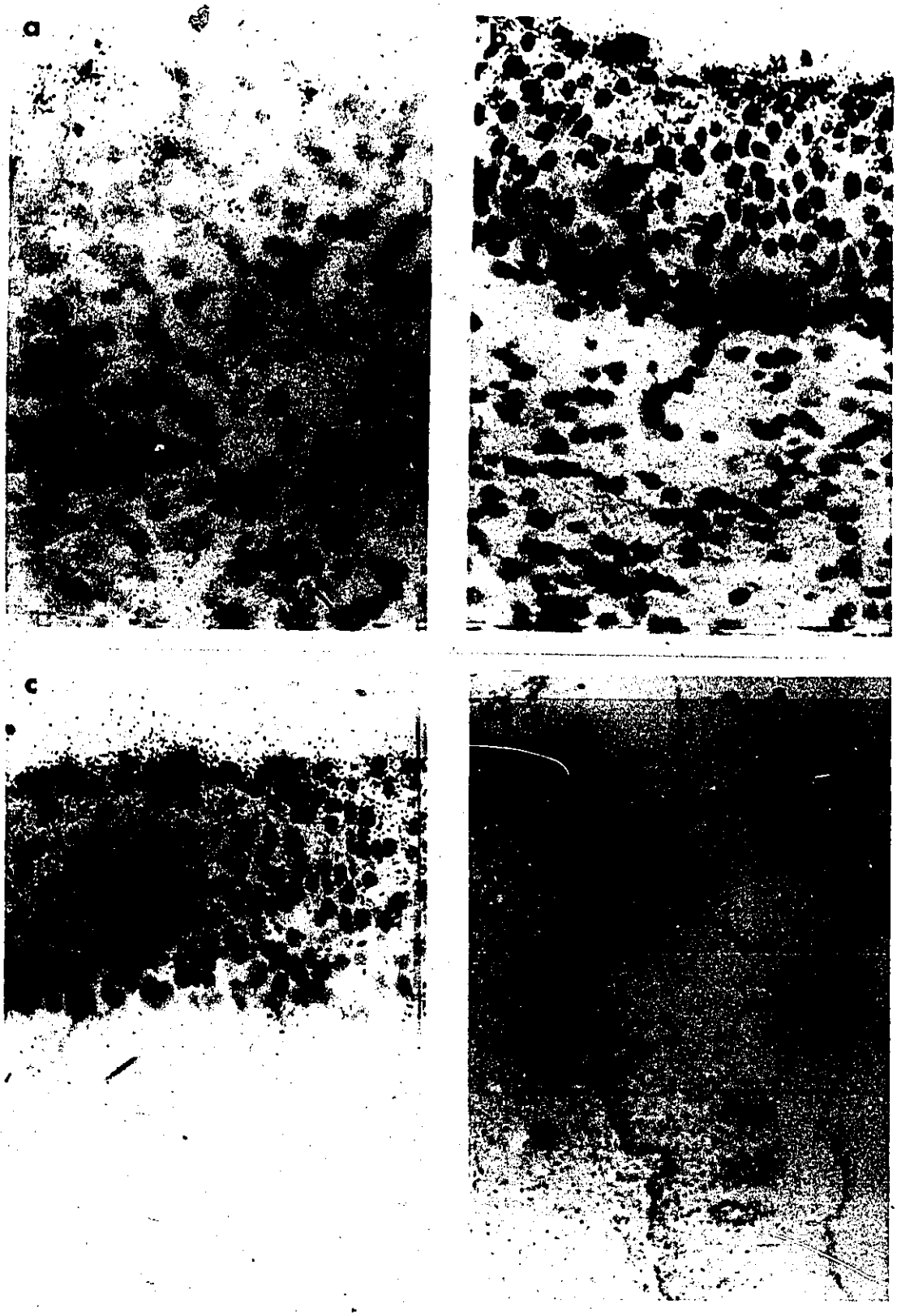

d
F2

copies of HPV genomes respectively (Yee et al., 1986). The Caski cells were positive(Fig II.7.a) but the Siha cells were negative(Fig II.7.b). Thus, the lower limit of detection of this method was estimated to lie between 10 and 500 copies of HPV genomes per cell.

From these observations, the conditions of the tissue in situ hybridization assay were optimized as follows. The probe DNA was labelled by the nick-translation procedure and the hybridization reaction was carried in 20% formamide (Tm-42^{o}C) containing 10 mM DTT. Excess probe was washed off in high wash buffer at room temperature and the hybrid exposed for 3-4 weeks before developing. When the tissue in situ hybridization method was used for the detection of HPV sequences in samples from condylomatous lesions, the positive silver grains were observed to be unevenly distributed in the different layers of the epithelium. The signals were weakest in the basal layers and progressively increased in intensity towards the terminally differentiated epithelial cells (Fig II.7.c) and the koilocytes (Fig II.7.d), which showed the strongest signals. The type of hybridization signals observed in CIN lesions exhibited some interesting variation according to the disease category. Specimens from CIN 1 lesions showed a weak hybridization signal (Fig II.8.a). By contrast, stronger hybridization signals were observed in lesions from CIN 2 (Fig II.8.b) and CIN 3 (Fig II.8.c). In the CIN lesions, like in the condylomatous ones, the most intense nuclear staining was seen in the superficial, well differentiated areas usually containing koilocytic atypia. Even in the cases where the positively staining nuclei were present in a few cells, the signals were still located in

Figure II.8. **Illustration of autoradiographic signals seen in samples from lesions of cervical intra-epithelial neoplasia (CIN) and histologically normal cervix.**

Tissue sections from paraffin-embedded biopsy samples were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K, and hybridized with ^{35}S-labelled HPV 16 probes. **Panel a** illustrates that the signals observed in CIN 1 lesions were weakest. **Panel b** illustrates the stronger hybridization signals in CIN 2 lesions. **Panel c** illustrates that the pattern of signals seen in CIN 3 lesions. **Panel d** illustrates that sample from histologically normal cervix did not show any significant hybridization signal. The final magnifications for the photomicrographs were X400 for panel a; X250 for panels b and c and X80 for panel d.


a
b
c

the most superficial epithelium where maturation is more likely to occur. However, in contrast to the condylomas, the intensity of staining in some areas varied from cell to cell even within the same layer of epithelium (Fig II.8.c).

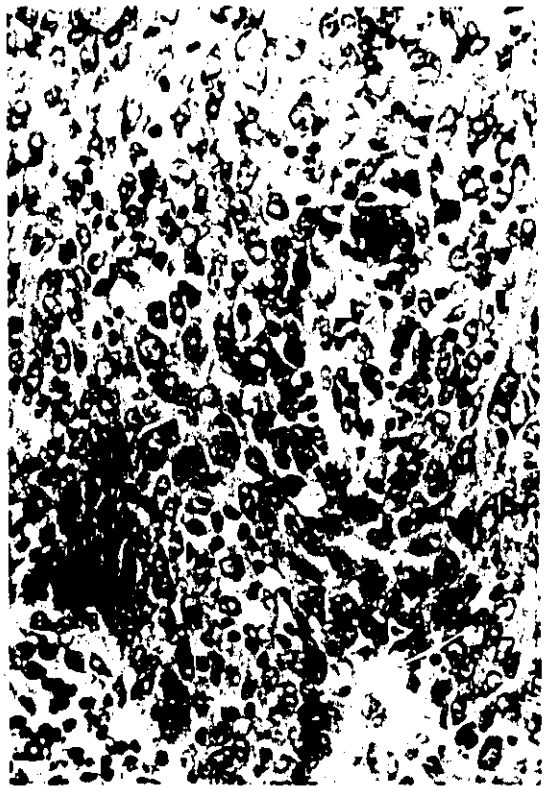
Essentially no hybridization signal was seen in the majority of tissues from histologically normal patients (Fig II.8.d); the morphologically normal epithelium of the columnar cells or the underlying stroma from condylomatous and CIN patients. Occasionally, focal positive signals were seen in the underlying stroma of some samples from GIN lesions. In such instances, the location of the hybridization signals was noted and separately analyzed. However, these specimens also reacted with more than one HPV probe and the vector probes but not against the unrelated adenovirus probes under stringent conditions of hybridization and wash.

Sometime sections from cancer cases appeared to show strong reactivity in and about cells at the periphery of the invasive squamous cell carcinoma islands, but most of the invasive cells were negative. This pattern was seen with invasive cells from occult-invasive (Fig II.9.a), microinvasive (Fig II.9.b) or clinical invasive samples (Fig II.9.c). However, marked focal positive signals were often seen in the underlying stroma and in area of inflamatory infiltrates (Fig II.9.d). Many of these specimens also reacted against the heterologous pBR-322 probe. The location of the hybridization reaction was noted as stromal or epithelial for each case and separately analyzed.

An important issue pertaining to the specificity of the

Figure II.9.. **Illustration of autoradiographic signals seen in samples from invasive lesions.**

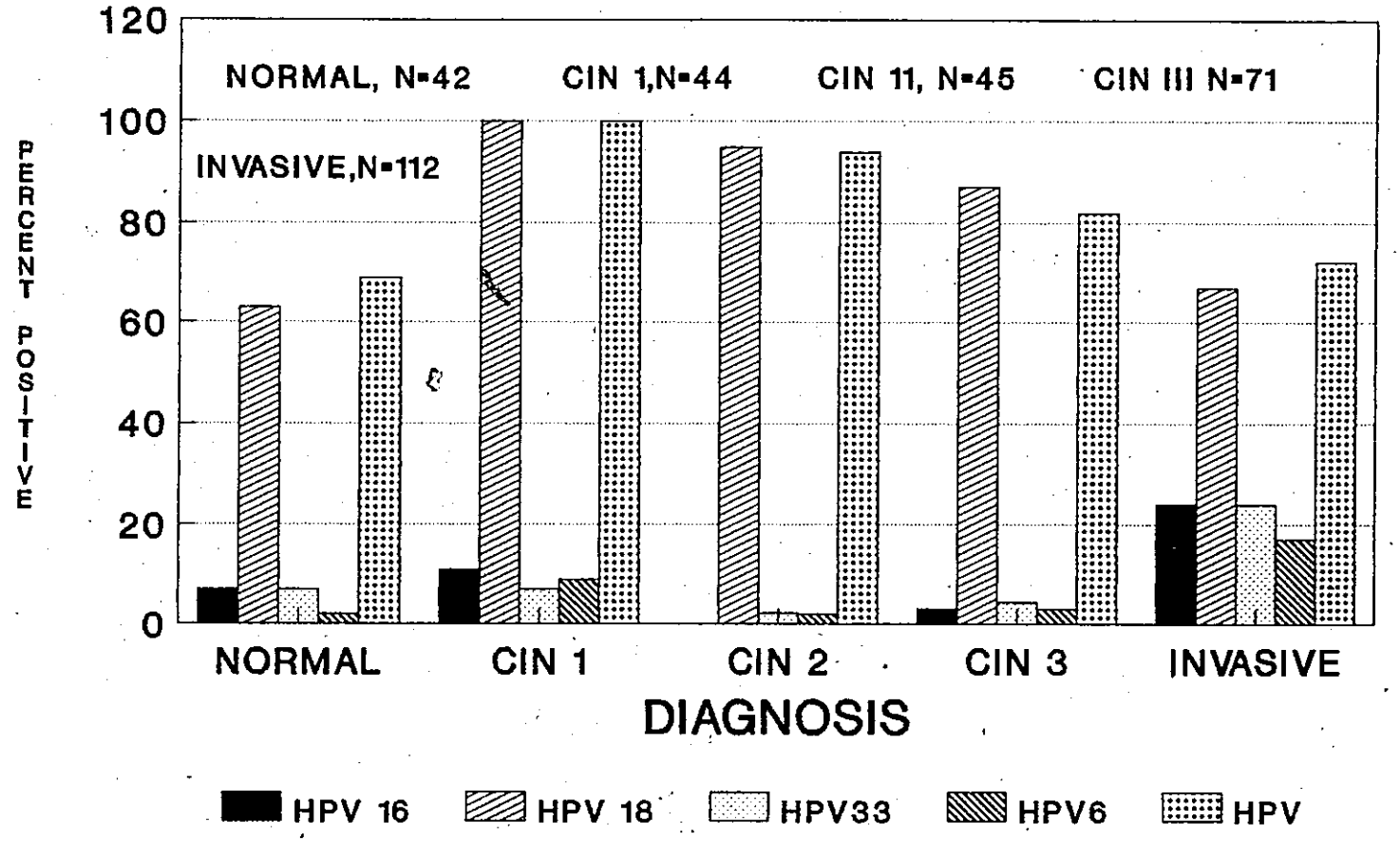
Tissue sections from paraffin-embedded biopsy samples were treated by standard histologic technique of deparaffinization and dehydration in varying grades of alcohol. The DNA was denatured by treatment with HCl, the sections digested with Proteinase K, and hybridized with ^{35}S-labelled HPV probes. Panel a and panel b respectively illustrate that samples from microinvasive and occult invasive cancer did not show any significant hybridization signals. Panel c illustrates that the pattern of signals seen in invasive lesions with the signals being located in cells at the periphery of the cancer and the bulk of the invasive cells were negative. Panel d illustrates the marked focal positive signals often seen the stroma or the inflamatory infiltrates from invasive lesions. The final magnification for all photomicrographs was X250.



hybridization reaction was the nature of the stromal reactions. Their distribution according to disease categories and HPV types is shown in Fig II.10. Preliminary Chi Square analysis showed that the association between the distribution of the stromal reactions and the diagnosis of the patients was not due to sampling variation. In the case of HPV 18, a significant association was also noted with the age of the patient. Therefore, the relationship between these three variables were investigated by the use of log-linear modelling- a statistical technique that can simultaneously examine the relationship between more than two variables (Everitt,1977). The results are summarized in Table II.4. When all disease types were considered together,it was found that all HPVs combined (i.e. types 16/18/33/6/11) were strongly associated with the diagnosis of the patients (p=0.006) and a significant interaction was noted between the age of the patient and the diagnosis of the patients (p=0.01). When this association was partitioned into specific types of HPVs, the following three observations were made: In the case of HPV type 6 both the association with diagnosis and the interaction between age and diagnosis disappeared. In the case of HPV 16 and 33 the association with diagnosis still persisted but the interaction with age and diagnosis disappeared. For HPV 18, the original association with diagnosis and the interaction between age and diagnosis were still noted. Interestingly, when the subset of the invasive patients was excluded from the analysis, the stromal reactions associated with HPV types 16, 6 and 33 disappeared altogether. The analysis, thus, suggested that the effects with these HPV types were associated with

Figure II.10. This figure illustrates the frequency distribution of positive hybridization reactions that were located in the stroma of tissues from cervical neoplasia. It was observed that hybridization with probes of HPV 16 or 33 gave minimal reactions in contrast to hybridization with probes of HPV 6 or 33.

FIG II.10.DISTRIBUTION OF STROMAL REACTION BY DISEASE TYPE



HPV=ANY GENOTYPES OF HPV 16/18/33/6/11

Table II.4. Summary of log-linear modelling for the association of stromal reaction with cervical neoplasia.

Disease	HPV Type	Parameter	Chi-Square	df@	p values
ALL HISTOLOGIC TYPES	All	age	1.93	1	0.1645
		diagnosis	14.22	4	0.006
		age*diagnosis	12.53	4	0.0138
	16	age	1.20	1	0.2735
		diagnosis	14.71	4	0.0053
		age*diagnosis	1.11	1	0.8924
	18	age	3.33	1	0.680
		diagnosis	19.07	4	0.0008
		age*diagnosis	11.21	4	0.0243
	6/11	age	0.34	1	0.557
		diagnosis	6.41	4	0.1707
		age*diagnosis	2.56	4	0.6337
	33	age	1.46	1	0.2273
		diagnosis	10.96	4	0.0270
		age*diagnosis	2.95	4	0.5665
CIN ONLY	All	age	2.65	1	0.1035
		diagnosis	9.31	3	0.0254
		age*diagnosis	2.99	3	0.3926
	16	age	0.72	1	0.3959
		diagnosis	4.09	3	0.2516
		age*diagnosis	1.10	3	0.7775
	18	age	3.94	1	0.441
		diagnosis	8.09	3	0.047
		age*diagnosis	2.10	3	0.5512
	6/11	age	0.01	1	0.9408
		diagnosis	2.62	3	0.4544
		age*diagnosis	0.78	3	0.8551
	33	age	0.00	1	0.9821
		diagnosis	1.520	3	0.3123
		age*diagnosis	0.02	3	0.9122

@df = degree of freedom

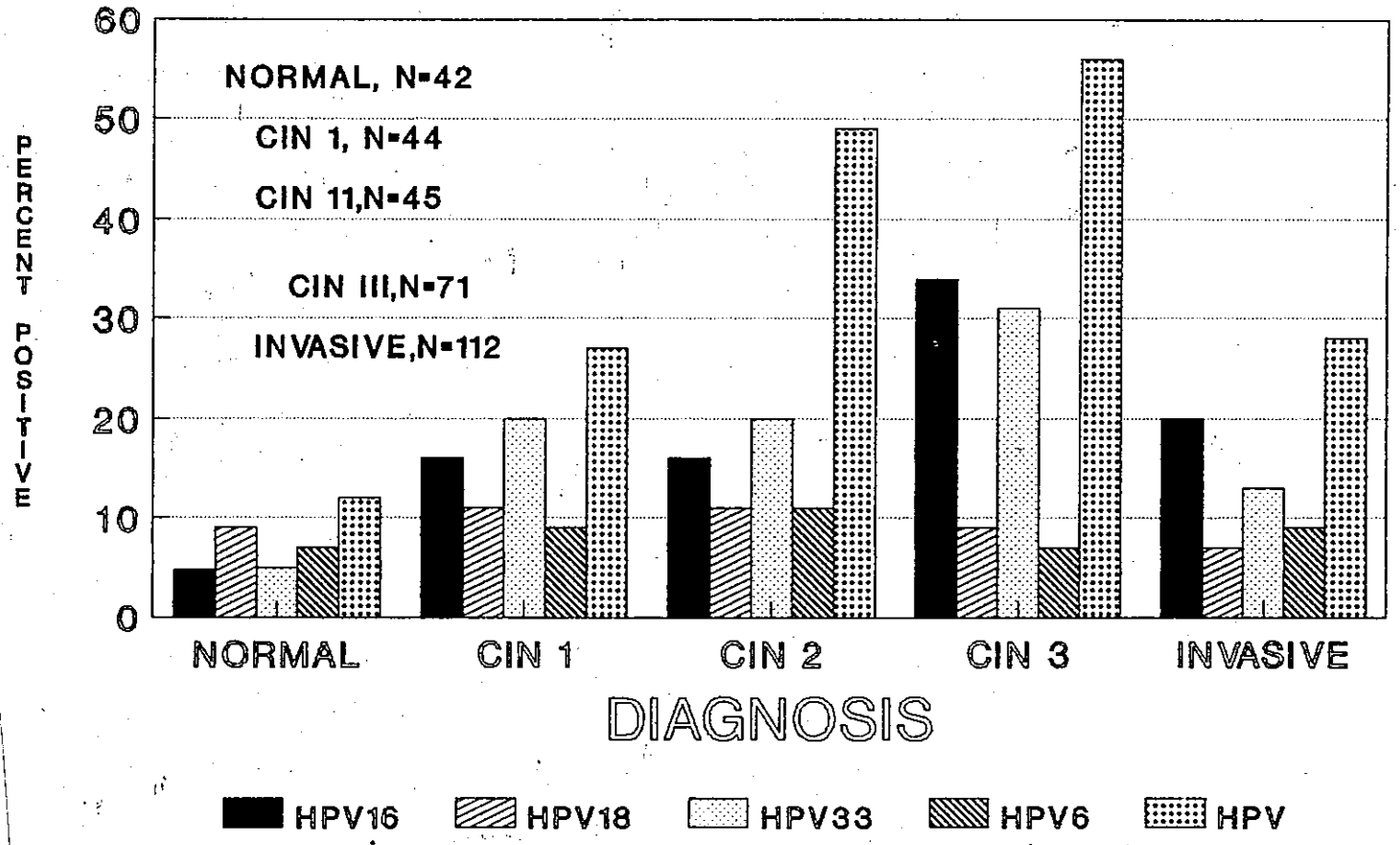
the diagnosis of invasive cancer. For HPV 18 the interaction between age and diagnosis disappeared. Since the association with diagnosis still persisted for HPV 18, in contrast to the remaining viruses, it was concluded that this was a pecularity of the HPV 18 probe. Therefore, the location of the autoradiographic signals were carefully noted during reading the results of the tissue in situ assay. However, in the results that follow only the epithelial reactions were considered.

As shown in Table II.3., data from the tissue in situ assay demonstrated that 72% of condyloma samples from patients in Hamilton contained HPV sequences. Among these, 52% were of type 6/11; 12% of type 16/18 and 8% of mixed types. In histologically normal cervices of patients from Hamilton, 6% of samples were positive for HPV 6/11 sequences. It was also observed that 30% of samples from 23 invasive cases of Panamanian patients were positive for HPV sequences; 7% being HPV type 6/11; 11% being HPV type 16/18 and 11% being of mixed types.

When the tissue in situ assay was used to detect HPV sequences in the population sampled from British Columbia, it was seen that the occurrence of HPV increased as did the severity of the CIN lesions and then decreased in the invasive lesions. This is illustrated in Fig II.11. Thus, HPV 16 sequences were found in 4.8% [95% CI 0,16%] of samples from histologically normal cervices; in

Figure II.11. This figure illustrates the frequency distribution of specific HPV types in different histologic tissues of cervical neoplasia from British Columbia. The prevalence of HPV 16 or 33 increased with the severity of the grade of CIN, in contrast to that of HPV 6 or HPV 18 that segregated independently of CIN grades. The detection rates of all HPV declined in the invasive category.

Fig II.11 DISTRIBUTION OF EPITHELIAL REACTION OF HPV BY DISEASE TYPE



HPV=ANY OF THE GENOTYPES OF HPV 16/18/33

16% [95% CI 6,30%] of samples from either CIN 1 or CIN 2 lesions and in 35% [95% CI 23,46%] of samples from CIN 3 lesions. However, they were found found in only 19% [95% CI 8,36%] of samples from microinvasive lesion, 18% [95% CI 6,35%] of samples from occult lesions and 21% [95% CI 10,36%] of samples from invasive lesions. By contrast, the occurrence of HPV 18 sequences seemed to be independent of the severity of the CIN lesions: It was observed that 9% [95% CI 2,22%] of samples from histologically normal cervices contained HPV 18 sequences as did 11% [95% CI 3,24%] of those with CIN 1 and CIN 2 lesions and 9% [95% CI 3,18%] of those with CIN 3 lesions. Although, no HPV 18 sequences were detected in samples from microinvasive lesions, 3% [95% CI 0,11%] and 14% [95% CI 5,28%] were respectively found in samples from occult and invasive lesions. The distribution of HPV 6/11 sequences showed a similar trend to that for HPV 18. It was found in 7% [95% CI 1,19%] of samples from histologically normal cervices; 9% [95% CI 2,21%] of those from CIN 1 lesions; 11% [95% CI 3,24%] from those with CIN 2 lesions and 7% [95% CI 2,16%] of those from CIN 3 lesions. However, it was found in only 6% [95% CI 0.7,19%] of samples from microinvasive disease; 12% [95% CI 3,28%] from those with occult invasive disease and in 9% [95% CI 2,22%] of those with clinical invasive disease. The distribution of HPV 33 paralleled that of HPV 16, although a different rate was observed. HPV 33 was found to occur in 5.2% [95% CI 0.6,17%] of samples from histologically normal cervices, in 20% [95% CI 9,35%] from those of CIN 1 or CIN 2 lesions and 30% [95% CI 20,43%] from those with CIN 3 lesions. In the invasive group only 11% [95% CI

3,26%] of samples from those of microinvasive or occult invasive and 16% [95% CI 6,30%] of samples from clincal invasive disease were positive for HPV 33 sequences. The collective occurrence of all the HPV types steadily increased with the severity of the CIN lesions. For instance, 11% [95% CI 3,25%] of samples from histologically normal cervices contained any HPV, as did 27% [95% CI 14,42%] of those from CIN 1 lesions; 48% [95% CI 34,63%] of those from CIN 2 lesions and 56% [95% CI 40,68%] of those from CIN 3 lesions. When all CIN lesions were considered together, it was observed that HPV 16 was found in 23.75% of the samples; HPV 18 in 10.1% of the samples; HPV33 in 24.8% of the samples; HPV 6/11 in 10.6% of the samples and all these HPV combined together accounted for 46.3% of the positivity of the CIN lesions. The rate of detection of HPV in invasive lesions was relatively constant, being 27% [95% CI 13,44%] in samples from microinvasive or those from occult invasive and 30% [95% CI 17,46%] in those from clinical invasive disease.

II.2.4.0 Sensitivity and specificity of the various hybridization assays.

The sensitivity and specificity of each hybridization method were first computed with respect to disease. The sensitivity of a test is defined as the proportion of subjects with the disease and a positive test result, while the specificity of a test is defined as the proportion of subjects without a disease and a negative test result (Fleiss,1981). In the computation of these test parameters,

Table II.5 Sensitivities and specificities of different hybridization assays in detecting HPV sequences in various clinical conditions.

Hybridization Assay	Diagnosis	Source	n tested	Sensitivities (%)	Specificities (%)
Southern blot	Condyloma	Hamilton	38	82	87
	Invasive	Panama	43	70	87
Filter in situ	Condyloma	Hamilton	32	62	90
	Invasive	Panama	39	89	90
Tissue in situ	Condyloma	Hamilton	38	72	94
	Invasive	Panama	39	30	94
	Invasive	B.C.	43	30	88
	Micro-Invasive	B.C.	37	27	88
	Occult Invasive	B.C.	36	27	88
	CIN 1	B.C.	44	27	88
	CIN 2	B.C.	47	48	88
	CIN 3	B.C.	71	56	88

the analysis was restricted only to autoradiographic signals located in the epithelium. The results of such an analysis are summarized in Table II.5. The Southern blot method exhibited a sensitivity of 82% and specificity of 87% in condylomatous tissues from Hamilton; in invasive samples from Panama it showed a sensitivity of 70% and specificity of 87%. The sensitivity of the tissue in situ hybridization varied according to the disease condition in all of the population examined. Thus, in the case of clinical invasive disease from Panama or B.C. it had an estimated sensitivity of 30% with a specificity of 94% in Panama and 88% in B.C. Furthermore, in microinvasive and occult invasive its sensitivity was 27%. In the case of condylomatous samples from patients in Hamilton its sensitivity was 72% and its specificity was 94%. Interestingly, the sensitivity of this assay varied directly with the severity of the CIN lesions increased in the target population in B.C. In CIN 1 lesions it had a sensitivity of 27%; in CIN 2 lesions it had a sensitivity of 48% and in CIN 3 lesions it had a sensitivity of 56%. The specifity of the assay in this population was estimated to be 88%.

Wherever possible, the relative sensitivity, specificity and reliability of the hybridization methods in detecting HPV sequences were estimated. Using the Southern blot method as the standard, the filter in situ method had a sensitivity of 66% and a specificity of 88% and showed significant agreement beyond chance (Kappa = 0.54, $p<0.0001$). With respect to the Southern blot method, the tissue in situ method has a sensitivity of 61% and a specificity of 86% and showed significant agreement (Kappa = 0.50, $p<0.0001$). Pairwise

comparison of the filter in situ and tissue in situ methods similarly showed significant agreement (Kappa = 0.55, $p<0.0001$).

II.2.4.1 Characteristic of the tissue in situ hybridization assay in the target population

The primary aim in characterizing the tissue in situ hybridization method was to validate it in the target population with respect to prevalent types of HPV. Hence further statistical analysis was undertaken to characterize whether or not the association between HPV and the CIN lesions was due to sampling variation. The results are summarized in Table II.6. Chi Square analysis showed that there was a significant association between cervical neoplasia and HPV types 16 ($p=0.015$); HPV 33 ($p=0.026$) or any HPV tested ($p=0.00$). The occurrence of HPV 18 and that of HPV 6/11 was observed to be independent of the CIN lesion or invasive disease. In those instances where a significant association was noted, a Chi Square for trend analysis was done to see if there was any dosage effect with increasing severity of disease. Since the sensitivity of the tissue in situ assay was found to be low in samples from the invasive diseases, such samples were excluded from the trend analysis. A significant linear trend was detected for HPV 16 ($\chi^2_{1df}=13.9$, $p=0.00$); HPV 33 ($\chi^2_{1df}=8.879$, $p=0.003$) or any type of HPV ($\chi^2_{1df}=26.96$, $p=0.00$). Hence, it was concluded that the occurrence of these types of HPV increased steadily with the severity of CIN lesions.

The reliability of the tissue in situ hybridization assay in

Table II.6. Summary of Chi-square analysis for the association of HPV with cervical intraepithelial neoplasia (CIN and cervical invasive disease.

Diagnosis	HPV type	Chi-Square	Degree of freedom	p values
CIN lesions only	16	14.781	3	0.002
	18	0.142	3	0.986
	33	10.074	3	0.018
	6/11	0.563	3	0.905
	All	27.609	3	0.000
CIN and invasive	16	15.803	6	0.015
	18	6.578	6	0.362
	33	14.612	6	0.026
	6/11	1.588	6	0.953
	All	31.900	6	0.000

CIN grades I,II and III were considered for CIN only, and all the CIN grades and invasive diseases were considered for CIN and invasive.

Table II.7. Reliability of the tissue in situ assay in sampling cervical biopsies for HPV

Sampling method	Number tested	Number positive	Percent positive
1 Biopsy	170	70	41.2
2-3 Biopsies	29	8	27.5

χ^2=1.920, p=0.166.

detecting HPV sequences was investigated on multiple samples collected on the same patients. Table II.7. shows that of the total 199 patients that had more than one biopsy, 170 had a single biopsy and 29 had two to three biopsies. When the HPV positivity was compared in such samples, some surprising observations were made: Contrary to expectation, the positive rate for the category with one biopsy was 41.2% and that for the category with two to three biopsies was only 27.0%. Thus, increased sampling did not lead to a higher likelihood of a positive test result, probably reflecting the fact that a colposcopically directed biopsy was likely to capture an overt HPV lesion in a single sampling.

The clustering of HPV types in CIN lesions was investigated to identify which HPV types most frequently occurred together. It was noted that 2.4% of samples reacted with all HPV types. If this is taken as a non-specific reaction, and the remaining figure accordingly adjusted, it is observed that 1.9% of all positive samples contained HPV 16/18 sequences; 5.5% contained HPV 16/33 sequences; 1.3%, HPV 18/33 sequences; 0.3%, HPV 16/18/33 sequences; 0.4%, HPV 16/18/6/11 sequences and 0.3 %, HPV 33/6/11 sequences. Thus, the most common combination was HPV 16 and HPV 33. However, from the subset of patients whose specimens reacted with HPV 16 or HPV 33,it was fonud that 8.8% of samples contained HPV 16 sequences only and 7.5% contained HPV 33 sequences only. Since only 5.5% of the samples were found to contain both types of viruses together, it was concluded that these two viruses react differently, although clustering together more often than the remaining types.

Age could be an important confounder in the evaluation of an assay. The age profile of the study population was as follows The mean age of the whole study population was found to be 36.6 years; of those with histologically normal cervices was 40 years; of those with CIN 1 lesions was 32 years; of those with CIN 2 lesion was 35 years and of those with CIN 3 lesions was 30 years. Patients with micro-invasive diagnosis had a mean age of 39 years while those with occult invasive had a mean age of 37 years and those with clinical invasive had a mean age of 47 years. In order to detect any significant association between the age of the patients and the prevalence of specific HPV, the data were collapsed over CIN and the Chi Square analysis was repeated. No significant association was noted between age and the prevalence of any type of HPV. Since the Chi Square test had too many degrees of freedom and therefore it might have been insensitive to detect any association, a log-linear approach was undertaken. In order to have adequate sample size per cell and based on the modal age of the study population, the patients were divided into two age groups; one with less than 34 years and one greater than 34 years. The results of the log-linear analysis showed that there was no significant association with age and the prevalence of HPV. The only significant associations in this analysis were between the diagnoses of the patients and HPV 16 ($p=0.02$); HPV33 ($p=0.01$) or any HPV ($p=0.0001$), thus confirming the initial Chi Square analysis.

II.5.0 Discussion.

The aims of the laboratory investigation were to compare the sensitivities, specificities and reliabilities of molecular hybridization methods in detecting HPV sequences in various clinical conditions. Towards this end, the Southern blot, the filter in situ hybridization and the tissue in situ hybridization methods were applied to specimens of cervical swabs and biopsies from histologically "normal" cervices, condylomas, CIN lesions and invasive cervical cancers from patients in Hamilton, Panama and the target population in British Columbia.

The evaluation of a test accuracy rests on its relationship to some way of knowing whether the disease is truly present or not. The respective sensitivity and specificity of each of the assay were evaluated with respect to histologic evidence for disease. Considering invasive disease the filter in situ hybridization method gave the highest sensitivity (89%) followed by the Southern blot method (70%). In the invasive diseases, the least sensitive was the tissue in situ method (30%). A converse pattern was seen for the specificity; the most specific assay was the tissue in situ method (94%) followed by the filter in situ method (90%) and the Southern blot method (87%). The sensitivity of the tissue in situ method, however, varied between different disease categories: It was 72% for samples of condylomas; 27% in samples from CIN 1 lesions; 48% in those from CIN 2 lesions and 56% in those from CIN 3 lesions. In this instance the specificity of

the assay was estimated to be 88%. In the computation of these sensitivities and specificities histology was taken as the "gold" standard for assessing the presence or absence of diseases. Unfortunately, the reliability of histology in the diagnosis CIN lesions is prone to inter- and intra-observer variation (Koss, 1987). Furthermore, the assumption that all CIN lesions are caused by HPV may not be a valid one. As a consequence of this imperfect standard of validity, the assays were compared with each other using the Southern blot as the standard since this is considered superior by most investigators in the field (Munoz et al., 1988).

The accuracy of one method versus another was expressed in terms of relative sensitivity and specificity. When the Southern blot method was used as a reference to evaluate the other methods in the detection of HPV DNA, the relative sensitivity and specificity of the filter and tissue in situ methods were not different from each other. Percent agreement beyond chance, as computed by the unweighted Cohen's Kappa statistic, showed good agreement (Kappa = 0.50, $p<0.0001$). However, it is apparent that agreement was not achieved in a substantial number of cases. This residual lack of agreement could be explained by a variety of factors such as limit of detection of the method or observers and sampling variations.

Relative copy number of the HPV genomes in the various samples may seriously affect the limit of detection by the hybridization methods. A reconstruction experiment indicated that the Southern blot method could detect 0.2 pg of HPV DNA, corresponding 0.2 to 0.3 copies of HPV DNA per cell (Prakash et al., 1985). The filter in situ

hybridization method, on the other hand could detect 0.1 pg of HPV DNA, an amount that is not identifiable by the Southern blot method (Wagner et al., 1984, McCance et al., 1986a). This could account for a slightly higher positivity rate for samples from cancer cases by the filter in situ hybridization method than by the Southern blot method. It is difficult to directly estimate the limit of detection of the HPV genomes by the tissue in situ hybridization assay. Even though this method is theoretically capable of detecting a single positive cell containing multi-copy of the HPV genomes, it may be unable to detect 10 or less copies of HPV genomes per cell. In support for this argument is the observation that Siha cells, containing 10 copies of HPV-16 genome by the Southern blot method (Yee et al., 1986), were negative by the tissue in situ hybridization method. However, with the length of exposure used in this study, 500 copies of HPV genomes could be detected as observed by using Caski cells that have been estimated to contain 500 copies of HPV 16 genomes by the Southern blot method (Yee et al., 1986). This limit of detection is slightly better than that observed by Crum et al. (1987) who could detect 800 copies of HPV genomes in their system. Taken together, it could be concluded that the lower limit of detection of the tissue in situ hybridization is about 100-200 fold lower than that of either the Southern blot or the filter in situ hybridization method.

There are at least two other main types of errors that are commonly encountered in the assays which were investigated. These include sampling errors in which material representative of the lesion is not included in the specimen and interpretive errors in reporting

the results of the assay. There is a degree of subjectivity in interpreting the results of the hybridization techniques used and an attempt to minimize this was done by accepting the consensus opinions of three independent observers. The tissue in situ hybridization method showed that the HPV DNA is predominantly confined to the upper cell layers of the epithelium. For the filter in situ hybridization method, unlike the biopsy, the surface of the lesion is sampled; thus a endocervical swab should capture those positive cells. Any sampling method missing a lesion will yield a low positive rate, irrespective of the limit of detection of the hybridization method used for the testing of such samples. The tissue in situ assay is theoretically the most vulnerable to this sampling variation because the lesions can be missed both during the process of taking a biopsy from the patients and during sectioning the biopsy. Thus, to test the hypothesis that sampling variation could account account for disconcordant results, multiple samples from the same patients were analyzed for likelihood of obtaining a positive tissue in situ assay result. Surprisingly, it was observed that the rate of HPV positivity did not increase with the number of specimens examined. On the contrary, the positivity rate dropped with increased sampling. Other investigators have observed that increased sampling leads to higher detection rates when either the filter in situ hybridization (Peng et al, 1988) or the Southern blot method (Scheinder et al., 1988) has been used. One possible explanation for this apparently contradictory observation is that the colposcopically directed biopsies were able to capture an overt HPV lesion, in contrast to the situation of the other

investigators who might have been studying occult HPV infection (Nuovo et al., 1988).

Taken together, the sensitivity of the in situ asay can thus be taken to be a function of both the relative abundance of the HPV sequences and sampling variation. The Southern blot assay have shown that the rate of detection of HPV sequences steadily increases from increasing grades of CIN to invasive diseases (Reid et al., 1987, Burke et al., 1985). The limit of detection of the Southern blot was shown to be superior to that of the tissue in situ assay in this study as well as those of others (Crum et al.,1986). A direct corollary of these two observations is that the distribution of HPV sequences in invasive cancer is such that it limits the sensitivity of the tissue in situ assay. The present observation that a number of cancer cases were positive by the Southern blot method but negative by the tissue in situ hybridization method suggests that the majority of cancers in some lesions might be harboring less than 500 genome copies of HPV genomes per cell. Alternatively, the distribution of HPV genome may not be uniform in the tumour.

The specificity parameter of an assay dictates that the assay should be negative when disease is absent. This was essentially found to be the case with the tissue in situ assay. Nonetheless, contrary to the currently known biology of the virus (Orth et al.,1971), in some cases, the the signals were located in the stroma of the cervical tissue . The presence of such stromal reactions has been reported by Gupta et al. (1987) and Ostrow et al.(1985). However, these investigators found that these specimens reacted with all types of HPV

probes as well as with the heterologous pBR-322 plasmid probe. In the present instance, reaction to pBR-322 probes were noted but reactions to all HPV probes were not observed in every case. When log-linear modelling was used to characterize these stromal reactions, it was found that the invasive specimens were more likely to be positive for stromal reaction with HPV and pBR-322 probes, suggesting that the stromal infiltrates were probably responsible for such reaction. It is possible that a cross reaction was taking place between the pBR-322 probe and bacterial infection in the cancer cells as has been observed in the case of hepatocarcinoma cells (Barbacid, 1984,; Ambinder et al.,1986). However, some degree of non-specificity cannot be ruled out as the stromal reaction associated with HPV 18 probes was noted across all disease categories. Non-specific reaction, due to the sulfur moiety of the probes have been reported by Brandllow et al (1987) who investigated the cellular location of nerve growth factor synthesis by the in situ hybridization assay. To test the hypothesis that such reactions might have been due to the sulfur moiety of the HPV probe a completely unrelated adenovirus DNA was labelled with the same isotope and hybridized to random samples of specimen showing stromal reactions. The stroma remained negative, suggesting the absence of this possibility. While the exact nature of these stromal reactions deserves further elaboration, their presence must be carefully noted as they could give rise to a false positive reaction. An altenative approach could be the use of HPV probe that has been excised from the pBR-322 component. However, in such instances the signals were observed to be weaker, confirming an earlier observation

demonstrating the need for plamid linked DNA to form a lattice that enhances the visualization of autoradiographic signals (Singer et al.,1986).

The application of the tissue in situ hybridization method for the detection of HPV sequences in the population served by the CCABC led to some interesting observations. There is no previously reported large study using this technique on cervical neoplasias. The observation that only 56% of samples from CIN 3 population were positive for HPV sequences is slightly below that reported in studies using the Southern blot technique (Reid et al., 1987; Pater et al ., 1986). However, the present findings are not surprising in view of the observations that the tissue in situ hybridization technique is less sensitive than the Southern blot method (Caussy et al., 1988). A study using the tissue in situ hybridization method has been reported by Crum et al., (1986), who observed that 10 out of 18 (55%) of unspecified grades of CIN lesions to contain HPV 16 DNA. However, their samples were selected on the basis of koilocytosis and thus may not be comparable to the present study where no preselection criteria were used.

An interesting observation in the population sampled in British Columbia was the unusually high occurrence of HPV type 33 sequences in CIN lesions. The occurrence of HPV sequences shows apparent geographic variation and that of HPV 33 has been insufficiently characterized. The highest estimated prevalence of HPV 33 in cervival neoplasia has been found to be between 4-8% by the Southern blot technique (Beaudenon et al.,1986) and the joint

prevalence of HPV 16/33 has been found to be about 83% in penile intraepithelial lesions (Barroso et al.,1987). This cross-sectional study demonstrates that the estimated point prevalence of HPV 33 in the sampled population is different from previously reported ones. The HPV 33 viral sequence was found in 30% of samples from CIN 3 lesions and HPV 16/33 sequences were found in 35% of those from CIN 3 lesions. However, with respect to HPV 18, the finding of this study is corroborated with that of Pater et al., (1986) who found that HPV 18 sequences segregated independently of the grades of CIN lesions.

The effect of age on the prevalence of HPV has been insufficiently studied and is still an unresolved issue. Thus, de Villiers et al., (1987), using the filter in situ hybridization assay, noted the HPV detection rate to be independent of age in a sample of 9295 smears from histologically normal cervices, CIN and invasive lesions. However, Yoshikawa et al., (1985b),using the Southern blot method, observed that the occurrence of HPV 16 was higher in cervical cancer patients below age 60 than those above age 60. Similarly, Meanwell et al., (1987), using the Southern blot method, observed that HPV 16 sequences was more prevalent in cervical cancer cases or matched controls in patient less than 40 years of age than those above age 40. Finally, Reeves et al. (1987), using the filter in situ hybridization assay, found that the occurrence of of HPV 16 and 18 increased with increasing age in control women but was age independent in cervical cancer cases. The finding of the present study, based on the tissue in situ assay for the detection of HPV sequences, was that the prevalence of HPVs was independent of the age of the patients.

While this confirms the observation of de Villiers et al., (1987), a strict comparison of the various studies cannot be made because the sensitivity and specificity of the different assays are not known.

In conclusion, the tissue in situ hybridization method was found to have its advantages and disadvantages. The simplicity of this assay and its ability to detect HPV DNA sequences in paraffin-embedded sections lend itself readily to retrospective studies. Its chief drawback is that it may be of limited sensitivity in detecting HPV sequences in a substantial portion of invasive cervical cancers. However, the main research question in this study depends on the demonstration of HPV sequences in CIN lesions. In such instances the assay exhibited a sensitivity of 56%, while not ideal it is still of sufficient sensitivity to answer the main research question (Sackett and Holland, 1975).

CHAPTER III

CASE-CONTROL STUDY DESIGN

III.1.0. Hypothesis

The natural history of invasive squamous cell cervical carcinoma of the cervix is thought to involve a gradual progression from an initiation event through preinvasive stages to invasive cancer. Based on the body of evidence implicating particular HPV in the genesis of cervical cancer, together with the known oncogenic potential of these viruses, it is hypothesized that certain types of HPV initiate neoplastic changes which are more likely to progress to invasive cancer than those initiated by other HPV types. Available evidence suggests that a number of types of HPV may induce morphological changes of the cervix called CIN, but that only those changes associated with HPV 16, 18 or 33 are likely to progress to invasive cancer (Lorincz, 1987b).

III.1.1. Research question.

"Is the presence of HPV types 16, 18 and 33 sequences in the preinvasive cervical lesions of sexually active women predictive of cervical cancer development?"

The primary research question is based on the above underlying hypothesis derived from the body of evidence reviewed in section I.4.0. (page 38). A working hypothesis is usually defined as a predictive statement about associations between independent and dependent variables (Schlesselman, 1982). In this instance, exposure to the particular HPVs is defined as the independent variable and cervical cancer is the postulated dependent variable.

III.1.2 Design architecture.

A direct test of the hypothesis would be to identify a cohort of women with CIN lesions containing HPV-16/18/33 DNA (putatively oncogenic) and a cohort with CIN lesions containing HPV-6C/11 DNA (putatively non-oncogenic) and follow them to determine the rates of progression to invasive cancer. A study to directly test this hypothesis is, however, not feasible because it would be unethical to withhold treatment for women with advanced CIN and to permit the lesions to progress to invasive disease. The only ethically feasible design, then, is a study of women with CIN who have not received curative treatment through refusal, missed diagnosis, treatment failure or loss to follow-up. Therefore, a retrospective, case-control study was used to compare the occurrence rates of HPV-16/18/33 DNA in CIN lesions of women who subsequently developed invasive cancer (cases) with matched CIN lesions of women who did not progress to invasion (controls). The HPV specificity was controlled by determining the occurrence of HPV-6C/11 DNA in the lesions of both cases and controls.

III.1.2. Place of case-control studies in epidemiologic research.

Classically, a case-control study consists of the assembly of a group of patients who have the disease in question (cases) and a nondiseased or control group. Cases and controls are then compared for previous exposure to the suspected etiologic agent(s). The choice and implementation of a case-control study design are primarily governed by three important considerations. These include: (i)

internal validity and freedom from biases, (ii) external validity or generalizibility of the design and, (iii) the feasibility of the design (Kleinbaum et al., 1982).

The case-control study design has been widely adopted in epidemiology because of its rapidity and efficiency in evaluating a hypothesis. It also has greater feasibility and lower cost than a cohort study. However, a case-control study design may be of limited use in establishing temporality, as information on the risk factor is obtained after the diagnosis of cancer. Furthermore, the validity of this kind of design could be undermined by a number of factors chief among which are selection bias, information bias, confounders and sampling variation or chance.

A selection bias could result if the cases and the controls are incorrectly assembled from two different sampling frames (Sackett, 1979). The strength of a case-control study is dependent on the choice of an appropriate control group. The selection of control group is an ongoing debate and there seems to be no consensus on the best control group to use for a particular case group. (Lilienfeld and Lilienfeld, 1979; Stavraky and Clarke, 1983;). There are many epidemiologists who argue that cases and controls should be comparable i.e. free from selection biases (Miettinen, 1985; Cole,1980). On the other hand, some epidemiologists have argued that in achieving comparability the study subjects might be restricted thus leading to overmatching and lack of generalizibility (Axelson, 1985; Schlesselman, 1985; Spitzer, 1985;). To compensate for the potential overmatching problem and still achieve comparability it has been suggested that controls from two different sources be used whenever

possible. For instance, in a hospital based case-control study this could be achieved by the inclusion of a neighbourhood as well as a hospital control group (Feinstein, 1985b; Poole, 1986; Poole, 1987; Schlesselman, 1987).

The chief type of **information bias** is due to measurement error. Misclassification error due to measurement could result from either biased data collection or the application of incorrect diagnostic criteria. This is more likely to happen if the interviewers or data abstractors are aware of either the study hypothesis or the subject's case-control status. In addition, an honest coding error will also lead to misclassification. Fleiss (1981) has shown that misclassification errors result in biased estimates of the association in question. In general, random and independent errors due to misclassification tend to diminish an apparent degree of association between two variables. The net effect of such errors is to bias the odds ratio towards unity (Schlesselman,1982).

The term **confounding** refers to the effect of an extraneous variable that totally or partially accounts for the effect of the study exposure or that masks an underlying true association (Schlesselman,1982). A confounding factor is an extraneous variable that meets the following two conditions: (1) it is a risk factor for the study disease; and (b) it is associated with the study exposure but it is not a consequence of exposure. Thus, factors such as age, race, severity of disease and type of treatment could be potential confounders if they are related to both exposure to HPV and the risk of cervical cancer. The estimate of an association could also be

distorted as a consequence of random variation, or chance in sampling cases and controls. Therefore, taken together, these biases, confounders and chance could partially or totally account for an apparent increased risk for cervical cancer and exposure to HPVs. Alternatively, they could mask the association between cervical cancer and exposure to HPVs. While certain of these limitations are inherent in the design of the case-control architecture, the validity and statistical efficiency of the case-control study could still be enhanced if certain methodologic standards are applied to the design (Horwitz and Feinstein, 1979).

The methodologic standards that have been advocated to enhance the design of case control study are as follows. Clearly defined starting points (point-zero) and end-points; predetermined method for the selection of cases and controls; defined exposure to suspected etiologic factors; unbiased data collection; avoidance of unequal constraint in cases and controls; equal diagnostic examination; equal medical surveillance and equal demographic and clinical susceptibilities (Horwitz and Feinstein, 1979; Feinstein, 1985a). Additional factors which increase the strengths of the case-control design include adequacy of sample size and correct statistical analyses. Finally, matching is another feature that enhances the validity of a design. It is a type of potential constraint in the selection of one or more control for making the controls and the cases similar with respect to one or more potential confounding factors (Fleiss, 1981). However, once a given factor is matched in a design, that factor, should it be an important etiologic one, can no longer be examined. Alternatively, the statistical efficiency of the case-control

architecture can be enhanced, at the analysis stage, by either post-stratification or the use of multivariate analysis techniques.

The major strength of a carefully designed case-control study is that it enables one to gauge the strength of the association of an exposure variable and an outcome variable. Case-control studies do not directly measure the risk of developing an outcome in the exposed or nonexposed subjects. However, Cornfield (1951) has shown that the relative risk, the ratio of risk in the exposed and the unexposed subjects, can be approximated from the odds ratio (OR) of the case-control study. Since the OR is defined as the ratio of the "odds" of the exposed person developing the disease and the "odds" of the nonexposed developing the disease, this ratio will have a value of unity when the exposure and the outcome variables are not related. Alternatively, if the OR differs from unity then the exposure variable is associated with risk of disease. For OR greater than unity, the association is positive and for OR of less than unity the association is negative (Schlesselman, 1982).

III.2.0. Case-control study protocol for determining the predictive value of particular HPV in the development of cervical cancer.

II.2.1. Study population.

The study population was drawn from patients who resided in the Province of British Columbia. Women who had been evaluated for cervical disease, at some point of their lives, by the gynecological

cytology screening laboratory of the CCABC constituted appropriate cases and controls. The characteristics of the population in terms of referral pattern, methods of follow up, incidence of cervical neoplasia and other demographic variables have been described in section II.2.1.1. As described in section II.2.3.2, a pilot study was undertaken to estimate the prevalence of specific types of HPV in all grades of CIN lesions by the in situ hybridization technique. The pooled estimated prevalence rates from stratified sampling, without weighting for grade of CIN, were as follow: It was found that HPV 16 occurred in 23.75% of the samples; HPV33, in 24.8% of the samples; HPV 18 in 10.6% of the samples; HPV 6/11 in 10.2% of the samples and HPV16/33 in 37.6% of the samples and 46.3% of women were positive for more than one HPV type.

III.2.2. Definition of eligibility criteria for study subjects.

The definition of a "case" or "control" involves two distinct specifications: (1) a statement of predetermined eligibility criteria for the selection of individuals for the study; and (2) establishment of objective criteria for the diagnosis of cervical neoplasia (Schlesselman, 1981).

For the purpose of this study a "case" was defined as a woman with invasive cancer and who had a diagnosis of CIN made two or more years before the invasive disease was diagnosed. The eligibility criteria for the cases were as follows: (a) women who were post-pubertal female, between 15 and 75 years old, (b) had at least a two year lapse between the diagnosis of CIN and invasive cervical cancer

(c) had the preinvasive biopsy with the CIN lesions available for HPV sequence analysis and ,(d) had been reported to the Cancer Control Agency of British Columbia (CCABC).

For the purpose of this study a "control" was defined as a woman who had no recorded history of cervical cancer during the latent period of her case. The eligibility criteria for the controls were as follows: (a) women who were post-pubertal female, between 15 and 75 years old, (b) had been diagnosed at the same CIN stage at about the same time as her case and followed for the same duration, (c) had availability of the cervical biopies for HPV sequence analysis in CIN lesions and, (d) had been reported to the CCABC. Women were excluded from the study if they had undergone partial or complete hysterectomies subsequent to the diagnosis of CIN.

To ensure completeness of the histologic or cytologic diagnoses, a set of objective criteria were followed. These criteria are given in Appendix III.1. In instances where a given biopsy showed more than one grade of CIN lesion, the more advanced grade was used. To minimize interobserver variation all the histological biopsies were reviewed by Dr. A.J. Worth of the CCABC.

III.2.3. Selection of study subjects

The method used for the selection of cases and controls were as follows. A search of the records at the CCABC revealed that of the 3400 women who were treated for invasive cervical cancer over the past 20 years, 108 had a recorded diagnosis of CIN two or more years prior to the diagnosis of invasive cancer. A search for avaibility of these

CIN biopsies revealed that 54 patients had no tissue block available; 1 was from out of Province; 4 had no dysplastic lesions left in the tissue and 7 were initially misdiagnosed as having invasive cancer. This left only 47 of the 108 potential cases that met the eligibililty criteria.

The selection of controls was done after the cases were located. Thus, in an attempt to end up with at least two controls per case, three women who had the same grade of CIN diagnosed the same year as the CIN lesion of the case were initially selected as controls. However, not all the controls thus selected had a CIN biopsy that could be used for HPV testing. Hence, sometimes the case:control ratios were 1:1 or 1:2 or 1:3.

III.3.0. Ascertainment of exposure to specific HPVs.

To ensure standardization of exposure for cases and controls the presence of specific HPV sequences was determined by laboratory procedure. The availability of only formalin fixed biopsies that had been stored over the years seriously limited the choice to only the tissue in situ hybridization assay. This assay has been used for the detection of both animal and human papillomaviruses (Crum et al., 1986; Beckman et al., 1985; Gupta et al., 1985; Syrjanen et al., 1987; Orth et al., 1971; Caussy et al., 1988; McDougal et al., 1986; Nagai et al., 1987; Grussendorf et al., 1979). The relative sensitivity of the assay seems to vary according to the disease conditions (Caussy et al., 1988). As described in section II.2.4.0., the sensitivity of the assay in detecting any HPV was 27% for samples from CIN I lesions; 48%

for samples from CIN II lesions, and 56% for samples from CIN III lesions. Since only 11% of histologically normal samples from the B.C. population contained HPV, the specificity of the assay was estimated to be 89%.

Based on the result of the pilot, prevalence study the following specific HPV probes were used: (1) HPV 6C/11 as a mixed probe since these two viruses share extensive homologies (2) HPV 18, and, (3) HPV 16/33. The justification for using a combined HPV 16/33 probe was based on the fact that these two viruses share extensive homologies (Beaudenon et al., 1986) and they were the most common type of viruses clustering together in the prevalence study. The assay was performed under non-stringent condition of hybridization and washed under stringent condition as detailed in section II.2.2.9.

Each slide was read by three different readers, including a pathologist. The final result on a subject was based on the concordant results of at least two readers. In order to minimize inter-observer variation which could distort the results of the in situ hybridization, a set of objective criteria was developed for scoring each slide. The criteria, which are based on the number of positive cells and the location of the positive grains within a particular tissue are given in section II.2.2.11. The cut off point of positivity was any score equal to or greater than five; any score less than five was scored negative. These results were entered in the special worksheet developed for key-punching and entry in the computer (Appendix III.2).

III.3.1. Data collection.

The non-laboratory component of this study constituted an integral part of the planning and execution of this design.

The criteria for the selection of all subjects have been already discussed in section III.2.1. To achieve this end, the information was abstracted from the relevant patients' records and entered in the special work sheet that was developed for the purpose of this study (see Appendix III.3). Such information was maintained on a computerized data base system for easy retrieval and statistical analysis. Key information that was sought was related to risk factors known to be associated with cervical cancer as well as factors thought to influence progression of CIN.

In addition to identifying information, data were collected on age (birthday), parity, marital status, and number of marriages. For the latter variable information regarding change in surname or divorce status was abstracted. Medical information on oral contraceptive use and history of venereal infections were also collected. The date of diagnosis of the initial CIN lesion was recorded, as was the histologic type and severity of the disease (as originally diagnosed). The date and type of treatment administered was recorded as was information on the dates and outcome of follow-up gynecological examinations. In addition, for the cases, the date of diagnosis of invasive cancer was recorded as was the histologic type of lesion, clinical stage, treatment method and follow-up outcome. Most of this information was obtained from the computerized records of patients registered with the CCABC; others were obtained by contacting

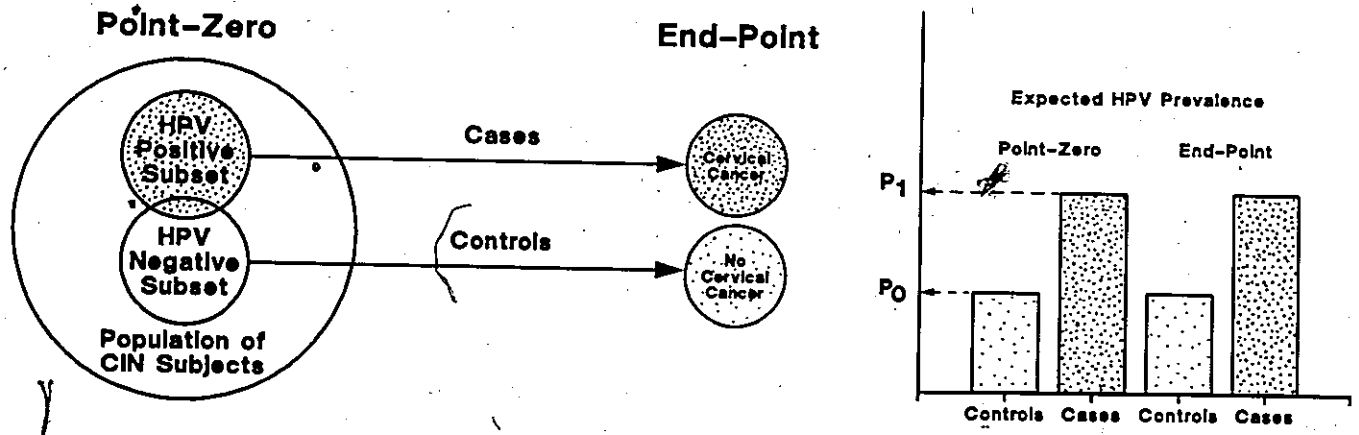
the family physicians either by phone or by mail.

III.3.2. Sample size consideration.

The association between particular HPVs and cervical cancer, as illustrated in Figure III.1, could take one of two forms: These HPVs could occur secondary to the invasive disease as opportunistic pathogens; or they could occur in the preinvasive stage of cervical cancer and be predictive of progression to invasive disease. If they are secondary to the cervical cancer, then the occurrence of these particular HPV sequences should occur with equal frequency in the CIN lesions of cases and controls since cases and controls are matched on grade of CIN. If they are predictive of progression to invasive disease, then it follows that the frequency of occurrence of these HPV sequences should be higher in the CIN lesions of cases than in those of controls. Thus, a comparison of the prevalence rate in the preinvasive biopsies of cases and controls will enable one to distinguish which of the two possibilities is true.

The main hypothesis to be verified was that the prevalence rate of particular HPV would be higher in the CIN lesions of cases than in those of controls. To statistically test whether or not the difference in these two rates (binomial proportions) are due to sampling variation, the chi-square statistic will be used. Furthermore, this case-control study will also enable one to determine if particular HPVs represent an increased risk for incurring cervical cancer. Consequently, the magnitude of the risk will be estimated by the relative risk. If exposure to particular HPV represents an

Possibility I: HPV Infection is Predictive of Cervical Cancer Development



Possibility II: Susceptibility to HPV Infection Increases With Cervical Cancer Development

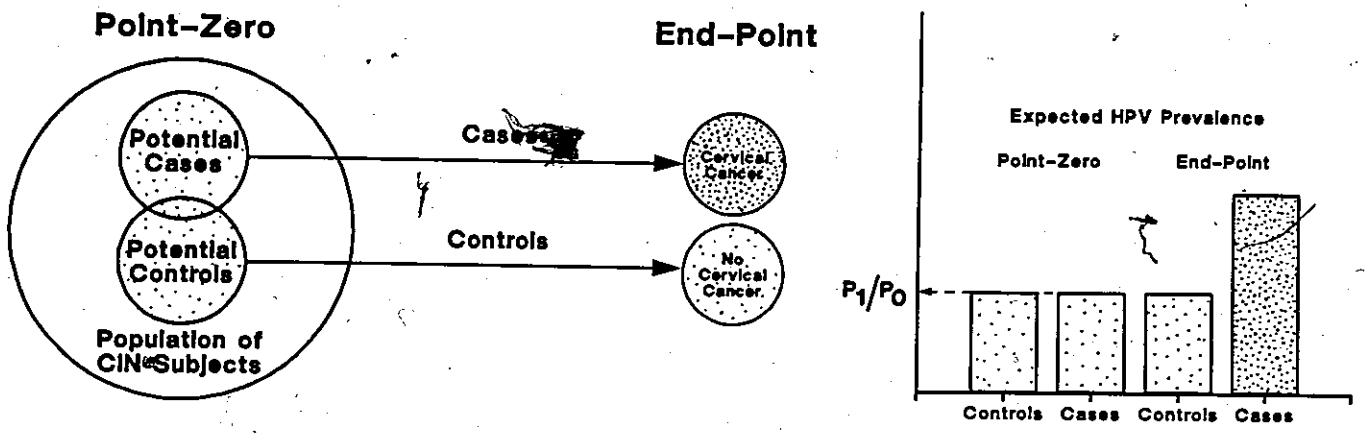


FIG III.1 TWO POSSIBLE MODES OF HPV ASSOCIATION WITH CERVICAL CANCER.

increased risk for progression to cervical cancer then the relative risk would be greater than unity. For both the binomial proportion and the relative risk estimate, in statistical terms, it was the **null hypothesis**, denoted by H_0, that was to be rejected. The null hypothesis for the binomial proportions would be one of no difference in the prevalence rate of particular HPV in the cases and the controls; the null hypothesis for the relative risk would be that relative risk for exposure to particular HPV and cervical cancer is 1.0. If the null hypotheses could be rejected, then the **alternative hypotheses**, denoted by H_1, can be accepted. The object of sample size calculation was to determine with what certainty the null hypothesis could be rejected or accepted.

There are two possible sources of errors. First of all, it is possible to reject H_0 when H_0 is true. Secondly, it is possible to accept H_0, when H_0 is not true. The probability of making the first type of error is called the **type I error** and is commonly denoted by "α" or "$P\alpha$" and is often referred to as the p-value in a significance testing situation. This kind of error is usually due to small sample size and random variation in sampling cases and controls. The probability of making the second kind of error is denoted by "β" or "P_β". This kind of error is commonly due to insufficient sample size. In either case, the probability of making the correct decision is given by the complement of these errors. Thus, $1-\alpha$ denotes the probability of accepting H_0 when H_0 is true; and $1-\beta$, also called the **power of the study**, is the probability of rejecting H_0, when H_0 is not true. In the present study, the sample size was constrained by the avaibility of 47 cases and 94 matched controls. The two parameters,

for which the power was calculated, were the binomial proportions (prevalence rates) and the relative risk.

The power for comparing two binomial proportions was based on the equation of Fleiss (1981). The determination of the power depends on four parameters: (1) the relative frequency of exposure among the controls in the target population (2) the meaningful difference in the prevalence rates to be determined (3) the desired level of significance, and (4) the available sample size. The equation for the power calculation is given below:

$$1-\beta = \frac{c\alpha/2 \quad 2PQ - |P_2 - P_1| \; n - |P_2 - P_1|^2}{P_1Q_1 + P_2Q_2} \qquad \ldots\ldots\ldots\ldots \; 3.1$$

Where $(1-\beta)$ = power of the study

$c\alpha/2$ = the critical value cutting off the proportion a/2 in the upper tail of the normal curve

P1 = exposure rate in the controls

P2 = exposure rate in the cases

Q = (1-P)

The power for the binomial proportions was determined based on the prevalence level of HPV in various grades of CIN in the target population and the expected positive rate in the invasive cancer cases from B.C. The prevalence of HPV6/11 or HPV18, in all combined grades of CIN lesions, was 10.1% and that of HPV16/33 was 37.6%. Since the tissue <u>in situ</u> assay is relatively insensitive in detecting HPV

sequences from invasive specimens, the prevalence of HPV in invasive cervical cancers from B.C. was assumed to be the same as, those reported by using the Southern blot method, on other populations (Prakash et al.; 1985, Durst et al; 1983). The best estimated figure is about 60%. According to the null hypothesis H_0 to be rejected here, the prevalence of all types of HPV at the earlier CIN stage in the cases are predicted to be 60%. Similarly, in the controls, it was predicted that the prevalence rate for HPV6/11 and HPV 18 would be 10.1%, while that for HPV 16/33 would be 37.6%. Based on these assumptions, and α=0.05, the power for calculating the binomial proportions was estimated to be as follows. For HPV 6/11, or HPV18, the study had 90% power to detect a difference of 50% in the respective prevalence rates. In the case of HPV16/33 the study had 65% power to detect a difference of 22% in the respective prevalence rates (Fleiss, 1981).

The power for calculating the relative risk was based on the equation of Schlesselman (1982). The determination of this power, like that for the binomial proportions, depends on the specification of four values: (1) the relative frequency of exposure among controls in the target population, P_0 (2) a hypothesized odds ratio to be determined (3) the desired level of significance α, and (4) the available sample size. These parameters are given by equation 3.2 (Schlesselman, 1982).

$$R = 1 + [AB' \pm (AB')^2 + 2(1 + c)\, AC']\ /C' \quad \ldots\ldots\ldots\ldots\ldots\ 3.2$$

where

$$A = (z\alpha + z\beta)^2$$

$$B = 1 + 2cp_0$$

$C' = 2cp_0[n(1 - p_0) - Ap_0]$

c = number of controls per case

p_0 = proportion of controls "exposed"

n = total number of cases

$z\alpha$ = unit normal deviate for significance test at level α for a one sided test.

$z\beta$ = unit normal deviate for test with power $(1 - \beta)$.

The term R represents the largest population relative risk less than 1.0, or the smallest population relative risk greater than 1.0, that can be detected for specified values of n, c, p_0, α and β.

To calculate the power for the relative risk, similar assumptions to the binomial proportions were made. It was estimated that the study had 80% power to detect a relative risk of 3.5 in the case of HPV6/11 or HPV18 and 80% power to detect a relative risk ratio of 4.5 for HPV16/33, at a significance level of 0.05 and one tail probability test (Breslow and Day 1987).

III.4.0. Data analysis.

Before the study was completed, at the planning stage, the following analysis strategy was envisaged. Since this was a matched design, it was necessary to use statistical techniques that maintained the matching scheme. Breslow and Day (1980) have shown that unconditional analysis of matched data could result in biased estimates of the test statistics. The study variables to be employed in the data analysis are summarized in Table III.1. The presence or absence of cervical cancer was identified as the sole dependent variable. The

Table III.1. Summary of study variables to be employed in data analysis

Variable name	Source of variable	Type of variable
I. DEPENDENT VARIABLE		
Cervical cancer	Histologic investigation	Nominal
II. INDEPENDENT VARIABLE		
Exposure Factors –		
HPV 16/33	In situ hybridization	Nominal
HPV 18	In situ hybridization	Nominal
HPV 6C/11	In situ hybridization	Nominal
History of venereal disease	Patient record	Nominal
Number of marriages	Patient record or interview	Ratio
Hormone use at time of smear	Patient record	Ratio
Confounding factors –		
Age	Patient record	Ratio
Parity	Patient record	Ratio
Severity of CIN lesions	Patient record	Nominal
No. of pap smears	Patient record	Ratio
Type of treatment at CIN stage	Patient record	Nominal

table includes 11 independent variables of which 6 were identified as exposure factors and the remaining as possible confounding factors. The test statistic to be employed would be the chi-square; the relative risk would be estimated. The chi-square would be used to reject the null hypothesis for the binomial proportion of no difference in the prevalence rates between the cases and controls, while the relative risk would be used to estimate the level of excess risk, if any, of incurring cervical cancer when specific HPV sequences are present in the CIN lesions. Next stratified analysis could be used to investigate possible confounding and the adjusted chi-square and odds ratio accordingly calculated. The data would finally be analyzed by multiple logistic regression techniques.

If particular HPV are predictive of cervical cancer, then the prevalence rates of HPV in the cases will be higher than those in the controls. This difference will be tested for statistical significance by the chi-square statistic, a procedure that will then permit the H_0 to be rejected in favour of H_1. With two controls matched for each case, the data may be tabulated in the form of matched case-control triplets (case, control 1, control 2); there are eight possible outcomes for a triplet when exposure to HPV is dichotomous (+ or −). The computation of this chi-square will be done by the method of maximum likelihood (Breslow and Day, 1980) using the MCSTRAT procedure of the SAS statistical package that first tabulates the appropriate matched pairs of cases and controls for each of the exposure variables. Similarly for the relative risk estimate, if particular HPVs are associated with cervical cancer then the odds ratio estimate will be greater than unity. However, various sources of bias such as

inappropriate selection of subjects or misclassification and confounders could also give rise to elevated chi-square or odds ratio values. Alternatively, these factors may completely mask a real chi-square value or an odds ratio greater than unity. Therefore, this possibility would be explored by stratified analysis.

A stratified analysis for various combinations of independent variables (risk factors) would be carried out if sufficient numbers exist for each stratum. From the contingency table of overall association, individual tables would be constructed for each stratum of the risk factors separately. To test the homogeneity of the odds ratio, appropriate component of chi-square would be computed by the Mantel-Haenszel procedure. The degree of confounding may be estimated from a comparison of the adjusted odds ratio with the crude odds ratio (Kleinbaum and Kupper, 1982).

The stratified analysis may be complicated by the inability to stratify on many variables at once, since this procedure can result in insufficient numbers in the respective strata. Furthermore, the existence of non-uniformity of the effect measure over the strata may also preclude the use of an overall chi-square test (Kleinbaum and Kupper, 1982). Attempts to circumvented these problems will be undertaken by using a multiple logistic regression approach that can control for several confounders simultaneously in a moderate sample size (Breslow and Day, 1980). The logistic regression technique also provides a direct estimate of the odds ratio. The logistic model specifies that the probability of developing cervical cancer (dependent variable) is linearly and additively related to a set of independent regressor variables denoted by x_1, x_2, x_3 ... x_p

(Schlesselman, 1982). The independent variables may represent potential risk factors for disease, confounding factors or interactions of these factors. The equation describing the logistic model for case-control study is given below (Schlesselman, 1982).

$$\ln p_x/q_x = \beta^1_0 + \beta_1 x_1 + \ldots + \beta_p x_p \quad \ldots\ldots\ldots\ldots\ldots\ldots (3.3)$$

where $\beta^1_0 = \ln \pi_1/\pi_2 + \beta_0$

p_x = probability of disease

q_x = probability of no disease

π_1 = number of cases

π_2 = number of controls

x_i = independent regressor variable

β_i = logistic regression coefficient

The log ratio of a disease is a linear function of the regressor variable x_1 .... x_p. Hence the model assumes that effect of any independent variable x_i does not depend on the remaining independent variables, a feature which enables the logistic regression technique to control for a baseline difference in a large number of variables at the same time. In order to estimate the odds ratio, the logistic regression coefficients would be estimated by the maximum likelihood using a standard statistical package such as SAS (SAS Inc., Carey, NC). The odds ratio for the regressor variable would be estimated by equation 3.4 below.

$$\psi = \exp(\beta_1) \quad \ldots (3.4)$$

In order to maintain the matching scheme it was proposed to use the conditional, stratified, logistic regression program MCSTRAT (from the SUGI library of the SAS Institute. Adjusted odds ratio for one level of a particular risk factor relative to another level would be

estimated by keeping the other regressor variable constant. This procedure would show if any confounding is present. If particular HPVs are associated with cervical cancer, odds ratio greater than unity should be observed. The precision of the odds ratio estimates will be gauged by computing the 95% confidence intervals. These values will indicate the lowest and highest true odds ratio that is likely to be compatible with the sample observation.

III.4.1. Resources needed.

The organizational structure of the study was as follows. Responsibilities were equally shared by the collaborators: Dr. Worth, the research co-ordinator at the CCABC, Dr. Rawls and Dr. Marrett and myself. This study examined the medical records, located the biopsies and recorded the laboratory results of about 141 patients and therefore had to be coordinated by a research personnel. The research coordinator, in consultation with Dr. Rawls and Dr. Worth, was responsible for doing the computer search and locating the relevant biopsies. Dr. Worth was responsible for verifying the presence of lesions in the biopsies prior to their being shipped to McMaster Univesity where they were analyzed for the presence of HPV sequences by me. The research co-ordinator was also responsible for abstracting the relevant demographic information from the patient history display sheets of the CCABC or by contacting the physicians' office. The patient material and records at CCABC were reasonably accessible. This included a careful follow-up of treated patients, thus selection of treated CIN patients who have not developed cancer or controls

could be accomplished with fair confidence. The facility of the Computational Services of the Health Sciences Department from McMaster University was used for data entry. The setting up and management of the patient data base as well as statistical analyses were done by me in close liaison with Drs. Rawls and Marrett.

A pilot study had also been carried in the target population and the prevalent types of HPV were known as well as the relative sensitivity and the specificity of the tissue in situ hybridization assay. No major technical difficulty was foreseen at the planning stage.

This study received the approval of the Ethics Committee of McMaster University and was financially supported by grants from Health and Welfare, Canada and the National Cancer Institute of Canada.

III.4.2. Significance

In general, demonstrating an apparent etiologic role of a virus in human cancers has two practical implicatons. These include the possible development of a vaccine and the use of viral markers to identify groups at which preventive measures can be directed. Both preventive measures have been used for the association of Hepatitis B virus and primary liver carcinomas (Beasley et al., 1981).

APPENDIX III.1. HISTOLOGIC CRITERIA FOR THE DIAGNOSIS OF PREINVASIVE AND INVASIVE CERVICAL CARCINOMA.

PREINVASIVE NEOPLASIA OF THE CERVIX.

MILD DYSPLASIA (CIN1).

- Two thirds of epithelium show good differentiation.
- Abnormality of the nuclei is mild and most marked in the basal third of the epithelium.
- Mitotic figures are present but not numerous.

MODERATE DYSPLASIA (CIN2).

- Upper half of the epithelium shows differentiation and maturation.
- Nuclear atypia confined to the surface.
- Abnormality of the nuclei is more pronounced than in CIN 1.
- Mitotic figures are present in the basal two thirds of the epithelium.

INVASIVE SQUAMOUS CELL CARCINOMA.

STAGE 1A MICROINVASIVE CARCINOMA.

- Early stromal invasion.
- Depth of invasion 3.0 mm or less below the base of the epithelia.
- No evidence of lymph-vascular space.

STAGE 1B OCCULT INVASIVE CARCINOMA.

- Frank invasion by confluent masses of neoplastic cells.
- Lesions usually unifocal.
- Lesions cannot be diagnosed by clinical examination.

GREATER THAN STAGE 1B.

- Lesions can be clinically diagnosed.
- Staging based on standard classification depending on extent of the primary tumour and metastases.

INVASIVE ADENOCARCINOMA.

- Carcinoma of the glandular epithelium of the cervix.
- Subclassification depends on types of cervical cells, mucin, endometrial clear cells or mixed adenosquamous type.

APPENDIX III.2. B.C. CASE-CONTROL STUDY HPV RESULTS FOR ALL Bx

A. INFORMATION COLLECTED FROM THE PATIENT HISTORY SHEET AT THE CCABC

	CODING COLUMNS
1.0 PATIENT NAME/CCABC NUMBER	1-8
2.0 HPV STUDY UNIQUE ID 001 case 002 control 003 control	9-11
3.0 DATE OF BIRTH DD/MM/YY 99 UNKNOWN	12-17
4.0 DATE OF FIRST TIME ZERO BIOPSY DD/MM/YY 99 UNKNOWN	18-23
5.0 DIAGNOSIS OF FIRST TIME-ZERO BIOPSY 0 NORMAL 1 CIN/1 2 CIN/2 3 CIN/3 4 CIS 5 MICROINVASIVE 6 OCCULT INVASIVE 9 UNKNOWN	24
6.0 DIAGNOSIS OF FIRST END POINT BIOPSY 0 NORMAL 1 CIN/1 2 CIN/2 3 CIN/3 4 CIS 5 MICROINVASIVE 6 OCCULT INVASIVE 7 INVASIVE STAGE 1 8 INVASIVE GREATER THAN STAGE 1 9 UNKNOWN	25

CODING SCHEME

First column subject id: 1 case, 2 control, 3 control.
Next two columns biopsy id: 1-5 time zero bx in numeric order.
6-10 end-point bx (6 = first, 7 = second, 8 = third, 9 = fourth, 10 = fifth, 00 = No specimen.
Next column HPV results (epithelial reactions).
Next column HPV results (stromal reactions).
HPV code: 0 = negative, 1 = doubtful, 2 = 1 cell in whole field, 3 = 2-3 cells in entire field, 4 = weak positive, 5 = positive >6 cells in field, 6 = positive more than 10 cells, 7 = classic positive, 8 = unsatisfactory specimen, 9 = missing or unavailable.

7.0 HPV 16 RESULTS	CODING COLUMNS
1	26-30
2	31-35
3	36-40
4	41-45
5	46-50
6	51-55
7	56-60
8	61-65
9	66-70
10	71-75

8.0 HPV 18 RESULTS	
1	75-80
2	81-85
3	86-90
4	91-95
5	96-100
6	101-105
7	106-110
8	111-115
9	116-120
10	121-125

9.0 HPV 33 RESULTS	CODING COLUMNS
1	126–130
2	131–135
3	136–140
4	141–145
5	146–150
6	151–155
7	156–160
8	161–165
9	166–170
10	171–175

10.0 HPV 16/33 RESULTS	
1	176–180
2	181–185
3	186–190
4	191–195
5	196–200
6	201–205
7	206–210
8	211–215
9	216–220
10	221–225

APPENDIX III.3. B.C. CASE-CONTROL STUDY QUESTIONNAIRE

A. INFORMATION COLLECTED FROM THE PATIENT HISTORY SHEET AT THE CCABC

	CODING COLUMNS
1.0 PATIENT NAME/CCABC NUMBER	1-8
2.0 HPV STUDY UNIQUE ID	9-11
001 case 002 control 003 control	
3.0 DATE OF BIRTH	12-17
DD/MM/YY 99 UNKNOWN	
4.0 DATE OF TIME ZERO BIOPSY	18-23
DD/MM/YY 99 UNKNOWN	
5.0 DIAGNOSIS AT TIME ZERO BIOPSY	24
0 NORMAL 1 CIN/1 2 CIN/2 3 CIN/3 4 CIS 5 MICROINVASIVE 6 OCCULT INVASIVE 7 INVASIVE 8 OTHER 9 UNKNOWN	
6.0 DATE OF END POINT	25-30
DD/MM/YY 99 UNKNOWN	

CODING COLUMNS

7.0 DIAGNOSIS AT END-POINT 31

0 NORMAL
1 CIN/1
2 CIN/2
3 CIN/3
4 CIS
5 MICROINVASIVE
6 OCCULT INVASIVE
7 INVASIVE
8 OTHER
9 UNKNOWN

8.0 HISTOLOGIC DIAGNOSIS AT END POINT 32

1 SQUAMOUS CELL CARCINOMA
2 ADENOCARCINOMA.
3 NORMAL
9 UNKNOWN

9.0 NUMBER OF PAP SMEAR (between point-zero/end point) 33-34

1-98 (actual number)
99 unknown

10 NUMBER OF COLPOSCOPIC EXAMINATIONS 35-36

(between point-zero and end point)
1-98 (actual number)
99 unknown

B. INFORMATION COLLECTED FROM THE PHYSICIAN'S OFFICE.

11 NUMBER OF PREGNANCIES 37-38

1-30 (actual number)
99 unknown

12 AGE AT FIRST PREGNANCY 39-40

(actual years)
99 UNKNOWN

13. USE OF ORAL CONTRACEPTIVE/DURATION 41-42

1 YES
2 NO
9 UNKNOWN

1 1-2 YEARS (short term)
2 3-5 YEARS (medium term)
3 MORE THAN 5 YEARS (long term)
9 UNKNOWN

	CODING	COLUMNS
	YES	NO
14. HISTORY OF GENITAL INFECTION		
1 GENITAL HERPES	43	44
2 SYPHYLIS	45	46
3 GONORRHOEA	47	48
4 OTHER	49	50
9 UNKNOWN	51	52
15. SMOKING HISTORY/DURATION		53
A. SMOKING AT TIME ZERO		
1 YES		
2 NO		
9 UNKNOWN		
B. EVER SMOKED		54
1 YES		
2 NO		
9 UNKNOWN		
C. DURATION IF EVER SMOKED		55
1 1-2 YEARS (short term)		
2 3-5 YEARS (medium term)		
3 MORE THAN 5 YEARS		
9 UNKNOWN		
16. HISTORY OF MEDICAL TREATMENT/DURATION		
A. CONE BIOPSY		56
1. YES		
2. NO		
9. UNKNOWN		
IF YES		57
1 AT TIME ZERO		
2 AT END-POINT		
3 IN THE INTERVENING PERIOD		
4 = 1 AND 3		
9 UNKNOWN		

CODING COLUMNS

B. CAUTERY 58
1. YES
2. NO
9. UNKNOWN

IF YES 59

1 AT TIME ZERO
2 AT END-POINT
3 IN THE INTERVENING PERIOD
4 = 1 AND 3
9 UNKNOWN

C. RADIOTHERAPY 60
1. YES
2. NO
9. UNKNOWN

IF YES 61

1 AT TIME ZERO
2 AT THE END-POINT
3 IN THE INTERVENING PERIOD
4 = 1 AND 3
9 UNKNOWN

D. CHEMOTHERAPY 62
1 YES
2 NO
9 UNKNOWN

IF YES 63

1 AT TIME ZERO
2 AT THE END-POINT
3 IN THE INTERVENING PERIOD
4 = 1 AND 3
9 UNKNOWN

E. PARTIAL HYSYTERECTOMY 64
1 YES
2 NO
9 UNKNOWN

IF YES 65

1 AT TIME ZERO
2 AT THE END-POINT
3 IN THE INTERVENING PERIOD
4 1 AND 4
9 UNKNOWN

CODING COLUMNS

F. TOTAL HYSTERECTOMY 66
1 YES
2 NO
9 UNKNOWN

IF YES 67

1 AT TIME ZERO
2 AT THE END-POINT
3 IN THE INTERVENING PERIOD
9 UNKNOWN

G. CRYOSURGERY 68
1 YES
2 NO
9 UNKNOWN

IF YES 69

1 AT TIME ZERO
2 AT THE END POINT
3 IN THE INTERVENING PERIOD
4 1 AND 3
9 UNKNOWN

H. OTHER (SPECIFY) 70
1 YES
2 NO
9 UNKNOWN

IF YES 71

1 AT TIME ZERO
2 AT THE END POINT
3 IN THE INTERVENING PERIOD
4 1 AND 3
9 UNKNOWN

I. MARITAL STATUS 72
1 SINGLE
2 MARRIED
3 WIDOWED
4 DIVORCE/SEPARATED
9 UNKNOWN

J FLAG FOR # F SEX PARTNERS 73
1 MORE THAN ONE MARRIAGE
2 PREVIOUS SURNAME
3 OTHER PARTNERS
4 NONE OF THE ABOVE
9 UNKNOWN

CHAPTER IV

ANALYSIS OF THE CASE-CONTROL STUDY

IV.1 Characteristics of cases and controls.

A search of the records at the Cancer Control Agency of British Columbia revealed that of 3400 women who were treated for invasive cervical cancer over the past 20 years, 108 had a recorded diagnosis of CIN two or more years prior to the diagnosis of invasive cancer. The procedures used for the selection of cases are summarized in Table IV.1. Of the 108 potentially eligible cases, 54 patients had no tissue samples left; 1 had moved out of Province; 4 had no dysplastic lesions left in the tissue and 2 were initially misdiagnosed as having invasive cancer. This left 47 cases who met the eligibility criteria. According to the design, each case was to be matched with at least two controls chosen for the same year of diagnosis and grade of CIN. In practice, there were 20 sets of 1:1 case: control; 31 sets of 1:2 case: control and 4 sets of 1:3 case: control. It is seen from Table IV.2 that 63.8% of cases and 69.1% of the controls had a histologic diagnosis of CIN grade 3 at the time of enrolment in this study. Although the original design consisted of selecting the index biopsy with a diagnosis of CIN, this criteria could not be met for 4.7% of the cases who had an index biopsy with a normal histologic diagnosis.

The demographic variables that were collected are summarized in Table IV.2. Approximately 53% of the controls could not be contacted due to diverse reasons such as change of family physicians, change of address and refusal or no response from the physicians. Therefore, except for information on age, no other demographic

Table IV.1. Summary of procedures used for the selection of cases

Procedure	Number	Percent
A. Initial record search for invasive diagnosis	3,400	
B. Initial identification of eligible cases by computer search	108	3.18 (of A)
C. Hospital contact for availability of biopsies	54	50.00 (of B)
D. Change of residence	1	0.92 (of B)
E. Misclassification due to original diagnosis	2	1.85 (of B)
F. No histologic evidence of disease	4	3.70 (of B)
G. Eligible cases left	47	43.5 (of B)

Table IV.2 Distribution of selected characteristics among 47 cases and 94 controls

Characteristics	Cases	Controls
Histologic diagnosis of index biopsy		
Normal	4.3(%)	0.0(%)
CIN I	12.8(%)	20.2(%)
CIN II	19.1(%)	10.6(%)
CIN III	63.8(%)	69.1(%)
Mean age at diagnosis of index biopsy, year ± SD	38.5 ± 12.8	36.1 ± 11.1
Marital status		
single	12.8(%)	18(%)
married	60(%)	30(%)
widower/separated/divorced	23(%)	6.0(%)
unknown	4.3(%)	45.7(%)
Mean age at first pregnancy year±SD	21.8 ± 4.3	23.4 ± 4.5
unknown	19(%)	53.2(%)
Mean Number of pregnancy±SD	3.8±2.8	2.1±1.5
unknown	2(%)	45.6(%)

information were available on these patients. The mean age of the cases was 38.5 years and that of the controls was 36.1 years. The difference was not statistically significant. Based on the available information, the cases were more likely to be either married or have been married than the controls. Cases were also more likely to be pregnant at an earlier age or have a higher number of pregnancies than the controls. However, these differences were not statistically significant. In this study, 14.8% of cases were deceased and 4.3% of the cases did not consent to releasing the demographic variables.

IV.2.0. Univariate Analyses.

IV.2.1. Non-viral exposure factors

The overall associations between cervical cancer and non-viral exposure factors are described in Table IV.3. Information was collected on the following four variables; smoking, contraceptive use, history of genital infection and estimated probable number of sexual partners. Based on the information obtained, cases were more likely not to smoke or not to use oral contraceptive than the controls. However, due to the inability to obtain complete information on more than half of the controls, no solid conclusions could be drawn. This point is dramatically illustrated in the history of genital infections where such information was available on only 10 cases and 3 controls. Furthermore, the probable number of sexual partners was indexed by surrogate variables, such as previous surnames; more than one

Table IV.3. Overall association between cervical cancer and non-viral exposure factors in 47 cases and 94 controls

Characteristics	Values	Cases/ Controls	Chi squares	p values
Smoking history	ever	14/23	1.968	0.162
	never	21/18		
	unknown	12/53		
Smoking duration	1-5 yrs	3/3	0.058	0.810
	> 5 yrs	17/21		
	unknown	27/70		
Contraceptive use	ever	23/40	8.970	0.003
	never	10/2		
	unknown	14/52		
Contraceptive duration	1-5 yrs	12/9	2.385	0.122
	> 5 yrs	9/17		
	unknown	26/68		
History of genital infection	yes	1/1	0.965	0.326
	no	9/2		
	unknown	37/91		
Probable number of sexual partners	> 1	20/26	0.417	0.519
	at least one	26/26		
	unknown	1/42		

Table IV.4 Medical surveillance and treatment of 47 cases and 94 controls

Characteristics	Values	Cases/ Controls	Chi Squares	p values
No. of papsmear	0 - 8	29/81	8.317	0.004
	9 - 20	8/4		
	unknown	10/9		
Conization	yes	28/60	0.261	0.609
	no	17/30		
	unknown	2/4		
Any biopsy	yes	46/88	1.203	0.273
	no	1/4		
	unknown	0/2		
Colposcopic biopsy	yes	29/81	9.320	0.002
	no	18/59		
	unknown	0/4		
Cautery/cryosurgery	yes	2/11	2.282	0.131
	no	45/79		
	unknown	0/4		

marriage; more than 1 partner, and current marital status. Even after collapsing all the surrogate variables together, such information was still missing on 42 controls.

The relative medical surveillance or treatment of cases and controls is summarized in Table IV.4. It was observed that controls were more likely than cases to have 8 or fewer pap smears during the latent period of the cases ($p = 0.0004$). The only available complete information for medical treatment was that performed by biopsy. Cases and controls were similar with respect to conization; however, cases were more likely to have had colposcopic biopsies than the controls ($p = 0.002$).

IV.I.3.0 Viral exposure factors.

IV.I.3.1. Reliability of the tissue in situ assay in sampling cervical biopsies for HPV sequences.

An important issue concerning the ascertainment of exposure to HPVs is the reliability and validity of the assay used. The reliability of an assay, i.e. its degree of constancy when repeatedly applied to the same individual, can potentially affect selection in a case-control study (Fleiss, 1986). The tissue in situ assay could be prone to sampling variation. Due to the focal and occult nature of the HPV infection, one might miss an infected lesion either during sectioning or sampling at a time when the virus is not being expressed (Nuovo et al., 1988). The validation study done in Section II.2.4.1. showed

Table IV.5. Reliability of the tissue in situ assay in sampling cervical biopsies for HPV sequences

Sampling method	Number Available	Number* Positive	Percent Positive
One/two biopsies	79	11	13.9
Three biopsies	26	7	26.9
Four biopsies	27	5	18.5
Five biopsies	9	2	22.2

* On one or more occasions for the corresponding number of biopsies

that when multiple sections were taken from the same individual at the same time, the rate of positivity was not significantly different. This observation indicates that in a colposcopically directed biopsy the HPV lesion is likely to be diagnosed in a single biopsy. However, this observation does not exclude the possibility of intermittent secretion or expression of occult HPV viruses. The latter possibilty was investigated in the case-control study due to the availability of multiple biopsies collected from the same patients over a period of 2 to 5 years. The results are summarized in Table IV.5. It was observed that the rate of positivity, in one or more biopsies, increased from 13.9% for the category with one or two biopsies to 26.9% for those with three biopsies and then remained steady at about 22% in the remaining categories. Nonetheless, chi-square analysis revealed that the difference was not statistically significant. However, despite the chi-square test, there was a definite trend for increased positivity from 13.9% in those with 1 to 2 biopsies to 24.9% in those with more than 2 biopsies.

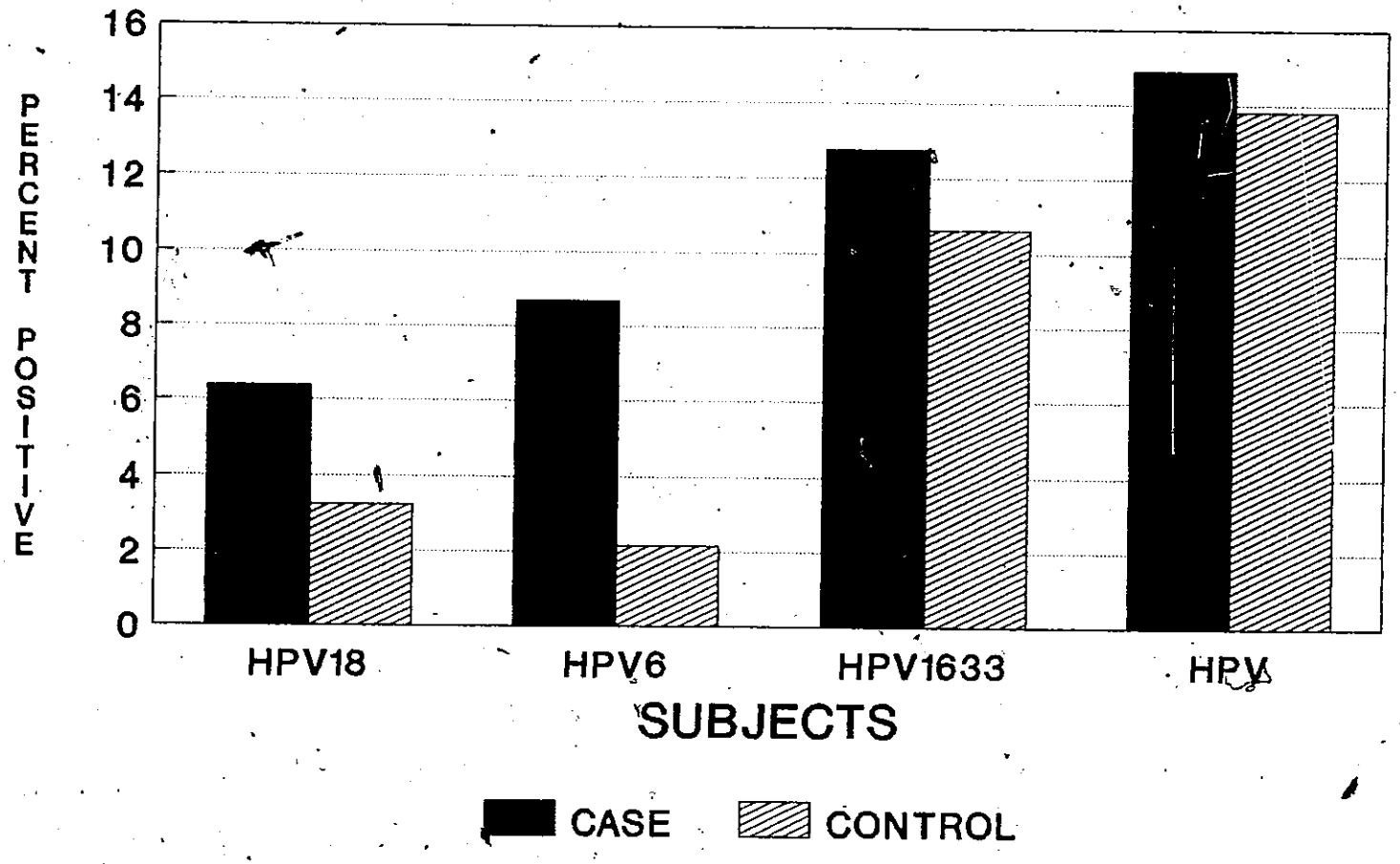
All the available biopsies were tested for exposure to HPV. This was based on the fact that increased sampling over time led to a moderate increase in the probability of a positive test result. Furthermore, as shown in Table IV.6, the distribution of biopsies in cases and controls was significantly different ($p=0.0001$). However, in order to maintain the original scheme of the case-control study design, two strategies of analyses were undertaken. The first consisted of restricting the analysis to the matched index biopsies in cases and controls. The second approach was to include the results of

Table IV.6. Distribution of multiple biopsies in cases and controls

Biopsy Number	Cases		Controls	
	Number	Percent	Number	Percent
One	0	0	1	1.1
Two	15	31.9	68	72.3
Three	10	21.3	16	17.0
Four	14	29.8	8	8.5
Five	8	17.0	1	1.1

FIGURE IV.1. This figure illustrates the frequency distribution of specific types of HPV in the index biopsies of cases and controls. Although the rates of positivity for specific HPV were different, Chi-square analysis showed that this difference could be accounted for by sampling variation.

FIGURE IV.1 DISTRIBUTION OF SPECIFIC HPV SEQUENCES IN INDEX BIOPSIES



HPV=ANY GENOTYPES OF HPV 6/11/18/1633

all the biopsies in order to control for the possible distorting effect of sampling over time. In the latter approach, appropriate steps were taken to adjust for the difference in the number of biopsies between cases and controls.

IV.I.3.2. Occurrences of HPV sequences in cases and controls.

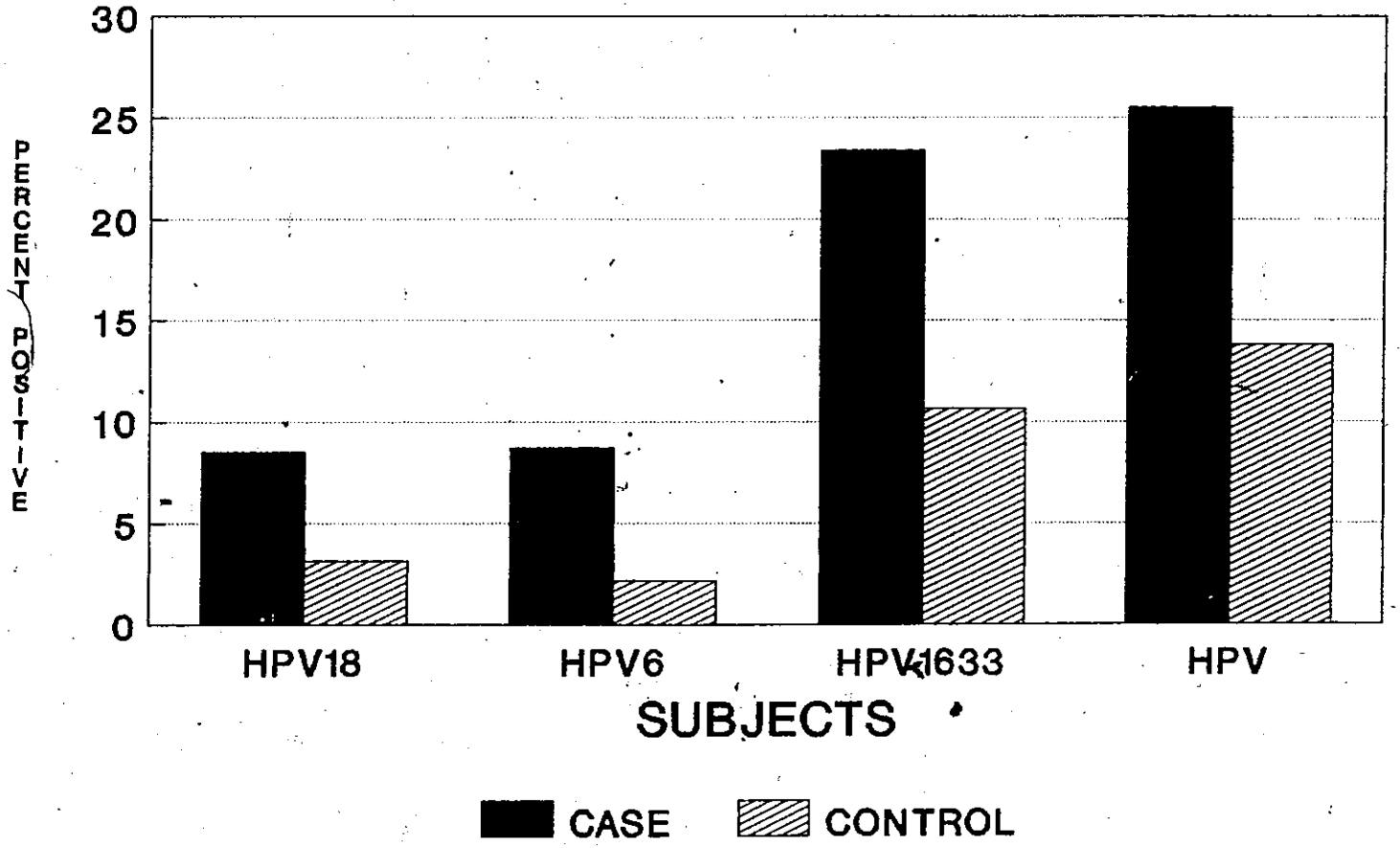
IV.I.3.3. Occurrences of HPV sequences in the matched index biopsies cases and controls.

The occurrences of HPV types in the matched index biopsies of cases and controls are illustrated in Figure IV.1. It is seen that 6.4% of cases and 3.2% of controls contained HPV18 sequences; 8.7% of cases and 2.2% of controls contained HPV6/11 sequences; 12.8% of cases and 10.6% of controls contained HPV16/33 sequences and 14.9% of cases and 13.8% of controls contained any HPV sequences.

The occurrences of specific HPV types in cases and controls, according to grades of CIN, were different at the time of enrolment. Five percent of samples from CIN 2 lesions and 5.3% of samples from CIN 3 lesions contained HPV18 sequences. By comparison, only 6.6% of samples from CIN 3 lesions contained HPV6/11 sequences. The occurrences of HPV16/33 sequences were found to be 4% in samples from CIN 1 lesions; 5% in those from CIN 2 lesions and in 14% those from CIN 3 lesions. The occurrences of all HPV types steadily increased as the severity of the CIN lesions increased. Thus, they were found to occur in 4% of samples from CIN 1 lesions; in 10% of samples from CIN 2 lesions and in 17.2% of samples from CIN 3 lesions.

FIGURE IV.2. This figure illustrates the frequency distribution of specific types of HPV in all the preinvasive biopsies of cases and controls. Although the rates of positivity for specific HPV were different, chi-square analysis showed that this difference could be accounted for by sampling variation.

FIGURE IV.2 DISTRIBUTION OF SPECIFIC HPV SEQUENCES IN ALL BIOPSIES



HPV=ANY GENOTYPES OF HPV 6/11/18/1633

IV.I.3.4. Occurrences of HPV sequences in all the preinvasive biopsies of cases and controls

The occurrences of HPV types in all the available preinvasive biopsies of cases and controls are illustrated in Figure IV.2. It is seen that 8.5% of cases and 3.2% of controls contained HPV18 sequences; 8.7% of cases and 2.2% of controls contained HPV6/11 sequences; 23.4% of cases and 10.6% of controls contained HPV16/33 sequences and 25.5% of cases and 13.8% of controls contained any HPV sequences.

The occurrences of specific HPV types in cases and controls, according to grades of CIN, were different at the time of enrolment. Ten percent of samples from CIN 2 lesions and 5.3% of samples from CIN 3 lesions contained HPV18 sequences. By comparison, only 6.6% of samples from CIN 3 lesions contained HPV6/11 sequences. The occurrences of HPV16/33 sequences were found to be 8% in samples from CIN 1 lesions; 10% in those from CIN 2 lesions and 17.2% in those from CIN 3 lesions. The occurrences of all HPV types steadily increased as the severity of the CIN lesions increased. Thus, they were found to occur in 8% of samples from CIN 1 lesions; in 15% of samples from CIN 2 lesions and in 20.43% of samples from CIN 3 lesions.

The comparison of HPV rates in the index biopsies with that in the multiple biopsies is also shown in Table IV.7. It is seen that increased sampling over time led to a moderate increase in the detection rates of HPV 18 and HPV16/33. However, the detection rate for HPV 6/11 remained the same by both sampling procedures. These observations indicate that occult HPV infection or intermittent HPV

Table IV.7. Comparison of HPV prevalence rates in the index biopsy and multiple biopsies of cases and controls

HPV type	Index biopsy			Multiple biopsies		
	CIN I (Percent Positive)	CIN II	CIN III	CIN I (Percent positive)	CIN II	CIN III
6/11	0	0	6.6	0	0	6 .6
18	0	5.0	5.3	0	10	5.3
16/33	4	5.0	14	8	10	17.2
HPV	4	10	17.2	8	15	20.4

Table IV.8. Comparison of HPV sequences in the case-control and the prevalence study

Population	Disease	Mean yr of birth	Mean age	Mean yr of diagn	HPV18	HPV6	HPV16/33	HPV (% positive)
Case-control	CIN I	1941	35.6	1977	0.0	0.0	8.0	8.0
	CIN II	1931	41.8	1977	0.0	0.0	10.0	15.0
	CIN III	1946	36.6	1981	5.4	6.6	17.2	20.4
	all CIN	1939	38.0	1978	5.0	4.4	14.5	17.5
	invasive	1939	38.0	1977	0.0	0.0	0.0	0.0
Prevalence								
	CIN I	1955	32.0	1987	11.0	9.0	27.2	27.0
	CIN II	1953	35.1	1987	11.0	11.0	40.4	48.0
	CIN III	1957	30.6	1987	9.0	7.0	53.5	56.0
	all CIN	1955	32.1	1987	10.0	8.8	37.6	46.2
	Invasive	1947	47.0	1985	6.6	9.0	25.0	28.0

expression was probably more common with HPV 18 and HPV 16/33 than with HPV 6/11.

IV.I.3.4. Comparison with an external referrent group.

The reports on prevalence of HPV have been found to vary widely according to disease type and geographic locations. However, the currently published observations are derived from undefined populations using unvalidated assays (Munoz at al., 1988). In the present study, the possibilty of variation in HPV rates with time was compared in the same population and the use of the tissue in situ hybridization assay which has been previously validated (Caussy et al., 1988). For this purpose, the unadjusted, overall rates of HPV in the cases and controls were compared with the HPV rates in the general populations from the prevalence study of section II.2.3.3. The results of such a comparison are shown in Table IV.8. In both populations it was seen that the prevalence of any HPV tested showed a similar trend of increasing rates with increasing severity of CIN disease. Furthermore, the most prevalent HPV types were HPV16/33 in both populations. However, the overall HPV rates for the case-control subjects were significantly lower than those for the prevalence ones. At least four possible explanations can be advanced for this observation. This could be due to an age effect, a cohort effect, a period effect or a decay of the detectable HPV sequences during long-term storage of the tissues.

An age effect is present when the HPV rate varies with the age

of the patients, irrespective of the year of birth. Log-linear modelling showed that there was no significant effect of age on the prevalence of HPV, confirming the observation made from the prevalence study of section II.2.4.1.

A cohort effect is present when HPV rate varies by year of birth, regardless of age. It is seen from Table IV.8 that for the case control study, the average year of birth for patients with CIN disease was 1939 whereas the corresponding one for the prevalence study was 1955. Thus, a cohort effect could be a possible explanation.

A period effect is present when the HPV rate varies by calendar year of diagnosis (period), irrespective of age or year of birth. Table IV.8 shows that, for the case control subjects, the mean year of CIN diagnosis was 1978 whereas the corresponding one for the prevalence subjects was 1987. Therefore, a period effect could also explain the observation. However, the possibility of a joint cohort and period effect cannot be ruled out. It is interesting to note that a previous ecologic study to determine the time trend prevalence of HPV did not observe any period effect (Armstrong et al., 1986). The latter study relied on the use of koilocytes as a marker for HPV infection. However, such markers are relatively insensitive indicators of specific HPV infection.

Storage of formalin-fixed biopsies over the years could potentially lead to a significant loss of HPV sequences. If this is true, then the detectable rates of HPV for the case-control subjects is bound to be lower than that of the general population because the

tissues for the case-controls subjects were stored for a period of approximately ten years.

IV.II.O. Subgroup analysis of HPV and other exposure factors.

In order to investigate whether the occurrences of HPV sequences were unevenly distributed over the various demographic and exposure variables, a subgroup analysis was undertaken. The results of such an analysis are shown in Table IV.9. It was seen that HPV18 was significantly associated with progression to invasion in women who were pregnant after age 18 ($p = 0.044$); had less than 9 pap smears ($p = 0.009$), and used contraceptives for 1 to 5 years ($p = 0.002$). By contrast, HPV 6/11 sequences occurred more frequently in smokers ($p = 0.054$); in women who were pregnant after age 18 ($p = 0.009$) and, those who used oral contraceptives for 1 to 5 years ($p = 0.000$). Interestingly, HPV16/33 sequences were significantly associated with women who used oral contraceptives for 1 to 5 years ($p = 0.008$). However, no great biologic importance can be attached to these associations since relevant demographic informations were missing from over 50% of the control subjects.

Table IV.9. Subgroup analysis of occurrence of HPV with other risk factors in 47 cases and 94 controls.

Factor	Value	HPV type	Case/ Control	Fisher's Exact Test	p values
Smoking	no	18	3/2	1.175	0.278
		6/11	2/1	1.122	0.289
		16/33	7/7	1.378	0.240
	yes	18	3/0	2.714	0.099
		6/11	4/10	3.705	0.054
		16/33	8/5	0.137	0.711
Age of pregnancy	< 18	18	2/0	0.1885	0.170
		6/11	2/0	1.870	0.171
		16/33	3/3	0.042	0.88
	> 18	18	5/1	4.045	0.044
		6/11	5/0	6.735	0.009
		16/33	11/8	2.000	0.157
# pap smear	0 - 8	18	5/3	6.795	0.009
		6/11	3/2	3.466	0.063
		16/33	9/17	1.969	0.161
	> 8	18	3/0	1.587	0.208
		6/11	2/0	1.833	0.176
		16/33	4/0	1.1000	0.294
Duration of contra-ceptive use	1-5 yr	18	8/3	9.226	0.002
		6/11	9/2	13.411	0.000
		16/33	17/17	7.012	0.008
	>5 yrs	18	1/0	1.096	0.296
		6/11	0/0	not computable	
		16/33	1/11	0.000	1.000

IV.III.0. Determining the predictive values of particular HPV to answer the main research question

IV.III.1 Chi-square analysis on the matched index biopsies of cases and controls

The main research question of the case control study design was to determine whether the rates of particular HPV occurrences in the preinvasive biopsies of cases were significantly higher than those in the controls. Substantiating this hypothesis would enable one to conclude whether the presence of specific HPV sequences is likely to be predictive of invasive cervical cancer onset. The hypothesis to be rejected was H_0 : of no difference in the prevalence rates of specific HPV in the cases and the controls. As explained in Chapter III, the test statistics was the chi-square for distribution of HPV in the cases and the controls. In order to maintain the matching scheme of the design, the appropriate matched sets were used for computing the chi-square statistic by the Maximum Likelihood Method (Breslow and Day, 1980)

The results of the chi-square analysis on the matched index biopsies of cases and controls are summarized in Table IV.10. It is seen that values of the chi-square statistic were not significant for any of the HPVs tested. Hence, the chi-square analysis did not permit the null hypothesis to be rejected. This observation translates into no detectable difference in the HPV prevalence rates between the cases and the controls. In turn, this indicates that particular HPVs could

Table IV.10. Summary of chi square analysis for determining the predictive values of specific HPVs in the index biopsies of cases and controls

HPV type	Matched chi squares	p values
HPV 6/11	2.922	0.096
HPV 18	1.24	0.88
HPV 16/33	0.1398	0.708
Any HPV	0.3052	0.586

not be predictive of invasive cervical onset in the sample of population that was investigated.

IV.III.2 Chi-square analysis on the all pre-invasive biopsies of cases and controls

The results of section IV.I.3.1. indicated that testing a single biopsy for HPV may slightly underscore the positivity of the tissue in situ assay. To overcome such possible bias, the predictive values were re-determined on all the available preinvasive biopsies. The crude chi-square values are summarized in Table IV.11. The values of the chi-square statistic indicated that the prevalence of HPV16/33 sequences was significantly different in the cases and the controls ($p = 0.0387$). Hence, in this instance, the results permitted the null hypothesis to be rejected in favour of the alternative hypothesis. By contrast, for HPV6/11, HPV18 or all HPV combined, the chi-square analysis supported the null hypothesis of no association between exposure to specific HPV and the onset of cervical cancer. This translates into no detectable difference in the prevalence rates of HPV between the cases and the controls.

Although the above crude chi-square test indicated that particular HPVs could be predictive of cervical cancer, this conclusion may be invalid because of the uneven number of biopsies in the cases and controls. The first attempt to adjust for the difference was to use the Mantel-Haenszel chi-square test after stratifying on two groups of patients. One group consisted of patients

Table IV.11. Summary of unadjusted chi square analysis for determining the predictive values of specific HPVs in all the preinvasive biopsies of cases and controls

HPV type	Matched chi squares	p values
HPV 6/11	3.114	0.0780
HPV 18	2.70	0.0918
HPV 16/33	4.27	0.0387
Any HPV	3.64	0.0563

Table IV.12. Summary of Mantel-Haenszel (MH) Chi square for adjusting the number of biopsies in cases and controls

HPV	Stratum (# of biopsies)	Number Tested/Number Positive case	control	Fisher Exact test	p. values	MH chi square	p values
18	1-3	1/15	0/64	0.633	0.43		
	4-5	3/32	3/25	0.0	1.0	0.0	1.0
6/11	1-3	2/14	0/64	4.34	0.04		
	4-5	2/32	2/25	0.0	1.00	0.64	0.43
16/33	1-3	1/15	9/64	0.118	0.731		
	4-5	10/32	1/25	5.057	0.023	1.64	0.2002
HPV	1-3	2/15	9/64	0.00	1.00		
	4-5	10/32	4/25	1.035	0.309	0.58	0.447

with one to three biopsies and the second group consisted of patient with four to five biopsies. The results are summarized in Table IV.12. It is seen that after such adjustment, the null hypothesis could not be rejected for any of the HPV tested. A direct corollary of these observations was that none of the HPV types could be predictive of cervical cancer onset for the sample of patients that was investigated. However, the validity of the stratified analysis in this instance could be questioned due to less than 5 subjects in some of the strata used (Kleinbaum and Kupper,1982). To overcome this problem, the number of biopsies was adjusted by means of the logistic regression technique which can control for possible confounders in a moderate sample size. The chi-square statistic was again computed by the maximum likelihood estimate (Breslow and Day,1980). The results are summarized in Table IV.13. It is seen that after such adjustment, the null hypothesis could not be rejected for any of the HPV tested. This translates into no detectable difference in the HPV prevalence rates between cases and controls. A direct corollary of this observation was that none of the HPV types could be predictive of cervical cancer onset on the sample size of patients investigated. This observation confirmed the finding of the stratified analysis and that obtained from the matched index biopsies. Furthermore, it demonstrated the fact that unadjusted number of biopsies was acting as a confounder accounting for the elevated chi-square.

Table IV.13. Summary of adjusted chi square analysis for determining the predictive values of specific HPV's

HPV type	Matched chi squares	p values
HPV 6/11	3.26	0.079
HPV 18	0.526	0.468
HPV 16/33	1.9016	0.167
Any HPV	1.009	0.315

IV.III.1. Risk estimates for various HPV types and cervical cancer on the matched index biopsies.

The failure to detect a significant difference between the rates of detectable HPV in cases and controls may be due to the small sample size and the limited power of the study. A useful parameter to estimate the strength of the association between an exposure variable and an outcome variable is the relative risk (Cole,1980). This parameter can be expressed as an approximated odds ratio which is defined as the ratio of the person exposed to HPV having cervical cancer versus a non-exposed person having the disease. The null hypothesis to be rejected was that the relative risk for exposure to HPV and cervical cancer was 1.0, i.e. there was no increased risk between exposure to specific HPV and incurring cervical cancer. A similar strategy to the chi-square analysis was adopted for the risk estimate: The results of the matched index biopsies were first analyzed followed by the results on all the preinvasive biopsies. Adjustment for the differences in the number of biopsies was done by the conditional regression technique, using the MCSTRAT procedure of the SAS package.

The results of the relative risks on the index biopsies, estimated separately and individually by univariate techniques, are summarized in Table IV.14. It is seen that relative risk values of greater than one were associated with the HPV 6/11 and HPV 18; however, for HPV 16/33 or any of the genotypes of HPV that were tested the risk was close to unity. Hence, the null hypothesis could be rejected for HPV 16/33 or any HPV. The precision of the relative

Table IV.14. Summary of relative risk estimates for specific HPV's and cervical cancer in the index biopsies of cases and controls

HPV type	Matched Relative Risk	95% Confidence Bounds
HPV 6/11	6.95	0.75, 64.17
HPV 18	3.77	0.32, 43.44
HPV 16/33	1.23	0.42, 3.62
Any HPV	1.05	0.35, 3.16

risks estimates was measured by computing the 95% confidence intervals. The lower 95% confidence limit of the relative risk for HPV 6/11 was 0.75 and the upper 95% confidence limit was 64.17; the corresponding ones for HPV 18 were 0.32 and 43.44 respectively, while those for HPV 16/33 were 0.42 and 3.63 respectivly. These wide confidence intervals reflect the imprecision of the estimates derived from the small sample size of this study.

IV.III.2. Risk estimates for various HPV types and cervical cancer on all the preinvasive biopsies

The results of the risks estimates obtained from all the preinvasive biopies of the cases and controls are summarized in Table IV.15. It is seen that relative risk associated with the HPV 6/11 remained the same whereas those associated with the remaining HPVs were raised. The relative risk for HPV 16/33 was 2.89 that for HPV 18 was 5.86, and that for all HPV types was 2.60. These results enabled the null hypothesis to be rejected in favour of the alternative hypothesis. A direct corollary of these observations was that there was an increased risk of incurring cervical cancer if particular HPV sequences were present in the CIN lesions. However, the values of the 95% confidence intervals for these risks were wide and reflect the degree of uncertainties in estimating the risks. The relative risk estimate for any HPV was relatively more precise than the rest because it had the narrowest 95% confidence intervals of 0.95 to 7.45.

Table IV.15. Summary of relative risk estimates for specific HPV and cervical cancer in all the preinvasive biopsies of cases and controls

HPV Type	Method	Relative Risk	95% Confidence Bounds
HPV 6/11	Crude*	4.29	0.75, 24.33
	Adjusted**	2.19	0.39, 12.43
HPV 18	Crude	5.86	0.59, 57.43
	Adjusted	2.45	0.22, 27.50
HPV 16/33	Crude	2.89	1.10, 7.96
	Adjusted	2.34	0.70, 7.86
HPV	Crude	2.60	0.95, 7.45
	Adjusted	1.87	0.55, 6.28

* Unadjusted risk, irrespective of biopsy number per cases and controls.

** Adjusted for differences in the number of biopsies between cases and controls.

The observed elevated risks could be due to the unequal distribution of biopsies in cases and controls. This difference was adjusted by partitioning the number of biopsies into three strata and the use of conditional regression technique. Based on the frequency distribution of Table IV.6., the first stratum consisted of 1 to 2 biopsies; the second stratum, of 3 biopsies and the third stratum, of 4 to 5 biopsies. As shown in Table IV.15, the overall relative risks, after adjusment for all strata, were 2.19 for HPV 6/11; 2.45 for HPV 18; 2.34 for HPV 16/33, and 1.87 for all HPV combined. The computation of the 95% confidence intervals indicated that the risk estimates were highly imprecise for HPV6/11 and relatively less imprecise for combined HPV. A comparison of the crude relative risks and the adjusted relative risks shows that the risks estimated by the two methods were not equal for HPV 6/11, HPV 18 and any genotypes of HPV. Hence the number of biopsies confounded the relative risk estimate for cervical cancer and HPV 6/11, HPV 18 and any genotypes of HPV. This confounding was not observed for HPV 16/33 as the risk estimates were the same before or after adjustment.

IV.III.3. Multivariate analyses.

The risk estimates by using separate HPV regressor variable indicated that a risk of above 1.0 was associated with all of the HPV types. To determine the possible independent prognostic effects of each type of HPV type a conditional multivariate logistic regression technique was employed (MCSTRAT, SAS, Institute Inc., Carey N.C.).

Table IV.16. Summary of multivariate analysis to determine the relative risk of specific HPV's for cervical cancer

HPV type	Matched Relative Risk	95% Confidence Bounds
HPV 6/11	16.69	0.78, 356.5
HPV 18	0.1883	0.01, 4.95
HPV 16/33	2.21	0.58, 8.42

However, if a high degree of colinearity exists between the regressor variables this may undermine the multivariate analysis (Breslow and Day, 1980). An analysis for the most common combinations of HPV occurring together revealed that of the subset of 18.5% of patients that was positive for any genotypes of HPV, 12.3% of specimens contained only HPV16/33 whereas 2.2% of samples concomitantly contained HPV6/11, HPV16/33 and HPV18. By contrast, 0.7% contained HPV 16/33/18; 0.7% contained HPV 18 only and 0.7% contained HPV 6/11 only. Thus, no high degree of colinearity existed. The results of a full model incorporating all HPV variables are shown in Table IV.17. It is seen that the values of the adjusted relative risk for HPV 6/11 was 16.69 and that for HPV16/33 was 2.21; however, the risk associated with HPV18 was reduced to 0.1883. It was concluded that the original observed effect for HPV 18 was probably due to the small number of positive samples that were also correlated with HPV16/33 positivity. The imprecision of the relative risk estimate for HPV6/11 was again reflected by the wide 95% confidence interval of 0.78 to 356.5. By contrast, the estimate for any HPV was more precise since the 95% confidence interval was from 0.58 to 8.42. The results of the multivariate analysis thus indicated that HPV16/33, and possibly HPV 6/11, were more prognostic of cervical cancer onset than HPV 18.

IV.IV.O. Discussion

The primary aim of the case-control study was to determine if particular type of HPV could be predictive of invasive cervical cancer

onset. Towards this end, a retrospective case-control study, consisting of 47 cases and 94 matched controls, was conducted in patients derived from the Cancer Control Agency of British Columbia (CCABC).

The main hypothesis was that particular HPVs are predictive of invasive cervical cancer, hence occurring at higher frequency in the preinvasive biopsies of cases than in those of controls. The results of this study indicated that the null hypothesis of no difference in the HPV prevalence rates of cases and controls could not be rejected for any of HPVs that was tested. Hence, the alternative hypothesis could not be accepted. However, this study also enabled one to estimate the excess risk associated with particular HPV and CIN lesions that are likely to become invasive. The the null hypothesis was that the relative risk would be 1.0, in contrast to the alternative hypothesis of the relative risk being greater than unity It was observed that the presence of HPV 16/33 and 6/11 was associated with excess risk for cervical cancer because the values of the relative risks were higher than unity. Hence, in this instance, the null hypothesis could not be rejected leading to acceptance of the alternative hypothesis. Furthermore, these relative risks increased in values as the number of biopsies tested increased. Multivariate analysis to determine the relative prognostic value of individual HPVs showed that the risks associated with HPV 16/33 and HPV 6/11 persisted while that associated with HPV 18 disappeared. Thus, it was concluded that HPV 16/33 may be more prognostic of cervical cancer onset than HPV 18. However,in the case of HPV 6/11, it could not be

unequivocally determined whether the risk was genuine or spurious. The number of sample being positive for this virus was small and zero value existed in some of the strata that was used for the multivariate analysis, this could easily lead to statistical artefact (Breslow and Day, 1980)

IV.IV.2. Limitations of the present study design

There are two main sources of difficulty in interpreting these data. The first is a diagnostic one due to the biology of the virus and the second is an epidemiologic one. The diagnostic one concerns the validity and reliability of the assay used to measure exposure to HPV. The epidemiologic limitations are due to the effects of play of chance when only small number of events are studied and the incompleteness of the available demographic data on many patients.

IV.IV.2.1. Diagnostic considerations

The ascertainment of exposure to particular HPVs was done by the tissue in situ hybridization technique. The validation studies of section II.2.4.1., on colposcopically directed biopsies obtained from the target population, revealed that the assay had an overall sensitivity of 46.3% in detecting HPV sequences in CIN lesions and a specificity of 88%. In order to estimate sampling variation, multiple biopsies from the same patients were examined. In the prevalence study, it was observed that, when biopsies were taken at the same

time, the first biopsy was positive in 46% of the samples and the subsequent biopsies were positive in only 22% of the samples. This was probably due to the fact that the biopsies were colposcopically directed and overt HPV lesions were likely to be captured in one sampling. However, in the case-control study, very few of the biopsies were colposcopically directed. Hence, a focal lesion could have been missed. Furthermore, in the case-control study, analysis of multiple biopsies collected from the same subjects over a period of 2 to 5 years showed that the detection rate increased from 13.9% for the first two biopsies to 24.5% for the subsequent biopsies. This probably reflected the pattern of virus expression or shedding from occult HPV lesions (Nuovo et al., 1988). Hence, the biology of these viruses may preclude their accurate detection.

The rate of detecting HPV sequences in the case-control study was considerably lower than in the prevalence study. While this difference is compatible with a cohort or period effect, a limitation of the assay cannot be unequivocally ruled out. It is possible that longer storage of biopsies of the case-controls subjects led to a loss of detectable HPV sequences. In order to minimize any inter-batch variation in the assay, the cases and the controls were tested in the same batch. Nonetheless, inter-batch variation between the prevalence study and the case-control study is still a conceivable possibility. However, the reactivity of the positive controls (standards) in all the batches renders this possibility rather remote.

Taken together then, the inherent limitations of the assay could partially account for the failure to detect a difference in the

prevalence rates of cases and controls. Such an effect could be due to misclassification of the HPV exposure status. However, such misclassifications are likely to affect cases and controls equally and therefore could not exclusively explain the results of this study.

IV.IV.2.2. Epidemiologic Considerations

The major sources of errors in studies of this type are: chance; biases in the selection of cases and controls; bias or general inaccuracy in the information about the patients medical history and confounding.

The play of chance can affect the findings of this study. As explained in section III.3.2. of chapter two, there are two main types of errors in a hypothesis testing study. In a **type I error**, one rejects a true null hypothesis and in a **type II error**, one could accepts a null hypothesis that is not true.

Type I error, usually called the **significance** of the test is commonly due to small sample size and random variation in sampling. In the present study, cases and controls were chosen from the same sampling frame. Except for potential confounders, cases and controls were unlikely to have different likelihood for exposure to HPV. The null hypothesis of no difference between HPV prevalence rates of cases and controls could not be rejected for any of the HPV at a significant level of 0.05 and a one tail probability. The probability of this being the correct decision was 92% for HPV6/11; 83% for HPV 16/33; 47% for HPV18, and 68 % for any HPV. Hence, it is seen that the play of

chance on a small sample size could account for the failure to reject the null hypothesis. However, for the null hypothesis of no increased risk in incurring cervical cancer and exposure to HPV at the preinvasive stage, the relative risks value indicated that the null hypothesis could not be rejected at a significance level of 0.05, with a one tail probability test.

Type II error is usually due to insufficient sample size and a high exposure rate to HPV in the control population. The power of a test predicts the certainty with which one can accept the alternative hypothesis as being true. With the sample size of 47 cases and 94 matched controls and the various exposure rates to specific HPVs, this study had only 60% power to detect the projected difference in the prevalence rates of HPV16/33 between cases and controls. It had less than 50% of power to detect a similar difference in the prevalence rates of HPV6/11 or HPV18 (Fleiss, 1981). Hence, it is not surprising that the null hypothesis could not be rejected in this study.

The present study had 60% power to detect a relative risk of 2.5 for HPV16/33 and 80% power to detect risk of greater than 3.5 for HPV18 and HPV6/11 (Breslow and Day, 1987). These risk estimates were realized by univariate conditional logistic regression technique; however, the 95% confidence intervals indicated that these risks estimates were highly imprecise. Thus, the most imprecise estimate, after adjusment for the number of biopsies, was that for HPV 6/11 with a lower 95% confidence interval of 0.39 and an upper 95% confidence interval of 12.42 Therefore, it is likely that the risk associated with HPV6/11 was a statistical artefact due to the play of chance on

the small number of samples being positive for this virus. By contrast, the lower 95% confidence interval for HPV 16/33 was 0.70 and the upper confidence interval was 7.86, and the most precise estimate was for any HPV with a lower 95% confidence interval of 0.55 and an upper 95% confidence interval of 6.28. Hence, the relative risk estimates for these viruses appear relatively more reliable.

Selection bias occurs when cases and controls are assembled from two different sampling frames (Sackett,1979). In the present study, both cases and controls were derived from the registry of the CCABC that serves patients in the entire Province of British Columbia. The composition of this population has been detailed by Boyes et al., (1982). In 1969, the proportion of women ever screened by the Agency was assessed to be approximately 78% of all women over the age of 20. In 1983 it was estimated that 48% of the female population of B.C. was screened annually (CCABC annual report, 1983). Out of approximately 3400 patients that were treated for invasive cervical cancer over the past 20 years, only 108 (3.176%) eligible cases were found. Eligible case and controls not included in this study were excluded for diverse reasons such as unavailability of preinvasive biopsy containing CIN lesions; misdiagnosis or move away from the region. It is unlikely that such reasons led to any substantial difference in the exclusion of eligible cases compared with controls. Furthermore, the comparability of cases and controls was ensured by matching for grade of CIN lesions and year of diagnosis. This matching could control for a cohort or a period effect in the prevalence of HPV. Comparison of the HPV rates in the subjects from the case-control study with that

from the general population of the prevalence study showed that the rates of HPV were not similar. This lack of comparability could be explained by a cohort or period effect, although a previous report has not documented this phenomenon (Armstrong et al., 1986).

Alternatively, in trying to achieve comparibility of cases and controls in this study, the design might have resulted in **over-matching.** Based on the evidence reviewed in section I.2.0 of chapter 1, it is plausible to argue that the factors which cause CIN will also likely be causative for invasive cervical disease. A prevalent view among pathologists is that CIN is caused by HPV (Koss et al., 1987; Richart et al., 1987). Thus, by matching on grade of CIN, the exposure rates to causative HPV would have been equalized leading to overmatching. If true, this could also explain why no significant difference in the HPV prevalence rates could be established between the cases and the controls.

Information bias is due to general inaccuracy in measurement. To avoid such potential biases a structured questionnaire was used to collect demographic informations from the patients or the physician's office. In order to avoid any diagnostic suspicion bias during the reading of the tissue in situ assay, the identity of the cases and controls were concealed from the raters. Finally, as elaborated in section IV.IV.2.1, misclassification of HPV exposure due to limitation of the assay, though possible, is unlikely.

One situation where **confounding** may occur is when cases and controls are chosen from two populations that differ on domains other than HPV exposure. Factors such as age, severity of disease, type of

treatment and sexual life-styles could all potentially confound the finding of this study. As already mentioned, the severity of CIN disease was controlled for by matching, a process which also resulted in a fairly close match on the age of the patients. Similarly, the treatment of the cases and controls with respect to conization was not significantly different. However, the unknown differences in the sexual behaviour of the subjects remained a major potential confounding factor.

Various epidemiologic studies have shown that the attributes which predispose a woman to cervical cancer are early age of coitus and number of sexual partners (Rotkins, 1973; Kessler, 1981). Age of first coitus could not be directly estimated in this study. When age of first pregnancy was used as a surrogate to estimate age of coitus, it was observed that the cases were more likely to be pregnant at an earlier age than the controls. However, such information was missing from about 53% of the controls, making the validity of any such conclusion questionable. An attempt to estimate the probable number of sexual partners was made by indexing on prior surname, marital status, and other partners. However, the results were not very conclusive as such information was missing from approximately 45% of the controls. Therefore, different sexual behaviour of cases and controls remained the single most important potential confounder to distort the observed finding of the study.

In the present study, the unequal distribution of biopsies in cases and controls acted as a confounder for the computation of the chi-square statistic and the determination of relative risks. Attempt

to control the the degree of confounding was done by stratified analysis and logistic regression technique. However, it is conceivable that the high risk estimates in the stratum with 4 to 5 biopsies are due to residual confounding effect that could not be adequately controlled by categorizing the data into three strata. The possibility of an interaction between HPV and the number of biopsies could not be explored due to the small sample size of this study.

IV.IV.I. Implications for causality

The conclusion of the present study that the presence of specific HPV sequences in the preinvasive biopsies of cases may not be predictive of cervical cancer is corroborated by both the current biologic properties and epidemiologic features of these viruses. For the HPV 16/33 pair most of the knowledge is derived from studies on HPV16. However, the extensive genetic similarities between HPV16 and HPV33 (Beaudenon et al., 1986) enable them to be regarded as subtypes (Coggins and zur Hausen, 1979) that may share the same biologic properties.

The biologic properties of HPV16 are compatible with its being a potential oncogenic agent, albeit an incomplete one. Various studies have shown that HPV16 DNA can induce in vitro cellular transformation, a process that is analogous to tumour initiation. Among the cell types which can be transformed are NIH 3T3 (Yasumoto et al., 1986); primary human keratinocytes (Pirisi et al., 1987) and primary rat cells (Matlashewski et al., 1987). However, other studies

have shown that cofactors such as oncogenes (Matlashewski et al., 1987) or steroid hormones (Pater et al., 1988) can enhance the transformation efficiency of the HPV16 DNA. Similarly, there is evidence to suggest that HPV 6 also has potential oncogenic property. Thus, the E6 and E7 regions of HPV 6 has been reported to transform secondary rat embryo fibroblasts in co-operation with the v-ras oncogene (Chesters and McCance, 1988). Taken together, these observations support the argument that HPV is a probable co-factor rather than an independent etiologic one in the genesis of cervical cancer.

The epidemiologic evidence linking HPV to cervical cancer has been reviewed in section 1.3.5.0 of Chapter I. Although the evidence is compelling, serious gaps exist in linking HPVs with cervical cancer (Munoz et al., 1988). The presence of HPV 16 in cervical cancers has been demonstrated in virtually all the studies; however, considerable variation in the rates has also been detected. A dose-response relationship, at a population level, has not been adequately established (Kjaier et al., 1988). Although these viruses mostly segregate in cervical cancers, they also occur in other forms of neoplasias (zur Hausen,1985). Finally, the correct temporality for HPV 16/33 and cervical cancer has not been established. The epidemiologic evidence implicating HPV 6/11 in the genesis of cancer is circumstantial as HPV 6/11 have been infrequently associated with human carcinomas. HPV 6 was originally isolated from condyloma acuminata and HPV 11 was isolated from benign laryngeal papilloma (Pfister, 1984). Subsequently, there has been two lines of evidence to indicate that they may be involved in squamous carcinomas: (a) HPV

6/11 has been isolated from 18% of invasive cervical cancer (Gissmann et al., 1983); from verrucous carcinoma, a non-metastasizing invasive tumour of the cervix (Okagaki et al.,1984); from carcinoma of urethra in man (Grussendorf-Conen, 1986a); HPV 11 has been isolated from squamous carcinomas of the lung (Byrne et al., 1987), and a subtype of HPV 6 has also been isolated from invasive vulvar carcinoma (Rando et al.,1986), (b) previous epidemiologic observation has shown that women with genital warts, now identified to be associated with HPV 6/11, have a relative risk of 5.8 for CIN disease than appropriately matched controls (Franceschi et al., 1983). A direct corollary of all these observations is that these HPV sequences per se are insufficient to be predictive of invasive cervical cancer. However, taken together, the evidence does not rule out a role for HPV16/33 and HPV 6/11 as a risk factor for cervical cancer. Indeed it has been suggested that HPV per se may be insufficient in causing cervical cancer and other co-factors may be required to act in synergism (zur Hausen, 1982).

The strength of the association between HPV and cervical cancer, in this study, was calculated by univariate analysis after adjusting for the number of biopsies. The values of the relative risk was 2.34 [95% CI 0.70,7.86] for HPV16/33; 2.45 [95% CI 0.22,27.50] for HPV18 and 2.19 [95% CI 0.39,12.42] for HPV6/11. These values are rather modest as Cole (1981) has suggested that only relative risk above 5.0 may reflect a causal association in a case-control study. Multivariate analysis showed that the relative risk for HPV 16/33 was increased to 2.21 and that for HPV 6/11 to 16.69. However, the respective confidence intervals indicated that there were less

uncertainties associated with the risk estimate for HPV 16/33 than that for HPV 6/11. The wide confidence interval for HPV 6/11 may also reflect a statistical artefact due to the small number of samples that was positive for these viruses. If the causal relationship is favoured then, with the existence of subtypes of HPV 6 (de Villiers et al., 1981; Rando et al.,1986), the possibility of a prevalent oncogenic subtype in the B.C. population cannot be ruled out. Unfortunately, the tissue in situ hybridization cannot distinguish between subtypes of the virus.

It is equally plausible to argue that HPV 6/11 and HPV16/33 represent covariables of sexual behaviour. A number of prevalence studies point to the ubiquity of the HPVs in the male and the female genital tracts (Reid at al., 1987; McCance et al.,1987; de Villiers et a., 1986; Grussendorf-Conen et al., 1986b). The presence of HPVs also correlates with the presence of other venereally transmitted agents (Syrjanen et al .,1984). Several consort studies have also demonstrated that these viruses are transmitted from female to male (Stein, 1980.; Levine et al., 1984; Barroso et al.,1987). Finally, a case-control study to determine the risk factors for patients with genital condylomas has been recently published (Brisson et al ., 1988). In the latter study it was found that the risk for having genital condylomas increased from 1.0 to 2.0 as the number of sexual partners increased from 1 to 4 or more. If these HPVs are causally associated with cervical cancer then they must show an association much stronger than that for other sexually transmitted agents. Such information was, unfortunately, not available for all the study subjects, hence this

argument can be neither refuted nor substantiated. Thus, compelling as the evidence may be, the relative risk values remained confounded by possible differences in the sexual behaviours of the cases and controls.

IV.IV.3. Concluding Remarks

In response to the main research question, it is concluded that this study did not have sufficient power to show that the presence of HPV sequences in the preinvasive biopsies could be predictive of invasive cervical cancer onset. Nonetheless, this study identified a moderate risk of incurring cervical cancer associated with the presence of HPV 16/33 and with HPV 6/11 in CIN biopsies. The values of the relative risks are more consistent with these viruses being a risk factor rather than an independent etiologic one. However, due to the small number of samples reacting to HPV 6/11 it was not possible to unequivocally conclude if the risk associated with HPV 6/11 was real or spurious. In either case, these conclusions are potentially confounded by differences in the sexual behaviours of the cases and controls.

Future studies, possibly using pap smears for testing exposure to HPV is warranted. If the sample size is large enough one would be able to control for extraneous variables and determine the true predictive values of these viruses. Recently, a more sensitive assay for detecting HPV sequences in fixed tissues have been introduced (Shibata et al., 1988). Once the specificity of this assay is

determined, it could be used to increase the detection rates of HPV sequences in retrospective studies.

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