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DETERMINANTS OF INCIDENT PRECURSOR LESIONS OF CERVICAL CANCER

Sophie Kulaga
Department of Epidemiology and Biostatistics
Faculty of Medicine
McGill University

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STATEMENT OF SUPPORT

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STATEMENT OF ORIGINALITY

The research presented in this thesis consists of original work. I distinguish my work from that of the many researchers collaborating on this project by using the first person when referring to my direct role. On the basis of data gathered for the Ludwig-Mcgill Brazilian Cohort I have reported an incidence density rate for cervical precursor lesions in this population at high risk of cervical cancer and have identified some risk factors for cervical precursor lesions that have an independent association beyond the well established etiologic agent of HPV.

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While I share the credit with many to whom it is due, I alone accept responsibility for the shortcomings of this work.

ABSTRACT

Squamous intraepithelial lesions (SIL) are believed to be precursors of in situ cervical neoplasia and of invasive cervical cancer. Though the burden of this disease has been greatly reduced thanks to the availability of an effective screening test, the worldwide morbidity and mortality remain high.

On the basis of a classic approach to analysis of data from a prospective cohort study, I correlated HPV status at enrollment with subsequent risk of incident SIL during up to three years of follow-up among women of Brazil enrolled to the Ludwig-McGill cohort.

The risk of occurrence of a first instance of SIL among women was strongly associated with HPV infection. After adjustment for a number of socio-demographic factors that have previously been established as risk factors for cervical cancer, the magnitude of association with high-risk HPVs remained unchanged while the association with low risk types was dampened, furthering the evidence for the role of oncogenic HPV types in the development of cervical cancer.

Parity was found to have an independent positive association with cervical precursor lesions, after adjustment for HPV status, age, previous history of Pap screening, number of sexual partners, age at first intercourse, and income. Income was also positively associated with the development of lesions.

RÉSUMÉ

On pense que les lésions squameuses intra-épithéliales (LSI) sont les précurseurs de la néoplasie cervicale et du cancer invasif du col utérin. Même si le poids de cette maladie s'est considérablement allégé grâce à l'existence d'un test de dépistage efficace, la morbidité et la mortalité qui s'y rattachent à l'échelle mondiale n'en restent pas moins élevées.

Sur la base d'une méthode classique d'analyse des données, à partir d'une étude prospective de cohorte, j'ai établi la corrélation entre la détection du VPH (virus du papillome humain) au début de l'étude et le risque subséquent de LSI incidente. Cette étude porte sur les trois premières années du suivi dont la cohorte Ludwig-McGill, composée de femmes bresiliennes, a fait l'objet.

Le risque d'occurrence d'un premier cas de LSI est étroitement associé à l'infection par le VPH. Après ajustement en fonction d'un certain nombre de facteurs socio-démographiques qui ont été établis antérieurement comme facteurs de risque du cancer cervical, l'importance de l'association avec le VPH à haut risque est restée inchangée alors que l'association avec les types à faible risque a été amoindrie, soulignant le rôle des types de VPH oncogènes dans le développement du col utérin.

Une association positive indépendante a été obtenue entre le nombre d'enfants et les précurseurs du cancer du col de l'uterus après ajustement en fonction du bilan VPH, de l'âge, des antécédents liés au dépistage par le test Pap, du nombre de partenaires sexuels, de l'âge au premier rapport sexuel et du revenu. Une association positive indépendante a été aussie obtenue entre les précurseurs du cancer du col de l'uterus et le revenu.

Part I

INTRODUCTION

This report opens by summarizing the descriptive epidemiology of cervical cancer and providing some historical context. The important previously established risk factors of cervical cancer are then reviewed, with the understanding that precursor lesions would be expected to share some of these risk factors. Any risk factors that are specific to cervical cancer and not found in association with precursor lesions could prove to be those that determine whether a lesion in a particular women will follow a neoplastic course. This type of comparative analysis presumes characterization of the determinants and behavior of precursor lesions which are the focus of this report. The most important risk factor for cervical cancer and also for precursor lesions, infection with certain human papillomaviruses, is presented in some depth. Description of terms and procedures relevant to the full appreciation of the results presented are provided.

The Ludwig-McGill Brazilian Cohort Study (LMB), which is the source of the data analyzed and presented, has been designed specifically to measure exposure variables and ascertain cervical status over time. This ability is crucial to a study in which the central hypothesis is that persistent infection with certain types of human papillomaviruses (HPV) provides the first step of the etiologic process in the development of cervical cancer. For the purpose of this report a classic approach for the analysis of prospective cohorts has been taken. using only the baseline measure of HPV infection, a single determination of exposure with which to predict an outcome that is measured over time. The limitations of this approach are fully recognized. Within several months, the cohort will become vastly more informative allowing a richer analysis of exposures as they change with time. Such analysis will be the focus of future reports from the LMB Cohort Study Group.

Objectives

i) To measure the incidence of cervical precursor lesions in a population of women known to be at high risk for cervical cancer.

ii) To examine the point prevalence of cervical precursor lesions and the rate of regression, persistence or progression of precursor lesions.

iii) Using baseline data on HPV as the main predictor, the central objective of this work is to identify factors able to predict cervical precursor lesions out of a set of classic risk factors for cervical cancer.

Rationale

If precursor lesions and cervical cancer truly exist on a continuum of lesions of ever increasing severity, then some of the risk factors for cervical cancer must be shared by its precursors. Identifying those risk factors which maintain an independent association with precursor lesions in the face of adjustment for the main explanatory variables would contribute to understanding the causal pathway of cervical cancer.

In this cohort study I explore some risk factors of cervical cancer and their association with cervical precursor lesions in full consideration of certain HPV types as the etiologic agent.

Part II

BACKGROUND

Descriptive Epidemiology of Cervical Cancer

Worldwide, 440,000 women (Parkin, et al.,1993; Stern and Stanley,1994) are newly diagnosed with invasive cervical cancer each year. Eighty percent of these cases occur in the context of developing countries where cervical cancer is the most important malignancy in terms of both morbidity and mortality among women (Parkin, et al.,1993; Pisani et al.,1993). Globally, cervical cancer is the second most frequent cancer of women, representing 11.6% of all female cancers. Breast cancer, which is the leading cancer of women represents 19% of the world's burden of cancer among women (Parkin, et al.,1993). Despite the fact that cervical cancer is one of very few cancers for which an effective screening test exists, worldwide mortality continues to be high. In 1985, 203,000 women were estimated to have died as a result of cervical cancer (Franco,1996). Average worldwide incidence in that year was 18.1/100,00, ranging from a low of 5.1 in Western Asia to a high of 35.7/100,000 in Southern Africa (Parkin et al.1993).

There exist even greater disparities in incidence and mortality between regions within countries. In northern Brazil there are 80-100 new cases per 100,000 women per year. Here a woman's lifetime cumulative risk of cervical cancer can approach that of a North American woman's risk of breast cancer, 10% lifetime risk (Franco,1996).

On average, cervical cancer strikes at a younger age than does breast cancer and is characterized by a shorter survival time. The average age at diagnosis of invasive cervical cancer is 47 years, 17 years younger than the average age at diagnosis of breast cancer.

Where health care systems are able to provide mass screening, the major proportion of these neoplastic lesions are found at an early point while the disease is highly treatable and relatively few women will die as a result of this cancer or its metastases. In most regions of developed countries there has been an important decrease in both mortality and incidence of cervical cancer due to cytological screening,

but women everywhere with low socioeconomic status continue to be at high risk of cervical cancer or pre-malignant lesions. Organized cytological screening, under which targeted groups of the population are actively called in for screening, seems to be more efficient at reducing the incidence of, and mortality due to, cervical cancer than opportunistic screening which allows the patient or physician to request the screen (Gustafsson et al.,1995).

At least eighty percent of incident cases occur where resources do not permit a regular schedule of screening or adequate quality of cytology. Here cervical cancer will claim a high number of those presenting with late symptoms.

Though insufficient access to screening and absence of screening programs are widely cited as the reason for high incidence and mortality, there is some evidence that even in those developing areas where such programs have been implemented, the expected decreases are not being observed, in spite of high participation and compliance rates (e.g. Costa Rica). Reasons for lack of impact seem to be related to the quality of various aspects of the program (Herrero et al.,1997).

In the United States, cervical cancer remains an important cancer among African-American, Native and Hispanic populations. Though statistics are reported for the general population, the 14,500 new cases of invasive cervical cancer and 4,800 deaths due to cervical cancer per year in the US (Parkers et al.,1997) arise largely among women from these high-risk groups. In developed countries, the size of the population of women who truly bear the risk is much smaller than the greater female population.

Cervical Cancer in Canada

In Canada, the estimated age-standardized incidence rate of cervical cancer for 1995 was 7.8 per 100,000 population (Gaudette and Lee,1995) representing 1300 incident cases (a decrease from 1493 in 1990). The estimated age-standardized mortality rate due to cervical cancer in the general female population is 2.2/100,000 population (women), representing 370 deaths in 1995 due to cervical cancer among

Canadian women (compared to 368 in 1992). This compares to a mortality rate of 24.3/100,000 native women for the period 1973-1984 (Hislop et al.,1994).

Cervical cancer is one of three cancers for which incidence and mortality in Canada have decreased, leading the National Cancer Institute of Canada to conclude that the "decline in mortality is due mainly to the decline in incidence". In Canada, incidence of cervical cancer since 1969 has decreased from 21.6 to 7.8 per 100,000 population in 1995; mortality due to cervical cancer has decreased from 7.4 (1967) to 2.2 (1995) per 100,000 population (Gaudette and Lee,1995).

Current Trends in Cervical Cancer

The decline in incidence and mortality in the US observed from 1987-91 has slowed and a trend of increased incidence of this cancer in women less than fifty years of age is appearing in the North American and European populations (Franco,1996). In the United Kingdom, though overall mortality has declined between 1979-1992 due to screening programs, the age-specific mortality in women younger than 40 has increased markedly (Monsonego,1997). There is also an apparent slowing in improvement of the five-year survival rate following a diagnosis of cervical cancer (Franco,1996).

In some populations (notably England and Finland) an increase in the incidence of invasive cancer among young women (age 20-35 years) has been reported. This raises a concern for future incidence rates if this increase is related to a cohort effect (Miller et al.,1991).

Etiologic Agent: Human Papillomavirus

There is overwhelming biological, experimental (Zur Hausen,1994) and epidemiological (Schiffman et al.,1993) evidence to indict human papillomavirus (HPV), a very common cervical infection, usually transmitted sexually (Bauer et al.,1993), as the main etiologic factor for pre-malignant and malignant lesions of the uterine cervix. The International Agency for Research in Cancer (IARC), in its 1995 Monograph of HPV states, "*there is sufficient evidence in humans for the carcinogenicity of human papillomavirus (HPV) types 16 and 18; there is limited evidence in humans for the*

carcinogenicity of some other HPV types; there is evidence suggesting lack of carcinogenicity to the cervix in humans of HPV types 6 and 11" (IARC,1995). HPV can be found in 90% of high grade cervical lesions (Eckert et al.,1995) and it is possible that the few cervical cancers found without HPV might reflect either the imperfect sensitivity of the detection techniques, the eventual degradation of viral DNA (Franco,1995), or causative factors other than HPV.

HPV is ubiquitous in human populations with some believing that this obligate parasite has developed in co-evolution with its human host since earliest times (Arrand,1994; Gissman and Muller,1994). HPV infection is increasingly being looked upon as a necessary cause of cervical cancer (Franco,1997), but just as certainly, it is not a sufficient cause (Stern and Stanley,1994). Only a relatively small proportion of infected women develop cervical cancer. All estimates of prevalence of HPV infection are far greater than the prevalence of cervical cancer. It is likely that HPV represents the etiologic "first step" (Schiffman and Brinton,1995). What are the co-factors which, in the presence of an HPV infection, will set the HPV infected epithelium on a course to development of lesions that will not regress, but rather will progress towards truly neoplastic characteristics? HPV type, viral load and persistence; cell mediated immunity, reproductive factors (particularly parity), nutritional factors, co-infections are all risk factors for progression that are under study (Schiffman and Brinton,1995). They can be classified in three main categories: viral factors, host factors and environmental factors. What are the critical events in the progression of the neoplastic process and what is their timing? What does the moment of integration of viral genome mark?

HISTORICAL PERSPECTIVE

Though long postulated to be a sexually transmitted disease, with epidemiological studies showing that cervical cancer behaves as a venereal disease (La Vecchia et al.,1986), only recently has the virally-induced carcinogenesis of cervical cancer become widely accepted (IARC,1995).

In the 1840's Rigoni-Stern published relative frequencies of cancer by site according to marital status. Stern was looking for an explanation of his finding that

women were eight times as likely to have died of a cancer as were men. He found that " *cancers are more frequent among nuns than among all other women in the ratio of 5 to 1*" (de Stavola,1987) Though this is an often quoted reference in introductions to the epidemiology of cervical cancer, Stern's conclusions focus on the increased incidence of breast cancer among nuns as the main contributor to these elevated odds for nuns. It is of historical interest that he notes an increased incidence of cancers with increasing age, and suggests the necessity for age-adjustment ("*it is better instead to compare their different frequencies relative to age*"). Stern's most interesting finding with respect to cancer of the uterus, (which would have included cervical cancer, as this site was not, at that time, anatomically defined as distinct from the uterus) was that this cancer "*is already very frequent between age 30 and 40*", while breast cancer is very rare at that age, occurring with greatest frequency in women after the age of 60.

In 1886, Sir John Williams "*commented on the presence of noninvasive epithelial abnormalities adjacent to invasive squamous cell carcinomas of the cervix*" (Wright et al.,1994). Williams is credited with being the first to observe and report that cellular changes might be prognostic indicators of eventual development of carcinomas (Correa,1991).

In the late 1920's Pemberton and Smith reported on the histological characteristics that would allow them to differentiate between carcinoma *in situ* (CIS) before it had broken through the basal membrane, and invasive cervical cancer (Pemberton and Smith,1929). Others credit Broders (1932) with this discovery (Parkin,1997).

The 1940's saw the recognition of the existence of precursor lesions and in 1943, Papanicolaou and Traut reported that exfoliative cytology could be used in the diagnosis of cervical cancer (Parkin,1997). Dysplasia was the term used for those lesions that had some of the cytological features of CIS, but to a lesser extent (Wright and Kurman,1994). In the 50's and 60's when the natural history of dysplasia was studied, researchers found that only a small proportion of dysplasias progressed to invasive cancers, while most regressed.

In 1954 Wynder reported findings from a case-control study that related sexual activity to cervical cancer (Wynder et al.,1954). In 1967 Rotkin found that an early age of initiation of sexual activity and promiscuity are strong risk factors for later development of cervical cancer (Stern and Stanley,1994) .

Results of epidemiological research in the 70's suggested that cervical cancer was associated with an infectious agent that was transmitted sexually, thus providing an explanation for the previously determined associations with sexual behavior (Kessler II,1977). Studies of virgins and women in predominantly monogamous cultures show low incidence rates of cervical cancer (Parkin et al.,1993). Female partners of men with penile cancer were more likely than controls who were married to men without penile cancer to be diagnosed with cervical cancer. The risk increased as their partners had more sex partners or had previously been married to a woman who had died of cervical cancer (Kessler II,1977). In 1976 Zur Hausen found a parallel incidence of cervical cancer and genital warts, leading him to conclude that the wart-causing agent (HPV) might also be the cause of cervical cancer (IARC,1995). In the early 1980's work began to identify this sexually-transmitted agent. After eliminating many sexually transmitted diseases as well as human sperm, two viruses remained the most likely candidates as etiologic agents: human papillomavirus (HPV) and herpes simplex virus II (HSV2) (Kjaer et al..1990; Franco,1991). By the early 1990's researchers were reporting that HSV was not associated with cervical cancer (Peng et al.,1991).

In the late 80's, as reviewed by Franco (1991), some epidemiological studies returned unexpected results. Sexual behavior continued to be strongly associated with the development of cervical cancer in all studies, but HPV infection was not showing the expected association with sexual activity variables, which was necessary for the sexual activity-HPV-cervical cancer relationship to hold. The cause of this apparent lack of association turned out to be misclassification of HPV exposure status due to measurement errors of early molecular laboratory techniques (Munoz et al.,1992). When polymerase chain reaction (PCR) was first used in the detection of HPV DNA of cervical specimens, the prevalence of HPV infection was much higher than that expected of an etiologic agent. More recent laboratory methods, such as PCR protocols

that require the use of consensus primers, or the hybrid capture assay, have reduced the problem of misclassification and the expected association between etiologic agent and disease is again being observed.

Case-control studies of the early 90's using new generation hybridization assays (PCR, Virapap, and Southern blot hybridization) with protocols that minimize contamination have reported odds ratios of large magnitude for the association between certain HPV types and cervical cancer (Munoz et al.,1992).

PCR studies conducted in different populations do not consistently report a given magnitude of association between sexual activity and prevalence of HPV infection. The source of the variability in these results may reflect the differences between populations in the prevalence of certain HPV types or of some important co-factors required for carcinogenesis (Franco et al.,1995; Richardson,1996). On the other hand, cervical cancer specimens from all over the world consistently exhibit a high prevalence of HPV infection, though there appears to be a great deal of geographic variability of HPV types (Bosch et al.,1995).

In 1991 Zur Hausen concluded that "papillomavirus infection of the genital tract can lead to squamous cell carcinoma, particularly of the uterine cervix".

Recently, Franco (1996) reviewed several case-control and cohort studies that demonstrate the link between HPV infection and risk of cervical neoplasia. The combined relative risk (RR) for CIN obtained from pooling PCR results of those studies was 19.8 (95%CI: 15.2-25.8). The risk is even higher for invasive cervical carcinomas (ICC). Studies using PCR to determine viral status show that women infected with HPV have almost 35 times the risk of developing ICC (RR of 34.5, 95%CI: 21.5-55.4) when compared to women who are not so infected.

HPV is now widely accepted as the main etiologic agent in cervical neoplasia. Zur Hausen has said, "experimental as well as epidemiological data leave scarcely any room to doubt causality" (Zur Hausen,1994). It is believed that the natural history of

cervical cancer is characterized by a continuum of increasingly severe precursor lesions, beginning with those lesions caused by cervical HPV infections right up to invasive cancers (Schiffman and Brinton,1995). Several case-control studies have shown that certain HPV types (i.e. high risk HPV 16 and HPV 18), are more often found in women with cervical pre-malignant and malignant lesions than in those without (Davies,1994).

The relevant Bradford-Hill criteria for causality have been met (Franco,1996; Schiffman and Brinton,1995): strength (magnitudes of relative risks in the range of 20 - 70; consistency of results among studies (all studies showing positive associations); correct temporal sequence (early results of large prospective studies of cytologically normal women are showing high RRs of HSIL within a few years of viral detection, thus demonstrating that viral infection precedes lesion development), specificity (HPV causes carcinomas of mucocutaneous epithelium); coherence (between animal studies showing the malignant potential of papillomaviruses and experimental evidence in which the ability of certain HPV types to transform human cell lines in culture has been demonstrated) and (HPV epidemiology paralleling existing epidemiological knowledge of cervical cancer). Recent studies have shown an association with viral load, which would satisfy the requirement for a demonstrable biological gradient (Ho et al.,1995; Villa et al.,1996).

Zur Hausen has proposed several criteria of causal relationships specifically for the case of tumours caused directly by persistent infectious agents (Zur Hausen,1994). These represent a case-specific update of Koch's postulates and are summarized as follows:

1) Existence of epidemiological evidence of particular infection representing a risk factor for a given tumour.
2) The genome of the infectious agent is "regularly" found in the tumour.
3) Proliferation of cells in tissue culture can be induced by the transfection of the genome of the infectious agent.
4) This proliferation and the subsequent malignancy of the tumour are the result of the actions of the DNA of the infectious agent.

Several on-going cohort studies, including the one that is the focus of this report, are underway to elucidate the natural history of this viral infection and to fully characterize its role in carcinogenesis. The majority of the studies that have contributed to the evidence for the association with HPV are of the case-control type, in which by design, a temporal ambiguity with respect to timing of exposure and onset of disease exists. Those who are concerned that neoplasia might precede the exposure to HPV propose that lesions might be permissive for viral infection or proliferation. Results from prospective cohort studies show that the timing of the exposure to HPV is critical to an individual developing cervical cancer. Questions that consider the occurrence of disease in relation to the moment of exposure can only be addressed by prospective cohort studies designed specifically to study exposures that change with time. The prospective cohort is ideally suited to resolving the issue of temporality.

In spite of the limits to our knowledge, our understanding of the pathogenesis of this cancer is far greater than for that of most other malignancies. Thanks to the collaboration between molecular biologists and epidemiologists in which very precise molecular-based tests used to measure exposure are combined with classic risk factor analysis (molecular epidemiology), it seems likely that this will be one of the first human malignancies for which the molecular and cellular events will be fully characterized (Zur Hausen,1994).

THE UTERINE CERVIX

Anatomy Relevant To Cervical Cancer

Eighty percent of cervical cancers are squamous cell carcinomas, as distinct from adenocarcinoma and adenosquamous carcinoma (Schiffman and Brinton,1995; Wright et al.,1994) . The epithelium of interest in cervical lesions is the non-keratinizing stratified squamous epithelium of the female genital tract. It is a keratinocyte lineage made up of a basal layer (from which stem cells migrate), the stratum spinosum and the granular layer (Campo,1994).

The human cervix is made up of two epithelial surfaces - the ectocervix of stratified squamous epithelium and the endocervix, a single layer of mucin-secreting

epithelium The transition between the ectocervical and endocervical epithelia is at the squamous columnar junction - the transformation zone. Several developmental events characterize the squamocolumnar junction. During the fetal, perimenarchal and first pregnancy periods, the junction undergoes squamous metaplasia (Campo,1994). It is in and around this transformation zone of metaplastic epithelium that the majority of cervical cancers occur (Buckley,1994). It may be that this transitional epithelium is particularly susceptible to oncogenic agents.

Developmental Events

In a female neonate endocervical cells are present on the vaginal portion (exocervical or portio surface) of the cervix, forming the cervical ectopy, the size of which is determined by the extent of inward migration of vaginal squamous epithelium. At one year, the squamocolumnar junction moves towards the external os. The size and distribution of the cervical ectopy is affected by hormonal and physical factors. Pregnancy and menarche are characterized by the enlargement of the cervix and the eversion of the endocervical epithelium onto the portio. During pregnancy and parturition the cervix undergoes morphologic changes related to the effects of elevated steroid hormones (Ferenczy and Wright,1994).

After puberty the columnar epithelium of the ectopy is replaced by metaplastic squamous epithelium forming the functional squamocolumnar junction. The transformation zone is the region between the original (neonatal) squamocolumnar junction and this new squamous junction. Almost all cervical squamous neoplasia begins at the post-pubertal squamous junction and cervical cancer precursors occur almost exclusively within the limits of the metaplastic epithelium of the transformation zone. During the reproductive years (as well as during pregnancy), the transformation zone is located on the exposed part of the cervix (Ferenczy and Wright,1994).

Pathogenesis of HPV related cervical cancer is now understood as a 3-stage disease process: (1) HPV infection (2) increasingly severe grades of cervical intraepithelial neoplasia (CIN 1-3) and (3) invasive cancer (IARC,1995).

RISK FACTORS FOR CERVICAL NEOPLASIA

Work prior to the elucidation of the role of HPV had established certain risk factors for cervical cancer. In the late 1800's and early 1900's various groups were reporting that 90% of cervical cancers occurred in women who had previously delivered babies. The thinking of the time was that the healing process somehow triggered the eventual cancers (Pemberton and Smith,1929). Risk factors for cervical precursor lesions are very similar to those risk factors for cervical cancer (La Vecchia et al.,1986). Women with low socio-economic status are at greater risk of cervical cancer than more advantaged women (Franco et al.,1988). Marriage at a young age was established as a risk factor by early epidemiological workers, corroborated by studies which found that women who initiated sexual activity at an early age or had more lifetime sexual partners were at increased risk of cervical cancer (Brinton et al.,1987; Herrero et al.,1990). Over the past 25 years epidemiological studies consistently show that certain sexual behaviors are the most important determinants of cervical cancer: lifetime number of sexual partners, age at first intercourse, and sexual behavior of the woman's male partners are considered the classic risk factors for cervical cancer. Parity has been shown to have a "dose-response" relationship with increasing number of live births associated with an increase in cervical cancer risk (Brinton et al.,1989).

Use of Oral Contraceptives

Most studies that consider oral contraceptive use (OC) as a risk factor for cervical cancer show moderately elevated risk, particularly among women who are HPV-positive (La Vecchia et al.,1996). Others find that the timing of this exposure is crucial to the relationship: first use at an early age (Daling et al.,1996), current or recent use (Zondervan et al.,1996), or increasing risk with increasing duration of use (Kjaer et al.,1993).

Interpretation of the association of oral contraceptive use (OC) with cervical cancer is confounded by sexual activity (of which OC use is also a measure) and the increased number of detection opportunities (Pap smears) as a result of more frequent gynecologic examinations. OCs would likely act as a promoter in carcinogenesis and so lifetime exposure, which is what most studies measure, may be less relevant than

exposure once a lesion has become established at the cervix. The biological action of OC could be the proliferation of epithelial cells, including those which have presumably undergone transformation, thereby hastening the process (Clarke et al.,1985). An alternative mechanism by which OC might be involved is the interactive effect (effect modification) of OC on HPV and in this case measures of recent OC use, or OC use concomitant with exposure to HPV would be of greatest relevance. It is also possible that OCs have a protective effect by stabilizing physiologic levels of the relevant hormones and maintaining hormonal balance (Williams and Weisburger, 1991).

Smoking

In 1966, Naguib and co-workers reported that the proportion of smokers among cervical cancer patients is greater than among controls without cervical cancer (Naguib et al.,1966). In 1977, Winkelstein identified a correlation between the distribution of smoking-related cancers and cervical cancers, leading him to suggest an association between cervical cancer and smoking (Winkelstein,1977). He updated this view in 1990 by reviewing all published epidemiological studies. Winkelstein concluded that smoking is a risk factor for cervical cancer (Winkelstein,1990). As smoking is a behavior known to be associated with sexual behavior, to establish smoking as an independent risk factor its effect must be fully adjusted for sexual behavior, which is difficult to do in practice (Phillips and Smith,1994).

Tobacco smoking provides a source of carcinogens and the cervical mucus of smokers has been shown to be mutagenic (Holly and Petrakis,1986). In 1995, a British group of basic scientists confirmed that the number of DNA adducts in exfoliated cervical cells was higher in smokers than in non-smokers, but that there appeared to be no interactive effect between smoking and high-risk HPV types (HPV16) (Simons et al.,1995). Smoking either acts as a direct carcinogen or by lowering local immunocompetence.

Smoking is thought to be capable of inducing changes in the immune system, reducing IgA, IgG and IgM levels and contributing to the suppression of T-cell function (Holt,1987). That host immune status plays a role in HPV infection and in progression

to cervical lesions has been evidenced in patients with immune deficiency. Study of incidence and progression to carcinoma of condylomata (skin warts) in immunosuppressed patients (e.g. renal transplant patients) argues in favor of the role of the immune system in carcinogenesis. Women infected with human immune deficiency virus (HIV) have a high prevalence of HPV infection (Judson,1992) and cervical cancer has been added as one of the clinical criteria for diagnosis of HIV. Studies of squamous intraepithelial lesions (SIL) from US, UK, Kenya, Zaire and Italy show a higher prevalence of cytological abnormalities among women infected with HIV (12.5-fold) than amongst sero-negative controls (Wright,1997). All forms of immunosuppression are permissive for both the virus and the replication of infected cell, and consequently, for the persistence of lesions (Campo,1994). Spontaneous regression of the HPV infection among the majority of immunocompetent people is the norm (Schiffman and Brinton,1995; Kiviat,1996).

Socioeconomic Status

Invasive cervical cancer is known to occur more often in women with low socio-economic status (SES) (de Sanjose et al.,1996): women with low education are three times more likely to have invasive cervical cancer than better educated women. Low SES, as measured by education or income, is associated with higher prevalence of HPV infection, but also with nutritional deficiencies, multiparity, and concurrent genital infections, all of which may contribute to the elevated risk (Schiffman and Brinton,1995). Education level has also been found to be a strong indicator of cervical cancer risk. In case-control studies in Colombia and Spain, HPV DNA prevalence was found to decrease with increasing education (de Sanjose et al.,1996). Although in Columbia and Spain women with lower education report fewer sexual partners than do women with higher education , their partners report being more likely to have ever used prostitutes.

An alternative, but non-exclusive explanation of the association between SES and cervical cancer is that SES is a proxy measure for nutritional status, with women in lower SES categories suffering more often from deficiencies of certain nutrients. For example, nutritional deficiencies of vitamins A, C and beta-carotene have been shown to be a risk factor for cervical cancer (Eckert et al.,1995; VanEenwyk et al.,1992).

History of Attending Pap Screening

Among the most important variables predictive of cervical cancer is a lack of regular Pap smear screening, explaining a great part of the higher incidence of cervical cancers in under-served regions. There exists a strong negative correlation between the intensity of Pap smear screening in a population and the invasive cervical cancer risk of its women (Hakama et al.,1991).

Increasing level of education is thought to be protective with respect to screening behaviors. An inverse dose-response relationship has previously been shown between level of educational attainment and both precursor cervical lesions and cervical cancer (Parkin,1991).

Cervical HPV Infection

By far, the strongest risk factor for all cervical precursor abnormalities, including low-grade squamous intraepithelial lesions, high-grade intraepithelial lesions or CIN I-III, is infection with certain types of genital HPVs, with some finding a stronger association between certain HPV types and cervical cancer than between CIN I (considered a precursor lesion) and cervical cancer (Kiviat,1996). Sexual activity affects the risk of acquiring HPV infection, although other factors likely influence the susceptibility of the individual's getting that infection (Rohan et al.,1991).

In 1986 La Vecchia concluded that "the infectious agents implicated in dysplastic lesion probably differ to some extent from those causing invasive cancer" (La Vecchia et al.,1986). We now understand that it is largely oncogenic (high risk) HPV types that will, under certain circumstances lead to cervical cancer and that low and high risk types exhibit functional differences.

The classic risk factors for cervical cancer are now under re-evaluation in consideration of the role of HPV. Those previously established risk factors for cervical cancer are either correlates of HPV infection, co-factors interacting with HPV infection or truly independent risk factors for cervical cancer (Schiffman and Brinton,1995).

In a case-control study, established risk factors for CIN or SIL shown to be associated with cervical HPV infection are lifetime number of sexual partners, age at first intercourse, level of educational attainment, family income, smoking, use of oral contraceptives and parity (Schiffman et al.,1993).

HUMAN PAPILLOMAVIRUS

Human papillomaviruses are strictly epitheliotropic, exclusively intra-epithelial viruses (Stanley,1994) whose life cycle has evolved to be well-suited to the host cell that it targets, the keratinocyte. It has not been observed in vitro, as only differentiating cells of the epithelium provide the necessary environment for HPV infection, replication and assembly (Stanley,1994). In HPV-containing lesions, the viral DNA is found either in extrachromosomal, episomal form or integrated into the host genome.

HPV DNA is initially present in the host cell as an extrachromasomal episome, and eventually integrates into the host genome. In early cervical lesions the HPV DNA is most often found in the form of an episome; in cancer it is more often integrated (Cullen et al.,1991). This two phase intra-cellular involvement is corroborated by molecular epidemiological studies of viral load---the early infection in which the virus is proliferating produces a high number of viral particles per cell. Results from our own study show that once the virus has been integrated into the host genome, it is no longer at the proliferating stage and the number of viral copies per cell is far lower in high-grade lesions (Villa et al.,1996) . Increasingly, the mechanism of carcinogenesis is considered to include, and possibly even require, genomic instability (either inherent or acquired via oncogenic agent) which is permissive for the progression of events that eventually result in the "neoplastic phenotype" (Morgan et al.,1996).

HPVs are a family of the wider class of papillomaviruses, some of which are oncogenic-inducing lesions in humans and animals(Stanley,1994). Though all (except for BPV-1) infect epithelial cells, they are highly host-specific (IARC,1995) and even human types exhibit marked tissue specificity and are associated with particular disease manifestations. There is now reference in the literature to 'genital papillomaviruses', those found to be associated with lesions of the anogenital tract (Thierry,1996).

Early clinical studies focused on four HPV types: two with low oncogenic potential (HPV 6 and 11) and two with high oncogenic potential (Types 16 and 18). Campion and co-workers (Campion et al.,1986) conducted a follow-up study in which 26% of those with mild dysplastic lesions went on to develop CIS within 30 months—85% of these women were HPV-positive. In 1992 Lorincz and co-workers suggested that HPV types be divided into low-risk HPV 6 group; intermediate-risk HPV type 31 group; high-risk HPV 18 group and high-risk HPV 16 group, depending on the frequency with which they are found in carcinomas (Lorincz et al.,1992). HPV types 16 and 18 are most often associated with severe dysplasia and invasive cancers (Jochmus and Altmann,1996) .

Most recently, seventy-seven different HPV genotypes have been identified (van Ranst et al.,1996) on the basis of sequence homology and are being classified into three distinct groups based on the frequency with which they occur in malignant tumours (Franco.1996): low-risk (HPV 6,11,42,43,44); intermediate-risk HPV (31,33,35,51,52,58) and high-risk (HPV 16,18,45,56).

HPVs are non-enveloped, closed circular double-stranded DNA viruses with a genomic length of around 8000 bp (base pairs) (Eckert et al.,1995). Early open reading frames (E-ORFs) encode the proteins involved in regulation of viral DNA replication and cell growth stimulation; late ORFs (L-ORFs) specify the proteins of the viral capsid (Gissman and Muller,1994).

Genetic variability within HPV types is restricted mostly to point mutations, so type classifications are considered "tight". The genomic evolution of a DNA virus is relatively slow (compared to RNA viruses) and genomic variability may be as low as 1% over 100,000 years (Bernard et al.,1994) suggesting that HPV types have been nearly identical since the beginning of the human species. The degree of DNA homology between types varies considerably and classification of a new type is made when there is less than 90% sequence homology in the E6, E7 and L1 genes to any known HPV types(Arrand,1994; Gissman and Muller,1994). A sub-type is an isolate whose genome is closely related (up to 10% variability) to a previously defined type. A further

refinement of sub-type is the molecular variant which is defined if there is 2% or less variance within a type.

Epidemiology of HPV Types and Infection

HPV 6 is most often found in CIN 1 or LSIL. HPV 16 and 18 are thought to be the most prevalent HPV types worldwide; these are also the types that are most often found in cervical cancers. The measure of prevalence of HPV infection varies mainly according to the age and sexual habits of the women tested, but also according to the sampling method used (swabs, scrapes, brushings) and according to the DNA detection method used (Schiffman and Brinton,1995).

Sexual transmission of genital HPV infection is undoubtedly the method of transmission that is of interest with respect to cervical cancer. There is, however, evidence for another important mode of infection: vertical transmission from mother to baby during birth (Syrjanen,1996).

The Joao Pessoa survey from Brazil has shown that the association of HPV infection with sexual activity varies with oncogenicity; HPV infection with oncogenic type is strongly associated with markers of sexual activity, while non-oncogenic types are not (Franco, et al.,1995). A recent study of HPV infection in university students in Montreal (Richardson,1996) confirms that oncogenic and non-oncogenic HPV types differ in their ability to be transmitted sexually.

Between 15 and 40% of sexually active women have cervical HPV infections, most of which are transient; a small proportion of these infections are thought to persist and it is likely that it is only these infections that have the potential to lead to carcinogenesis (Franco,1996).

The single most important determinant of HPV infection is age (Franco,1996). Using various methods of detection and studying different populations, there consistently appears to be a decrease in prevalence of HPV infection as the age of the women increases, peaking in women age 16-25 (Schiffman and Brinton,1995) and being

found rarely in menopausal and post-menopausal women (Woodman,1994). It is most frequently detected among sexually-active women between the ages of 15-25 years (Kiviat,1996) with a decrease in prevalence in women after the age of 30 (Schiffman and Brinton,1995). This lower HPV prevalence is due either to immunologic clearance and/or suppression or to less exposure to new types, with a decrease in the number of new sexual partners. HPV infections are extremely common and usually transient in younger women, with spontaneous clearing within a few months to a few years (Schiffman and Brinton,1995). It is for this reason that, presently, adjuvant testing for HPV infection would likely lead to high levels of over treatment of lesions that would regress on their own. Kiviat has reported that the majority of HPV infections amongst young women presenting to STD clinics resolve within two years (Kiviat,1996), without serious consequences.

Biological Evidence Regarding Oncogenicity of HPV

More than ninety percent (with some finding up to 98% of cervical cancers positive for HPV (Meijer et al.,1997)) of cervical cancers biopsied contain HPV DNA, and protein products of the E6 and E7 gene can be found in all HPV DNA-positive cervical cancers (Zur Hausen,1994).

No in vitro system exists that allows the complete infectious cycle of HPV, i.e. the virus does not undergo in vitro replication (Stanley,1994), but in vitro studies have shown that transformation and immortalization of cells can be mediated by HPV DNA (Vousden,1994). In vitro expression of E6 and E7 is considered to be sufficient for immortalization of cultured keratinocytes and cervical epithelial cells (Eckert et al.,1995). Though this does not prove that the virus necessarily induces carcinogenic changes in vivo, it argues strongly as to the biological plausibility of a carcinogenic effect.

These in vitro studies led to the understanding that viral DNA has the ability to transform and immortalize cultured cells. The viral oncogenes that code E6 and E7 proteins express viral protein products that interact with and inactivate the products of the p53 and retinoblastoma (Rb) genes (respectively) of the host genome. P53 and Rb are tumour suppressor genes, involved in cell cycle regulation. A disruption of this

regulation can interfere with normal cellular differentiation, senescence or apoptosis (Vousden,1994). HPV DNA has been found to be integrated into the host genome in most cervical tumours (Eckert et al.,1995). Though much of viral genome becomes lost once integrated into the host genome, the E6 and E7 genes are maintained and expressed in cervical cancers. The production of E6 and E7 may even be increased due to the disruption (during integration) of E2, a viral mRNA regulator (Eckert et al.,1995).

METHODS FOR DETECTION OF CERVICAL LESIONS AND HPV INFECTION

Diagnosis of Cervical Cancer

The cellular changes characteristic of cervical precursor lesions, carcinoma *in situ* (CIS) and invasive cervical cancer (ICC), can be detected cytologically, histopathologically or by colposcopy. HPV-related changes can also be seen upon cytological analysis. HPV infection and intra-epithelial neoplasia are usually first suspected following a cervical Pap smear. The Pap smear is a screening test designed to detect squamous intraepithelial neoplasia, or pre-invasive lesions. The test also detects HPV infections and the presence of cervical carcinoma.

The nuclear abnormalities caused by neoplasia are present in the full thickness of the epithelium, so SIL (CIN) may be detected by scraping the surface of the lesion. The likelihood of detection is reduced by a poorly taken smear, by the withdrawal of the transformation zone into the endocervical canal (usual in women of 35 years of age or older) or as a result of a small lesion. Ideally a Pap smear contains both squamous cells and endocervical or squamous metaplastic cells, confirming that the transformation zone has been sampled (Kurman and Solomon,1994).

Though screening programs using the Pap smear have been highly effective in reducing morbidity and mortality over the past 30 years, it is of historical and epidemiological interest that the Pap test has never been subjected to a randomized clinical trial (Parkin,1997). Pap screening programs operate at a high cost to the supporting health care structure and have been criticized for failing to incorporate the knowledge of the role of HPV in cervical cancer pathogenesis (Kiviat,1996).

Colposcopy and Cervicography

If a woman is found to have some evidence of cellular abnormality on Pap screen, her cervix may be further evaluated by colposcopy. Colposcopy is the direct examination of the cervix (under magnification) which allows a directed biopsy of the abnormal area. If a lesion is suspected, visualization of the squamocolumnar junction is sometimes possible; if not, a cone biopsy may be required to assess the cervix. A cone biopsy is both a diagnostic and therapeutic procedure during which a conical piece of tissue, with its base on the ectocervix (around and including external os) and its apex extending into the endocervical canal, is removed.

Cervicography is a cervical assessment technique that uses a colposcope connected to a camera. This is a technique for remote evaluation of cervical status. A cerviscope is used to visualize and photograph the endo and ecto cervix. The film is shipped to a central lab for reading by expert colposcopists. This procedure was first introduced by Stafl (1981) and would offer an excellent solution to the problem of lack of high-quality cervical screening to under-served populations where the cost of cytological screening is prohibitive or for women in isolated regions.

Detection of HPV Infection

Most HPV infections are asymptomatic and are thought to be cleared by an intact immune system (Stern and Stanley,1994). A latent HPV infection is not detectable by clinical examination, colposcopy, cytology, nor histology, since the virus does not cause any morphological abnormality in the infected tissue. In sub-clinical HPV infection, the woman is usually asymptomatic on clinical examination but the virus produces flat lesions that can be seen by colposcopy or may cause cellular changes that can be diagnosed cytologically. Clinically apparent infections are visible to the naked eye and may cause symptoms in the patient, but are usually related to low-risk HPV types.

The manifestation of an HPV infection that is most likely to be apparent on clinical examination is condyloma acuminatum, a condition known as genital warts (exophytic papillary lesions) which are usually the result of HPV types 6 and 11. HPV

infection can also cause a non-condylomatous wart (flat condyloma) that can only be detected by cytological analysis. Hallmark morphological changes of the epithelial cells caused by HPV is koilocytotic atypia (Buckley,1994) in which the nucleus is enlarged, hyperchromatic, with irregular outline.

Viral culture cannot be used to diagnose HPV infection, because viral production depends on the differentiation of the epithelium (Walboomers et al.,1994). Another method for diagnosis of viral infection is identification of the genome of the pathogen or one of its protein products.

Nucleic Acid Hybridization

HPV genetic material can be identified by nucleic acid hybridization (NAH) (Gissman and Muller,1994). NAH uses the pairing ability of complementary single-stranded DNA (or RNA) to form double-stranded hybrids and is considered the best diagnostic tool available for HPV detection. Efficiency of hybridization depends on the extent of homology in nucleotide sequence between probe and target DNA and on the reaction conditions. These conditions can be varied from high-stringency (under which only totally homologous strands form hybrids) to low-stringency, under which less homologous strands also hybridize (Villa et al.,1995).

Colposcopy, histology and cytology can detect the clinical and sub-clinical manifestation of HPV infection, but not the virus itself. Koilocytosis is the main cytological and histological criterion for diagnosis of HPV infection and the "HPV" colposcopic pattern. Neither cytology, histology nor colposcopy can distinguish between HPV types. Methods now considered very sensitive and specific in HPV detection are those that identify nucleic acids directly, including Southern blot, dot blot, hybrid capture, Virapap, as well as methods that first amplify the nucleic acids and then detect the amplification products, particularly polymerase chain reaction (PCR) (Schiffman and Brinton,1995). PCR is the most sensitive of these methods and involves the enzymatic amplification of small amounts of target DNA, followed by hybridization to known probes.

PCR is conducted in three main steps: the denaturation of target DNA, the annealing of primers to the opposite strand of denatured target DNA and primer elongation. PCR products can be further analyzed by digestion with restriction enzymes in a technique known as restriction fragment length polymorphism (RFLP) analysis for typing or identification of novel HPV types. Advantages of PCR include its high sensitivity and specificity, the need for very small amounts of target DNA and the possibility of using a crude cell suspension. Material from cervical Pap smears or archival paraffin-embedded tissue can also be used. On the other hand, due to its high sensitivity, PCR is susceptible to contamination which can give false positive results. To avoid such contamination the different PCR steps (sample preparation, electrophoresis, and PCR solution preparation) need to be carried out in three separate rooms under strict adherence to the procedure that minimizes the risk of contamination.

Serology

The humoral immune response triggered by exposure to an infectious agent can provide a measure of lifelong exposure. In the case of HPV, however, the impossibility of cultivating the virus under experimental conditions means that antigenic material for serological assays is difficult to obtain (Gissman and Muller,1994). Also, since HPV production occurs only in the differentiating part of the epithelium, the humoral immune response is not very strong. Early viral proteins are produced in small quantities, (Gissman and Muller,1994) and there is some question as to whether the antibodies produced are relevant to mucosal infection (Stern and Stanley,1994). Another complicating factor in most immunological studies is that infection with high-risk HPV types is found with equal frequency in symptomatic patients and asymptomatic controls (Davies,1994). This could mean that sub-clinical HPV infections are extremely common and not necessarily transmitted strictly by the sexual route. HPVs can also infect the oral mucosa, thereby eliciting an immune response that is irrelevant with respect to the genital tract findings. Another explanation may be that the humoral immune system might not be capable of recognizing HPV and mounting an effective immune response. A further complexity is the fact that different HPV types might carry different levels of immunogenicity; high-risk types maybe less immunogenic than low- risk types (Davies,1994), making the immunological effect of high-risk HPVs difficult to study.

CERVICAL PRECURSOR LESIONS

Cytological Classification of Cervical Precursor Lesions

Precursor lesions represent cellular changes on a continuum from those caused by productive viral infections (particularly HPV) through increasingly severe grades of dysplasia right up to CIS and invasive cervical cancer.

The Pap screening test is designed to evaluate cervical status on the basis of cytological analysis of a sample of ectocervical and endocervical cells. The descriptive nature of this process requires that cellular characteristics observed be classified according to some agreed-upon system.

The strategy of using cytological markers as prognostic indicators of cervical cancer has survived three incarnations of classification from the old World Health Organization (WHO) system using Papanicolaou Class I-IV to the more recent WHO system of classifying cervical intraepithelial neoplasia as CIN I, II, or III, all the way to the most recent Bethesda system of classifying lesions as either low-grade squamous intraepithelial lesions (LSIL: representing the cytopathologic signs of HPV infection and the equivalent of CIN 1) or high-grade squamous intraepithelial lesions (HSIL: representing true pre-malignant lesions, CIN 2 and CIN 3 including CIS (Schiffman and Brinton,1995; Kurman and Solomon,1994). The differing terminologies reflect the evolution of biological understanding, but all, from the earliest to the most recent, are founded on some underlying spectrum of cellular changes, with the differences being largely quantitative rather than qualitative (Wright and Kurman,1994). The important contribution of the recently introduced Bethesda system, is the recognition that similar morphological changes at the cellular level can be the result of either productive viral infection or neoplastic process (Gaudette and Lee,1995). Advances in biology are thus reflected in diagnostic criteria and can be translated into increasingly appropriate patient management.

Under the old WHO system lesions were classified as either dysplasia (mild, moderate or severe) or carcinoma *in situ* (including microinvasive carcinoma). This classification system was based on the assumption that intra-epithelial neoplasia is a

two-stage disease and that dysplasia might regress, persist, or progress while CIS will inevitably develop into microinvasive carcinoma (Syrjanen et al.,1992), see Table 1.

Cervical intraepithelial neoplasia (CIN) is graded according to the extent of cytoplasmic maturation of the cells of the squamous epithelium of the transformation zone. CIN I, CIN II, CIN III lesions are characterized by cells with decreasing degree of differentiation and increasing degree of nuclear abnormalities (Wright et al.,1994). The three grade CIN system was adopted on the basis of the conclusion that dysplastic changes occur on a single continuum of lesion severity. Richart and Barron had conducted a follow-up study (Richart and Barron,1969), from which they concluded that almost all cases of dysplasia would progress, becoming invasive cervical cancer given enough time and the absence of treatment. Richart and Barron followed 557 women with varying degrees of cytologicai abnormality for 36 months and on the basis of statistical modeling found that within 10 years, 90% would progress to a more severe lesion (Nasiell et al.,1983). This inference was made on the basis of the results of a study that, contrary to most other studies of the natural history of cervical lesions, found that CIN 1 lesions were unlikely to regress and likely to progress. Kiviat has shown that these results could be attributed to the latter study's specific entry criteria of a minimum three abnormal smears; women entered into the Richart study had all had multiple previous smears of CIN 1. It is known that CIN 1 is more likely to regress within the first year and that women with CIN 1 were, by previous gynecologic history, predisposed to lesions that would progress (Kiviat,1996). CIN 1 is widely thought to be a precursor to CIN 3. This belief is supported by the observation that most of those who are diagnosed with invasive cervical cancer were previously diagnosed with CIN 1.

This three-tier grading has been replaced in some health care systems by the Bethesda two-grade system, in which lesion severity is classified into the more common, low-grade squamous intraepithelial lesions (LSIL) and the rarer, high-grade squamous intraepithelial lesions (HSIL). The new classification is said to answer a need with respect to clarification of terminology, which should result in greater clinical usefulness (National Cancer Institute Workshop,1989). Kurman and Solomon, (1994) have said that the main contribution of the Bethesda system is the incorporation of

Table 1: Equivalence of Cytological Classifications[1]

OLD WHO	Cytomorphological Changes	WHO	Proportion of atypical cells of basal layer	BETHESDA
Pap I	normal	**normal**		**WNL**
Pap II	inflammatory	**normal**		**BCC***
Pap II	inflammatory	**normal**		**ASCUS/AGUS**
Pap IIIa	mild dysplasia	**CIN I**	1/3 of total thickness	**LG-SIL****
Pap IIIb	moderate dysplasia	**CIN II**	1/3-2/3 total thickness	**HG-SIL**
Pap IIIc	severe dysplasia	**CIN III**	2/3-whole thickness	**HG-SIL**
Pap IV	Carcinoma in Situ (CIS)	**CIN III**	2/3-whole thickness	**HG-SIL**
Pap V	Invasive Cervical Cancer (ICC)	**ICC**		**ICC**

* Benign Cellular Changes

**koilocytosis, HPV changes or condylomatous atypia

[1] Kurman and Solomon, 1993

specific criteria for specimen adequacy. They further propose that cytological evaluation should not be conducted by "blinded cytopathologists" but rather in the context of all pertinent clinical and previous history information.

The recommendation of the participants of the 1988 workshop at Bethesda, was that HPV related changes be included in LSIL, though they recognized that "HPV could be reported as a separate diagnostic statement" (National Cancer Institute Workshop,1989). The Bethesda system applies the knowledge of HPV involvement and is made on the basis of risk of progression, with LSIL carrying low risk, and HSIL carrying a high risk of progression. Others see the distinction as LSIL being a risk factor for cervical cancer and HSIL representing a true pre-cancerous or cancerous lesion (Schiffman and Brinton,1995). The Bethesda classification is supported by epidemiological studies that have found HPV Type 16 (a type considered to have high oncogenic potential) frequently in CIN II and III but rarely in CIN I. Cytological abnormalities that would previously have been classified CIN I would be classified by the Bethesda system as LSIL; those lesions previously classified as CIN II and CIN III would be classified as HSIL, those most likely to progress to carcinoma.

The Bethesda System puts equivocal results into a category termed atypical squamous cells of undetermined significance (ASCUS). This category includes cellular abnormalities beyond those attributable to reactive changes, but which do not meet the criteria for the definitive diagnosis of SIL (Kurman and Solomon,1994). A summary of the equivalence of cytological classifications between systems is provided in Table 1.

The US has adopted the Bethesda System of classification but Brazil, like Canada, continues to use the three-tier CIN classifications. The rationale for resisting the move to the new system is that though the Bethesda system presumes to better reflect current biological understanding of the development of cervical lesions, it does not solve the underlying problem with histopathological reporting, that is, lack of objectivity in assessing intraepithelial changes. Some (Walboomers et al.,1994), propose that Bethesda is simply an unsuccessful attempt to solve the poor inter- and intra- pathologist reproducibility in cytological scoring. There remains concern that a

proportion of LSIL (i.e. particularly that HPV 16-positive smears) will progress to carcinoma and that a classification system that relies on incomplete knowledge of the causal agent is inherently flawed. Particularly for classification in epidemiological studies, the Bethesda system has been termed best (Schiffman and Brinton,1995); but others maintain that a two-class system leads to loss of "diagnostically and prognostically valuable information" (Syrjanen et al.,1992). The SIL grading system is used for cytological analysis, while CIN continues to be used as the histopathological classification.

At The Level of The Individual

In Canada, using the WHO classification, a woman who has a cytological result of CIN I would have repeat Pap test within 6 months and every 6 months for the next two years (Miller et al.,1991). If all these follow-up Pap tests are negative, she can return to the usual screening schedule of once per year. If any of these are positive, she would be referred for colposcopy. Any cytologies of CIN II or CIN III would be referred immediately for colposcopic evaluation.

None of the classification systems proposed to date adequately assess the individual patient. Considering the heterogeneity of HPV types, a combination of cytology-HPV type screening would seem to be most useful in the assessment of an individual woman. Though the benefit of using HPV testing in clinical practice remains to be established, studies of HPV prevalence rates and cytomorphological results of cervical cancer screening in the Netherlands, where a good cancer registration system exists, suggest that a combination of HPV testing and cytology screening would allow an increase in the interval between Pap smears (Walboomers et al., 1994). Different treatments and follow-up regimens for women in different groups, with different combinations of cytological results and HPV types, could eventually be established. This approach could lead to better identification and management of high-risk women.

In 1992 a group of researchers used HPV testing to predict development of CIN in women who were referred to colposcopy on the basis of abnormal cytology, but for whom colposcopic evaluation showed no abnormality (Nuovo et al.,1992). Seventy-

eight percent of those who were HPV-positive were diagnosed with CIN within 12 months. Repeat cytology, however, was able to correctly predict 92% of the eventual histological diagnoses. In the Portland cohort, Schiffman (Schiffman and Schatzkin,1994) found that HPV was the most predictive factor with respect to lesion development and Kiviat has found that HPV infection is more predictive of CIN 2/3 lesions than CIN 1 lesions (Kiviat,1996).

Prevalence of Cervical Precursor Lesions

In the US SIL (CIN 1-3) was detected in 2.3% of patients of Planned Parenthood Clinics nationwide between 1981 and 1983. Prevalence was found to decrease with increasing age (Sadeghi et al.,1988). A Canadian study (Meisels,1992) found evidence of changes that would be classified as LSIL in 6% of the women screened. The National Breast and Cervical Cancer Early Detection Program recently reported (CDC, 1997) that 4% of Pap test are abnormal (CIN I, II, or III).

In some populations, prevalence has been found to be increasing. In Southern Australia, the prevalence of low-grade lesions has been reported to have risen from 0.6% in 1978 to 5.6% 10 years later (Evans and Dowling,1990).

Progression, Persistence, Regression of Precursor Lesions

Both LSIL and HSIL can and do regress; LSIL regresses more frequently, but 15-25% will progress to HSIL within 2-4 years (Schiffman and Brinton,1995). Regression is not considered a hallmark of neoplasia (Syrjanen et al.,1992). The rate of regression, progression and persistence of cervical lesions, be they LSIL or HSIL, is of interest with respect to the natural history of cervical cancer and is central to optimal management of women's lesions, allowing the development of a set of prognostic indicators able to predict the likelihood of disease progression in individual women. It is known that a high proportion of both low-grade and some high-grade lesions will regress without treatment, some will persist, yet others will progress. Modification of patient management protocols would require that the characteristics of regressing and progressing lesions be firmly established.

Richart and Barron found that 20% of mild dysplasias progressed to severe dysplasia in 1 to 3 years (Richart and Baron, 1969). Nasiell and co-workers found that 16% of CIN 1 (n=555) progressed to CIN 3 within 4 years (Nasiell et al.,1983). Flannelly and co-workers found that 35% of mild dyskaryosis progressed to CIN 3 (n=538) within 1-2 years (Flannelly et al., 1994). Hellberg and co-workers found that even among those who have an initial cytological result of CIN II (n=1466) but who were subsequently found to be normal by colposcopy and biopsy (n=328), 22% went on to develop histologically confirmed CIN within the 10-year follow-up period, making this a group at greatly elevated risk (Hellberg et al.,1994).

Nasiell and co-workers found that 55% of moderately dysplastic lesions regressed to normal and remained normal for at least 53 months (16% persisted, 30% progressed to more severe lesions) (Nasiell et al.,1983). She cautioned that most dysplastic lesions that eventually progress become normal transiently, and that the length of follow-up must be sufficient in order to apply 'regressed to normal' classification with any certainty. Nasiell recommends that the follow-up period to establish regression following abnormal (moderate dysplasia) cytology needs to be at least 2-3 years to be definitive. She also proposes a definition for persisting lesion as one that is seen on a minimum of three cytologies.

In a study of the behavior of mild dysplasia, Nasiell followed 555 women for an average of 39 months and found mild dysplasias regressing to normal in 62% of the women, progression to severe dysplasia, CIS or ICC in 16% and persistence of mild dysplasias in 22% of the women (Nasiell et al.,1983).

LSIL is clearly a risk factor for HSIL; Soutter and Fletcher found that women with LSIL were 16 times as likely as women with normal cytology to develop HSIL and invasive cervical cancer (Soutter and Fletcher,1994). High-risk HPV type was a better predictor of CINII/III lesion than CIN I, widely considered to be the precursor of CINII/III.

Determinants of risk of progression from LSIL to HSIL under investigation are: HPV type (low oncogenic vs. high oncogenic), viral burden, immune status, parity, use of oral contraception, nutritional (folate, carotenoid, retinoid or vitamin C) deficiency, and concurrent infections with certain sexually transmitted diseases.

An alternative explanation to the CIN I-II-III continuum, submitted by Kiviat, is that CIN I and CINII/III represent two separate diseases differing in likelihood of detection (Kiviat,1996). She proposes that CIN I lesions may be a marker of exposure to HPV and not necessarily a true precursor. She found that most CIN II/III develops 6 months after HPV DNA 16/18 has been detected. Since CIN II/III is a morphological classification, these changes can be the result of HPV infection which will resolve when the infection is cleared; others will persist and develop into true precursors. Even those who support the continuum of disease model question whether all cervical cancers necessarily pass through the precursor stages. All agree that the progressive potential of HSIL is high (though not inevitable).

In 1995 Ho and co-workers (Ho et al.,1995) re-analyzed data collected prospectively during a beta-carotene intervention trial to study determinants of persistent SIL. They looked at persistent HPV infection, viral load, and behavioral and demographic characteristics in the study of the natural history of CIN. Subjects were followed at 6-week intervals in the first 3 months and at 3-month intervals thereafter for a total of 15 months. Persistent SIL was defined by cytological or colposcopic diagnosis of SIL in 2 consecutive visits. The presence, typing and quantification of HPV DNA in cervical vaginal lavage samples was conducted by both PCR and Southern Blot Hybridization. These researchers analyzed the effect of both time-dependent and time-independent explanatory variables (HPV positivity, types) on the risk of lesions persistence or progression.

The analysis of Ho et al. indicated that women with persistent HPV infection had four times the risk of persistent SIL of those without and that there was no progression in the absence of high-risk HPV types. They also found evidence of interaction between persistent infection and viral load. A high level of type-specific persistent infection

confers highest risk for persistent SIL. They found that persistent HPV infection was associated with persistent SIL, whether the infection occurred before the occurrence of the lesions or at the same time.

In 1996 (Syrjanen,1996) published results from the Kuopio follow-up study: 28% of HPV lesions regressed within 25 months, 56% regressed within 57 months and 64% regressed within 83 months. Beyond 7 years there was no further increase in regression. Syrjanen showed that the regression of HPV lesions increases with length of follow-up. Though progression of HPV lesions to CIS does appear to increase with increasing follow-up time, Syrjanen reported that progression of HPV lesions to CIS plateaus at a rate of 14% by 15 months.

In a review of the literature since 1950, Ostar (1993) has summarized the results of 17 prospective follow-up studies that reported rates of progression, regression and persistence of low grade lesions (mild dysplasia or CIN I). His review found that overall, 57% of low-grade lesions (CIN I) regressed; 32% persisted and 11% progressed to CIN III.

Determinants of Incident Precursor Lesions

In 1992 Koutsky and co-workers conducted a prospective cohort study of incident CIN (Koutsky et al.,1992). They followed 247 women who had no evidence of CIN nor HPV-atypia on baseline cytological evaluation and who had no previous history of CIN. These women were at high risk of developing cervical lesions by virtue of presentation for evaluation of STD. They were followed prospectively one month after the first visit and every 4 months after that for an average follow-up time of 25 months. Demographic, sexual and reproductive history, and smoking history information was collected by standardized interview. The cervix was evaluated by cytology and colposcopy. HPV status and typing was conducted by the dot-blot method (for 1300 early specimens) and by Virapap (for 1389 later specimens). During follow-up 47 women developed CIN II or III as determined either by cytology or colposcopy, or CIN 1 by two smears, meeting the criteria for biopsy. The outcome was determined by histology in 41 of these women. The six women who refused biopsy all had HPV

infections (Type 16 or 18 in 33%; Type 31,33, 35 in 33% and untyped in 33%). The 41 who underwent biopsy had histological results as follows: 28 (68%) with CIN 2 or 3, 11(27%) had CIN 1 or HPV-related atypia, and 2 (5%) were negative on histology.

Koutsky's group found a 2-year cumulative incidence of 28% for CIN II or III from time of first positive HPV DNA test (or from time of enrollment for those women who remained negative for HPV), compared with a 3% cumulative incidence for women without HPV infection (Koutsky et al., 1992). Cervical HPV infection was the most important determinant of CIN II/III. They found that younger age at first intercourse, but not number of sexual partners, was associated with an elevated relative risk of biopsy confirmed CIN II or III. They also found that even after adjusting for HPV, other STDs were seen to have an independent association with CIN II/III. Koutsky suggests that this may be due to decreased ability to detect HPV in those with concomitant infection. Being of race other than white carried a RR of 1.7 (0.8 - 3.6), high school education was protective with a RR of 0.5 (0.2 -1.1), current smoking was protective compared with never smoking (RR 0.5, 95% CI (0.2-1.5)), and women who had given birth to at least one child were not at significantly higher risk, RR of 2.0, 95% CI (0.9 - 4.2) when compared with women who had never given birth.

The Link Between HPV, Precursor Lesions And Cervical Cancer

The epidemiological risk factors for HPV infection seem to be much the same as those for cervical cancer. This very fact provides evidence for the role of HPV in the causal pathway leading to cervical cancer. Determinants of precursor lesions are now the object of study. It is from greater understanding of these precursor lesions that, until the availability of an effective vaccine, public health programs stand to benefit most. Those who are cytologically normal but who test positive for high-risk HPV are at increased risk of developing severe dysplasias (Koutsky et al.,1992).

Current understanding of the causal pathway in carcinogenesis is that other factors in addition to HPV infection are needed to induce malignant transformation of the genital squamous epithelium (Walboomers et al.,1994). Specifically, HPV type and intensity of infection, cell-mediated immunity and reproductive factors (Schiffman and

Brinton,1995) and more recently, persistence of HPV infection with a high-risk type, are being studied as possible co-factors. The genetic constitution of the host is likely also among these co-factors (Campo,1994), as are factors related to host (systemic or local) immune response (Kiviat,1996). Other factors that have been suggested are number of sexual partners, age at first intercourse, infection with herpes simplex type 2 (HSV-2) and/or other infectious agents and other co-carcinogens such as smoking (Campo,1994) From a world-wide perspective, invasive cervical cancer seems to be consistently related to the integration of the HPV genome, but the important co-factors may eventually be found to vary by region (Schiffman and Brinton,1995), which might explain the disparities in incidence of cervical cancers between populations. Differential screening coverage and quality remain the main factors in determining differences in incidence of cervical cancers.

Incidence of Cervical Precursor Lesions

There are few studies which have been able to report incident rates of precursor lesions. I conducted a review of selected studies which reported incidence rates of precursor lesions. This review is summarized in Table 2. Only those studies which reported rates of both dysplasia and CIS are included. Dysplasia and CIS can in combination be considered equivalent to LSIL and HSIL (see Table 1).

The range of incidence rates of precursor lesions reported from these studies is a low of 150/100,000 woman-years in Los Angeles (Stern and Neely,1964) to a high of 233/100,000 woman-years in the Appalachia region of Kentucky (Friedell et al.,1992). Figures from the British Columbia Cohort are reported as a range, with a low of 140/100,000 woman-years to a high of 550/100,000 woman-years (Miller et al.,1997). All studies, except the one from the UK (Parkin et al.,1982), used histological ascertainment of lesions. The incidence rate of precursor lesions by cytological ascertainment in the UK study is 156/100,000 woman-years. All rates except those for Appalachia and San Francisco are crude.

Table 2: Studies Reporting Incidence Rates of Dysplasia and CIS

Population	Grade of Lesion	C/H*	Per 100,000 Woman-years
B.C., Canada[1] (1981-1985)	Dysplasia	H	90 - 300
	CIS	H	50 - 250
United Kingdom[2] (1976-1977)	Dysplasia	C	239/198
	CIS	C	69/59
Los Angeles, USA[3]	Dysplasia	H	110
	CIS	H	40
Appalachia, Kentucky[4] 1986-1987	Dysplasia	H	194.6
	CIS	H	38.2
San Fransisco, USA[5]	Dysplasia	H	83
	CIS	H	91

* Cytological (C) Histological (H) Ascertainment

[1] (Miller et al., 1997)
[2] (Parkin et al., 1982
[3] (Stern and Nealy, 1964)
[4] (Friedell et al., 1992)
[5] (Peritz et al., 1977)

Part III

METHODS

The Ludwig-McGill Brazilian Cohort Study

The data for this analysis comes from a large, on-going cohort recruited to study the natural history of human papillomavirus infection in the development of cervical cancer (LMB Cohort Study). The cohort has been assembled from among a low-income population of women residents of Sao Paulo, Brazil, considered to be at high risk for invasive cervical cancer on an international basis (Villa and Franco,1989). Sao Paulo, capital of Sao Paulo State, is located in Southeastern Brazil. The hospital center, Maternidade Escola Vila Nova Cachoeirinha (MEVNC), is a municipally run institution providing active screening programs to the population that it serves.

To conduct this analysis I used information that had been gathered on epidemiological risk factors, assessment of cervical status by cytology, and detection of HPV infection (presence and type) by PCR analysis from women enrolled in the LMB Cohort Study.

Using data available from the LMB Cohort as of May 1997, I linked the databases containing registration records, questionnaire data gathered at the first visit (see Questionnaire 1 in the Appendix), cytology results, and laboratory results. Questionnaire responses and laboratory results (PCR results for HPV testing and typing) are limited to those gathered at first visit. I used cytology data from up to the sixth visit, representing up to three years of follow-up.

The Cohort Design

The cohort design permits the study of the dynamics of cervical HPV infection in a given individual by taking multiple measures of viral status over time. This is crucial to the study of an exposure that is likely to change with time. Most cohort studies which follow women prospectively for cytological end-points, measure HPV status at baseline (t_0) only and use this status to predict lesion development. Due to the moment in the cohort at which I undertook this analysis, this report is limited to using only the baseline measure of exposure as well. The LMB cohort is designed to characterize the dynamic

nature of HPV infection and it will eventually be able to provide information on HPV transience and persistence; it will then be possible to determine whether any of these predispose a woman to high-grade lesions or CIS. Persistence of HPV infection is defined for the cohort at the molecular variant level and this will allow future analyses to determine whether persistence is in fact the necessary precursor event. Detection of the exposure is conducted by advanced molecular detection techniques such as PCR, molecular variant analysis, and measurement of viral load. Certain HPV types are expected to produce high-grade lesions, while others tend to produce low-grade lesions. Persistence of HPV infection by certain types is hypothesized to be a major determinant of the development of cervical neoplasia (Villa et al.,1996).

Study Population

The study started enrolling women in November 1993 and by March 1997 had enrolled 2528 women who will be followed over five years. Though recruitment was to have continued to the end of 1997, it has been stopped early thanks to the high compliance rate of the women enrolled to date. The resources of the study will now be applied to follow-up.

A random systematic sample of women outpatient clients of the family medicine, gynecology and family planning clinics of the municipally operated MEVNC clinics in Sao Paulo, Brazil were offered participation in the study. Participation in the study was subject to eligibility according to the following criteria: (1) age between 18 and 60 years, (2) permanent resident of the City of Sao Paulo, (3) neither currently pregnant, nor planning to become pregnant in the next year, (4) intact uterus and not currently referred for hysterectomy, (5) not having used vaginal medication in the previous 2 days, (6) not having been treated for cervical disease by electrocoagulation, cryotherapy or conization in the previous 6 months, (7) indicating interest in complying with scheduled returns for at least the next 2 years and (8) providing a signed consent form. Each woman who was approached for the study had her eligibility determined by the study nurse using a questionnaire before enrollment into the study.

Participation, Compliance and Follow-up

Of those initially approached 46% met the eligibility criteria, agreed to participate in the described study and could therefore be enrolled. The majority of losses to follow-up occurred after the first visit, with a high percent of those who returned to the first follow-up visit (i.e. visit two) continuing to return for the further scheduled visits. The compliance rate at 3 years has been 69% i.e. only 31% of the women initially enrolled have failed to return for all 6 scheduled visits expected by 3 years of follow-up.

Because of the importance to the study of being able to follow the study women for 5 years, a meal ticket incentive often used in Brazil as part of employee benefits packages was used in the study to compensate for costs (transportation, time away from work or school) and inconvenience caused by the clinic visits. This assures a high level of compliance with the schedule of visits.

Data Collection

The study was designed to accumulate six distinct types of data: (1) information on epidemiological risk factors, (2) assessment of cervical status by cytology, cervicography, colposcopy and histology where indicated, (3) detection of HPV infection by HPV testing and typing, (4) molecular variant analysis, (5) viral burden and (6) immunology/serology. Baseline and repeat biologic specimens and questionnaire information were actively collected during 8 visits at 0, 4, 8, 12, 24, 36, 48, and 60 months. At each visit cervical specimens were taken for Pap cytology and HPV testing, a 10-ml blood sample was drawn for HPV serology, and an interview based on a structured questionnaire was conducted. In addition each woman underwent at least two cervicographies during the first two years of follow-up. The data were entered and stored in four databases. Central records holds the woman's clinic record, follow-up data, dates of scheduled next returns and telephone reminders, and record of phone reminders if a visit was missed. To protect the confidentiality of the study subjects, this database is never linked to the other three. The visit-specific questionnaire database holds coded responses given in separate interviews. The laboratory results database holds the results of HPV positivity, typing and molecular variant analysis as well as serology. The cervical pathology database holds the results of local and review

cytological analysis and cervicography. The laboratory results and visit specific databases are all periodically transferred, via a computer link, to the Montreal office. The cervical pathology database is exclusively kept at McGill where the data sets are periodically linked for analysis.

Five different questionnaires in Portuguese are being used: four to be used during the first four visits and one that is used for each of four subsequent annual visits. The questionnaires were designed to return demographic, lifestyle and behavioral information that allow exploration of risk factors for HPV infection and cervical cancer i.e. sociodemographics, reproductive health, sexual practices, smoking and diet.

To obtain cervical specimens the Accelon sampler device (Medscand, Sweden) was used to collect a sample of ectocervical and endocervical cells at each visit. The Pap smear was prepared onto a glass slide and fixed in 95% ethanol. The sampler was then immersed in a tube containing Tris-EDTA buffer (pH 7.4), the cells released by shaking, and the sampler removed. The tubes with cell suspensions were kept at 4°C at the clinic for a maximum of 5 days. This is the cervical sample that was used for PCR analysis at the Ludwig Institute. The Pap smears were fixed, stained and first read at MEVNC cytopathology lab. The slides were then shipped to Montreal for coding by the Department of Oncology, Division of Epidemiology, McGill University, and forwarded for cytological review to the laboratory of Dr. Alex Ferenczy at the Jewish General Hospital.

In order to prevent possible between-subject contamination during the taking of the cervical specimens, the nurses who conducted the procedure were trained to use sterile methods and materials. Women who were found to have a moderate dysplasia or worse by MEVNC cytology, HSIL or worse by Dr. Ferenczy's laboratory, or any high-grade cervigram were recalled for colposcopy and treatment as per local and international standards of care.

All cervical specimens underwent PCR analysis at Dr. Luisa Villa's laboratory of the Ludwig Institute, to detect and type HPV DNA. The MY09/MY11 protocol used in this study is the one originally proposed by Manos et al.,(1989) with additional

modifications (Hildesheim et al.,1994). Typing was done by dot blotting of PCR products and hybridization with radioactively labeled oligonucleotide probes for 27 types and an additional HPV generic probe. Those samples found to be positive underwent further testing to measure viral burden (viral copies per cell) in the exfoliated cells (Caballero et al.,1995). A rigorous protocol was implemented to prevent inter-specimen contamination by the lab procedures used to analyze these specimens. Sample processing and reaction areas were physically isolated, solutions were autoclaved, reagents were pre-mixed and pre-aliquoted, laboratory personnel used disposable gloves, positive displacement pipettes, aerosol-free tips and "splash-free" tubes. Both negative controls (DNA from cultured human tissue) and highly diluted positive controls were used to alert to contamination and for quality control purposes.

The DNA from the cervical cells was extracted by proteinase K digestion and organic ethanol precipitation. Testing for HPV DNA was conducted using a PCR-based technique which targets a highly conserved 450 bp segment in the L1 viral gene (flanked by the MY09/11 primers).

PCR products were hybridized with a generic probe. Those samples which turned out to be positive with the generic probe were considered HPV positive, and were further analyzed for detection of HPV type. Typing of HPV positive samples was conducted using the Dot blot technique with type-specific HPV probes. If an HPV positive specimen did not hybridize with any of the type-specific probes, the PCR amplification products were subjected to restriction fragment length polymorphism (RFLP) analysis of the L1 fragment in order to characterize unknown types. This method allows detection of 40 genital HPV types. Further PCR analysis for molecular variant identification was conducted whenever two or more specimens from the same women were of the same HPV type.

Information gathered from the women was entered and held in four databases. Registration dates and dates of scheduled return visits is in the registration database. Cervicography, and cytology results from both local (Brazilian) cytology and the review cytology are held in the cervix database. Information gathered by questionaire at each

visit is held in a questionaire database. The laboratory results database holds results of each woman's assessment of HPV status, testing and typing by PCR.

Ethics

Each woman who entered the study was obliged to follow the consent process, culminating in the nurse administering an informed consent form. The study and the obligation of participants was described as fully as possible. The women were given time to make a decision before committing to participation. The study procedures and informed consent have been approved by the institutional review boards of the MEVNC, the Ludwig Institute for Cancer Research in Brazil, and the Faculties of Medicine at McGill University and University of Toronto.

Though clearly these women were being subjected to a level of testing far beyond that which they would require simply for their own care, in the absence of this study it is likely that many of these women would not have received the preventive attention they required. In addition the study serves as an educational vehicle in this population with respect to the importance of Pap screening: whether a woman participated in the study or not, the level of awareness of the importance of Pap screen is likely to provide indirect and residual benefit to the community.

Ascertainment of Outcome

In March 1997 it was determined that local cytology results should not be used for research purposes as, on "actuarial" analysis, HPV positivity at enrollment was not as predictive of local cytology as it was of review cytology. Since HPV is no longer questioned as the etiologic agent, I decided to use results from the cytology review as the outcome measure. Meijer and co-workers have even suggested that HPV testing can serve as a tool for quality control of Pap smear reading (Meijer et al.,1997). Review cytology is the cytological reading that was conducted expressly for research purposes and performed at the Jewish General Hospital laboratory of Dr. Alex Ferenczy. The cytological review classified lesions according to the Bethesda system, considered to be well-suited to epidemiological work.

After Brazilian cytologists had conducted their cytological readings, the slides were packaged and shipped to Montreal. The cytological review was conducted with the review cytologist blinded to the results of the local cytological analysis.

Women who were either negative or classified as ASCUS on baseline Pap smear according to review cytology, constituted the group who were 'at risk' of developing LSIL or HSIL and were selected for this analysis.

Cohort Context

The context of a cohort study that collects both questionnaire data and biological specimens is complex in that information from different sources accumulates at varying rates. The main hypothesis that the LMB cohort is designed to study is that it is *persistent* HPV infections with certain HPV types that have the potential to progress to cervical cancer. There therefore exists a hierarchy of importance with respect to processing of laboratory results. In order to provide the study with depth of information related to the issue of persistence, HPV testing and typing results for a woman for whom a PCR result from an earlier visit was already available is more informative than a first result from a given woman. A decision was therefore made that the PCR analysis of HPV specimens should not proceed on a strictly sequential basis, but that these analyses should be conducted preferentially for those women who already had at least baseline PCR results.

This decision affects this report to the extent that it restricts the number of women for whom information can be used in regression analysis. For bivariate analysis it was useful to include information from as many women as possible and I therefore conducted Kaplan-Meier (KM) survival analysis on those women who were negative on baseline cytology (irrespective of whether or not HPV results were available on their specimens). Multivariable regression always proceeds on the most restricted set of study subjects. In this case, the numbers were restricted by the availability of HPV results. So that measures of precision be comparable, I performed crude Cox regressions (containing only one explanatory variable) on the group of women who were negative on baseline cytology and for whom HPV positivity results were available. The

characteristics of study women presented in Tables 4 and 5 of the results section describe the three groups of women and attest to their similarity, save for the availability of HPV results and incomes.

Incidence Density

Incidence rate is a measure of the rate of occurrence of disease using the number of new events in a population at risk that occur within a certain period of time (Last,1995). Incidence density is a measure of the average occurrence of disease over time: the number of new events that occur over a cumulative amount of person-time from which these events have arisen (in this case woman-months). To establish an incidence-density rate for cervical precursor lesions I divided the total number of cases of incident lesions (SIL) that were found during visits 2-6 by the total number of woman-months of follow-up among women at risk (those who were found at baseline to have no cytological evidence of SIL).

Effect of Repeated Cytological Testing on Incidence-Density Rate

Though the protocol requires standardization of schedule of visits and recall efforts, there remains some concern that the intensive cytological follow-up of these women might lead to an inflated estimate of incidence density. Increased screening opportunities in the case of an outcome that is known to regress spontaneously increases the chances of finding that outcome. An analysis stratified by visit was therefore conducted to ensure that the incidence density rate of precursor lesions was not an artifact of the frequency of testing. A cumulative rate for each return visit was calculated by dividing the number of SILs found at that visit by the woman-months of follow-up from which these lesions arose.

Prevalence

The prevalence rate is the number of individuals with the condition under study, at a specific point in time, divided by the population at risk (Last,1995). In this case the event of interest was a LSIL or HSIL, the population at risk was all women enrolled to the cohort; the point in time is t_0, the moment that each woman attends the first visit. Women attending the first visit bring with them some level of prevalent lesions, which

was calculated by dividing the number of women with SIL by the number of women from whom the first Pap smears were read. I also calculated a point prevalence for subsequent visits.

Natural History of Cervical Precursor Lesions

As discussed previously, it is known that an important proportion of cervical lesions regress spontaneously with time. Using review cytology, I selected women who had completed at least two visits and analyzed the behavior of their lesions by comparing cytology results for pairs of consecutive visits. A woman with LSIL in a visit who also had such a lesion in the visit immediately following was termed a 'LSIL-persistor'. If in the visit immediately following the cytological result was normal, the woman was considered a 'LSIL-regressor'. If on that following visit the grade of lesions went to HSIL, the woman was considered a 'LSIL-progressor'.

Statistical Analysis

In order to study the temporal relationship between HPV infection and development of cervical precursor lesions, I focused the main analysis on incident cases of SIL, using information gathered from those women who had been found by baseline cytology to have neither HSIL or LSIL and who were therefore at risk of developing "incident" SIL. In this report the Bethesda classification of LSIL and HSIL is referred to in combination as SIL. Statistical significance is determined throughout at the 0.05 level.

To establish the predictive ability of a set of potential determinants, I first analyzed each independent variable in univariate form by classifying results into different categories. I then chose the best "functional form" for each in separate analyses using both significance testing based on log-rank results and univariate Cox regression. In the interest of parsimony and whenever appropriate biologically, when categories of a variable returned comparable hazard ratios (HR) along with 95% CI that overlapped adjacent point estimates, I combined the categories.

Kaplan-Meier Survival Analysis

Kaplan-Meier (KM) survival analysis provides a powerful and sensitive method for analysis of cohort data where the moment of occurrence of the event of interest is central to the understanding of the disease process. KM analysis uses information on all study subjects irrespective of whether the woman develops the outcome of interest or not (i.e. censored cases: those who fail to develop the outcome of interest during the study time or who are lost to follow-up). The assumptions that must be met in order to use KM analysis are a definite t_0 (as in this case Visit 1) and a well-defined study outcome (in this case the first cytologic result of SIL during Visits 2-6). Further requirements are that losses to follow-up occur independently of the occurrence of the outcome (i.e. random censoring) and that the risk of the outcome is independent of the moment in calendar time at which it occurs. KM analysis uses the first moment of occurrence of the event as the specific point at which to assess the event rate. Each step of a KM curve represents the moment of first occurrence of an outcome event (Kramer,1988).

In this study, time was measured in woman-months from the moment the woman entered the study (t_0). Each woman was followed and contributed information until the development of the outcome or until the latest visit attended. I created the dependent variable that measures time to SIL development for each woman by joining information from the cytological and registration databases. This represents the time to first occurrence of lesion in woman-months. Once a woman was found to have an SIL by review cytology, her contribution to the follow-up time ended; though she will continue to be followed for the cohort study she contributed no further information to this analysis. Censoring occurred at the time of the most recent visit attended for those who continue in the cohort without developing an SIL, or for those who were lost to follow-up.

The results of KM actuarial analysis for each candidate variable can be summarized either graphically (KM curves) or by appropriate statistical tests (i.e. log-rank). I compared cumulative rates of SIL development at 6 months and at 12 months in each group to the same rates in the contrast groups. This cumulative rate is interpretable as the probability for an individual study subject of developing SIL at a

given point in time. I first conducted univariate analysis using KM actuarial analysis on each of the risk factors under study. KM analysis summarizes the differences in hazards between groups with respect to the characteristics under study. The slope of the curve represents the instantaneous potential for the occurrence of the outcome. In this case rather than using survival curves, curves representing cumulative hazard were produced, using a rare disease assumption. I confirmed that this is an appropriate assumption for all variables by comparison with a 1-s(t) plot. The log-rank test is the method that is used for comparison of hazard curves when all events during the study period are to be equally weighted. I calculated this statistic for each variable to establish its overall ability to predict the outcome, time to development of lesions. The log-rank test compares the entire survival curve on the basis of observed number of events of interest in each group compared to the expected based on the total number of events. (Lee,1980)

Univariate actuarial analysis looks at the effect of only one variable on the outcome. Such results may be confounded by one or several covariates or the effects may be subject to modification by some characteristic. The multivariable regression model chosen to control for confounding and to assess interactions in this study was the Cox proportional hazards regression model. First introduced in 1972 (Cox,1972), the Cox model is best suited to the analysis of a situation in which the status of the dependent variable changes with time. It carries a fundamental assumption of proportionality of hazards that must be met by each variable to be used in the model. I produced log-minus-log plots to confirm that the proportionality assumption was met by each of the variables used in the model (SPSS,1996).

Cox regression uses the hazard function to estimate the relative risk of the event of interest. It represents the instantaneous potential of the occurrence of this event, given that the person remains at risk until that moment. A large estimate for hazard ratio (HR) means that there is a high risk of occurrence of the event relative to the reference category of a given variable (Kleinbaum,1995). In this analysis the crude HR for each variable was then adjusted for the three *a priori* explanatory variables age, exposure to HPV infection and previous history of Pap smear.

Cox Regression Model

The incorporation of a variable in the Cox regression model assures that each covariate included in the model is adjusted for all others. To be included in the model are variables in their most informative functional forms as well as any known confounders or covariates of biologic significance.

The approach to model building taken for this analysis was a type of backward elimination: starting with the most inclusive model, I removed, one at a time, those variables that were least predictive of the dependent variable. I considered covariates eligible for entry to the first model on the basis of a significant log-rank test from univariate analysis, not at the .05 level of significance, but rather at the 0.25 level according to the recommendation of Hosmer and Lemeshow (Hosmer and Lemeshow,1989). Both the approach to model building taken and this more liberal cut-off permitted assessment of each covariate in the context of all other covariates under study. The ability of the model to explain variations in the occurrence of SIL was evaluated by the likelihood ratio test, which compares the model to one with no covariates or to one with only a subset of covariates used in the larger model.

Part IV

RESULTS

Descriptive Results

At the moment that I conducted this analysis (May-June 1997) over 2500 women had been enrolled in the study . Cytology results (from review cytology) were available for the 2508 who had attended the first visit and for the 2035, 1651, 1325, 729, 158 women who attended Visits 2 - 6, respectively. At first visit 33 LSIL and 21 HSIL were found. I identified a total of 93 incident cervical precursor lesions (SIL), 75 (80.6%) incident LSIL and 18 (19.4%) HSIL during Visits 2 - 6.

The distribution of cytological results from Visits 1 - 6 are shown in Table 3. Incident lesions are those found during Visits 2 - 6 amongst those women who had been found by cytological review to be free of LSIL or HG lesions at baseline and are presented in bold in Table 3.

Sociodemographic, sexual behavior and reproductive history at baseline were documented using the first questionnaire; such information had been entered in the database for up to 1454 women. HPV testing (presence and typing) had been completed for 848 women selected for the incidence study. Figure 1 is a flow chart describing the status of the cohort at the moment of this analysis, summarizing the availability of results from the different sources of data in the study. Tables 4 and 5 summarize selected characteristics of the women enrolled in the cohort and compare them to the women who were lesion free at baseline and to those who were both lesion free and for whom PCR results were available.

I conducted KM actuarial analyses on the group of women who were free of lesions at baseline visit, in order to benefit from the information gathered from as many women as possible. Cox regression analysis was restricted to that group of women negative on baseline cytology and for whom HPV results were available, in order to obtain comparable measures of precision for each of the covariates in the models.

Table 3: Distribution of Cytology Results According to Scheduled Return Visit in the LMB Cohort*

Cytological Classification	Visit 1 t_0	(%)	Visit 2 4 months	(%)	Visit 3 8 months	(%)	Visit 4 12 months	(%)	Visit 5 24 months	(%)	Visit 6 36 months	(%)
WNL**	2403	95.8	1973	97	1598	97	1286	97	713	98	151	96
ASCUS	43	1.7	24	1	19	1.2	18	1.4	6	0.8	3	1.9
LG-SIL	33	1.3	24	1	28	1.7	13	1	6	0.8	4	2.5
HG-SIL	21	0.8	7	0.3	2	0.1	5	0.4	4	0.6	0	0
Inconclusive	6	0.2	7	0.3	4	0.2	3	0.2	0	0	0	0
Total	2506		2035		1651		1325		729		158	

* Excludes slides not yet read

** Within normal limits

Table 4: Sociodemographic Characteristics of LMB Cohort Study Women

Variables	Frequencies (Percent) Women Enrolled	Women With Negative Cytology*	Incidence Study Women**
Age (Years)			
18 - 24	289 (19.9)	282 (19.9)	157 (18.6)
25 - 34	584 (40)	562 (39.7)	334 (39.5)
35 - 44	408 (28)	400 (28.3)	257 (30.4)
45 - 59	173 (12)	170 (12.0)	98 (11.6)
Income (US$ per month)			
0-199	376 (26.4)	366 (26.4)	299 (36.2)
200-349	393 (27.6)	383 (27.6)	258 (31.3)
350-549	288 (20.2)	280 (20.2)	160 (19.4)
550+	368 (25.8)	357 (25.8)	108 (13.1)
Ethnicity			
White	958 (66)	930 (65.9)	549 (64.9)
Non-White	494 (34)	482 (34.1)	296 (35.0)
Education			
Some elementary	359 (25)	354 (25.1)	224 (26.5)
Completed elementary	835 (57)	803 (56.8)	462 (54.6)
High School	216 (15)	213 (15.1)	127 (15.0)
College/University	43 (3)	43 (3.0)	32 (3.8)
Smoking History			
Never	680 (47.0)	671 (47.5)	405 (47.9)
Ever	774 (53.0)	743 (52.5)	441 (52.1)

* WOMEN WITH NEGATIVE CYTOLOGY AT BASELINE VISIT

** WOMEN WITH NEGATIVE CYTOLOGY AND FOR WHOM HPV RESULTS FROM BASELINE VISIT ARE AVAILABLE

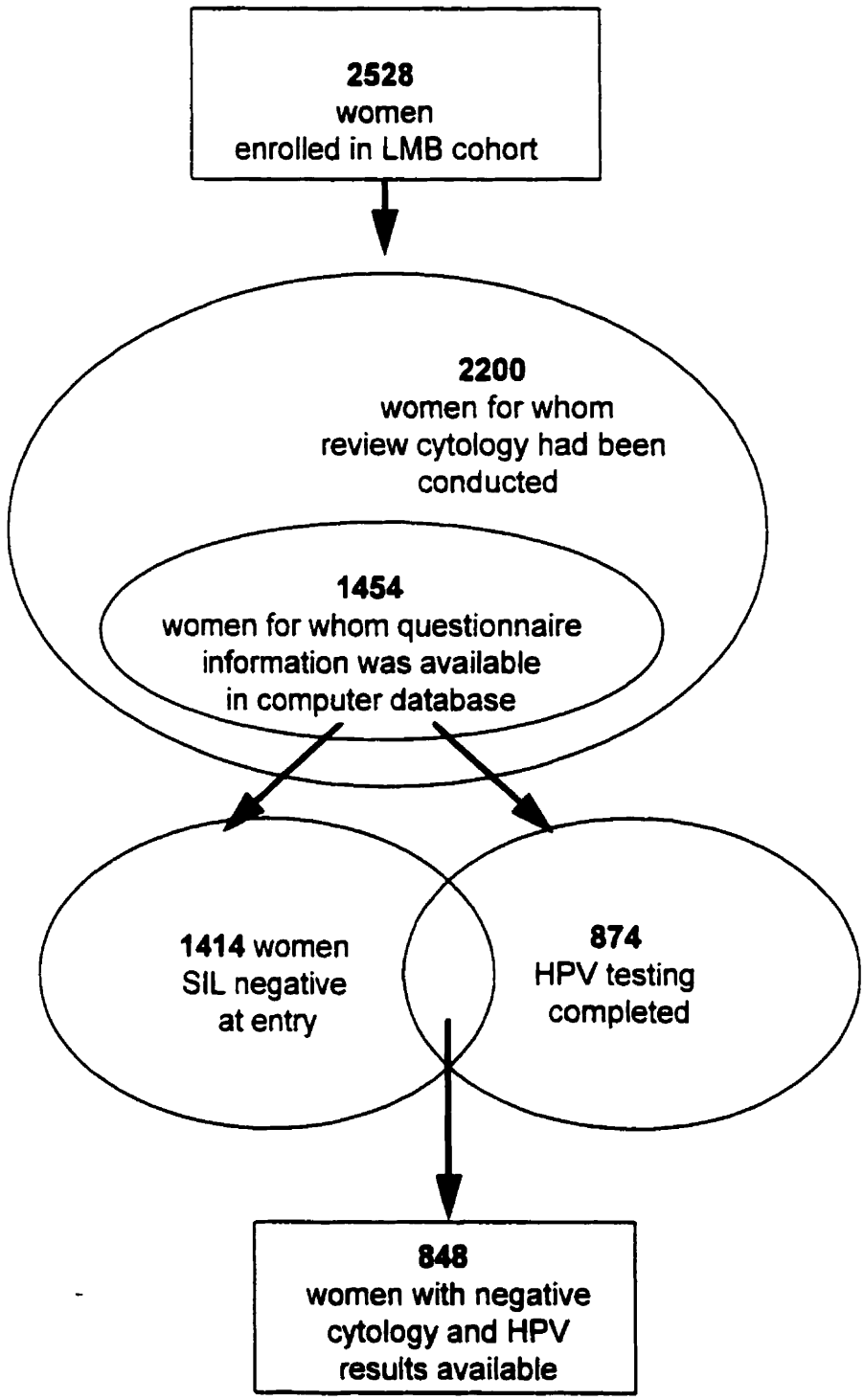
Table 5: HPV Positivity, Reproductive Factors, and Sexual Activity Characteristics of LMB Cohort Study Women

	Frequency (Percent)		
Variable	**All Women Enrolled**	**Women with Negative Cytology***	**Incidence Study Women****
Number of Previous Pap Smears			
Never	67 (2.7)	65 (4.6)	32 (3.8)
Ever	1385 (95.4)	1347 (95.4)	814 (96.2)
Age at Menarche (years)			
< 11	317 (22)	305 (21.6)	174 (20.6)
>= 11	1135 (78)	1107 (78.4)	671 (79.3)
Number of Pregnancies			
0 -1	237 (16.7)	231 (16.5)	122 (14.4)
2,3	623 (43)	614 (44.0)	382 (45.2)
4 to 6	428 (29.4)	409 (29.3)	241 (28.5)
7 +	149 (10.2)	143 (10.2)	85 (10.0)
Use of Oral Contraceptives			
Never	239 (16.4)	232 (16.4)	140 (16.5)
Ever	1215 (83)	1182 (84)	706 (83.5)
Age at First Intercourse			
<=20	1141 (79)	1105 (78)	654 (78)
20+	309 (21)	305 (22)	188 (22)
Lifetime Number of Sexual Partners			
0 - 1	643 (44.3)	631 (44.7)	378 (44.7)
2,3	491 (33.8)	474 (33.5)	290 (34.3)
4 +	319 (22.0)	308 (21.8)	178 (21.0)
Number of Sexual Partners (Past 5 years)			
0,1	1134 (78.0)	1111 (78.6)	665 (78.6)
2+	320 (22.0)	303 (21.4)	181 (21.4)
Number of Sexual Partners (Past year)			
0, 1	1371(94)	1335 (95.9)	785 (92.8)
2+	61 (4)	57 (4.6)	44 (5.2)
HPV Result			
Negative	746 (73.1)	739 (83.4)	739 (87.1)
Non-oncogenic HPV	53 (6.4)	52 (5.9)	52 (6.1)
Oncogenic HPV	75 (6.0)	57 (6.4)	57 (6.7)

*WOMEN WITH NEGATIVE CYTOLOGY AT BASELINE VISIT

**WOMEN WITH NEGATIVE CYTOLOGY AND FOR WHOM HPV RESULTS FROM BASELINE VISIT ARE AVAILABLE

Figure 1: Status of LMB cohort, May 1997



Socio-Demographic Characteristics

The mean (SD) age of the study women at enrollment was 33 (9.1) years; the median was 32 years. As presented in Table 4 the women are predominantly (60%) below age 34: 289 (20%) are 18-24 years of age and 584 (40%) are 25-34 years of age. Four hundred and eight (28%) are between 35-44 years of age, while only 173 (12%) are between 45-59 years of age.

During the course of the follow-up Brazil underwent a change in currency. For this analysis, the two currencies were each converted into US dollars and the income used for analysis is reported in US dollars per month. The income reported was household income, with the question specifically asking for the total income of all those living in the woman's house. The average income of the study women was US $465.61 per month; half the women reported monthly incomes below US $332.96 with incidence study women (those with normal cytology at baseline and for whom PCR results were available) appearing to be poorer.

Eighty-two (82%) percent of the study women (1212) had at most completed elementary school; 359 (25%) have some, but have not completed, elementary school; 835 (57%) have completed elementary school; 216 (15%) have completed or have had some high school education and 43 (3%) have gone on to college or university.

Ethnicity was established by the nurse interviewer who had been trained to classify the women according to the criteria used by social workers in Brazil. This is considered the most reliable way of reporting the race of the woman as well as the least disruptive in terms of rapport with the interviewer. The study population is 66% white and 34% non-white. In Brazil those defined as non-white are a highly heterogeneous group including Blacks, Indians or their descendants, and those of oriental descent.

Six hundred and seventy one (48%) of the study women never smoked, 481 (34%) were current smokers (at the time of the first visit) and 255 (18%) reported themselves as having been former smokers.

Reproductive And Sexual Activity Characteristics

As presented in Table 5, almost eighty percent of the women remembered being over age 11 at menarche, as defined by first menses. In the study population 305 (22%) of the women remembered having their first menses at less than 11 years of age; 1107 (78%) between 12-19 years of age.

In this study the mean reported age of first sexual intercourse was 18 (median 17) years; 380 (27%) of the women reported having had first intercourse before or at the age of 15; 353 (25%) at age 16 or 17; 296 (21%) at age 18 or 19; and 395 (28%) over age 20.

Of the women who responded to sexual activity questions, 1335/1392 (96%) reported either none or a single sexual partner in the past year; 1111/1414 (78%) reported either none or a single sexual partner in the past 5 years; and 631/1413 (45%) of the women reported either none, or a single sexual partner in their lifetimes. The mean (SD) lifetime number of male sexual partners of these women was 3.6 (21.4), with a median of 2.0.

In this study 231 (16.5%) of women report having never having been pregnant or having been pregnant once; 614 (44%) reported 2-3 pregnancies; 409 (29%) 4-6 pregnancies and 143 (10%) had been pregnant more than 7 times.

Oral contraceptive use by the study women was distributed as follows: 232 (16.4%) had never used any oral contraceptives (OC), 1182 (84%) had used some form of oral contraception during their lifetimes.

The women in the study reported on average (S.D.) 6.75 (5.8) lifetime number of Pap smears (median 5.0) prior to enrollment to the study. Sixty-five of the women (4.6%) report never having had a Pap smear taken; and 1347 (95.4%) reported having had at least one Pap smear in their lifetimes.

HPV results were available for 921 women. Seventy-three percent (73%) were negative at entry to the study; 6.4% were found to be infected with a low-risk type (defined as HPV Types 6, 11, 32, 34, 26, 40, 42, 53, 54, 55, 57, 59, 66, 67, 68, 69, 70, 72, 73 or some novel types awaiting official classification); 6% were infected with a high-risk type (defined as HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, or 58);and 3.4% had samples insufficient for analysis as evidenced by inability to identify human DNA (beta-globin negative).

As is to be expected among women selected for their absence of lesions at baseline, a larger proportion (83.4%) were HPV-negative at baseline visit; 5.9% were infected with low-risk HPV types; 6.4% were infected with high-risk HPV types (1.9% were beta-globin negative).

Prevalence of Cervical Precursor Lesions At Entry

The highest number (and proportion) of women with SIL (LSIL and HSIL) were identified at Visit 1. This provided a measure of prevalence of these lesions in this population. The (point) prevalence rate is the number of lesions found among the women attending the first visit. Fifty-four lesions were found amongst 2508 women who attended the first visit. This represents a prevalence rate of 2.2% cervical precursor lesions at baseline.

The prevalence of SIL in subsequent visits was as follows: 1.5% at Visit 2; 1.8% at Visit 3; 1.4% at Visit 4; 1.4% at Visit 5. Though some results were available for Visit 6, small numbers precluded the reporting of this prevalence.

Natural History of LSIL: Progression, Persistence or Regression

The finding that a high proportion of cervical precursor lesions regress spontaneously previously reported was corroborated by the experience of the women in this study. Sixty-seven (67) LSILs among women who attended at least two consecutive visits were found. Pairwise analysis of consecutive visits revealed that eighty-four percent (56/67) of these LSILs regressed by the subsequent visit; 7/67

(10%) persisted at least until the next visit and 4/67 (6%) progressed to HSIL by the next visit.

Incidence Density

As summarized in Table 3, the cytological review found 60 incident cases of LSIL or HSIL which arose from 31,052 woman-months of follow-up. This represents an incidence-density rate of 2.3/1,000 woman-years .

The sensitivity analysis confirmed that repeated cytological testing was not the cause of this apparently high incidence-density. The cumulative rates of SIL detection (reported per 1000 woman-months) appeared relatively stable for Visits 1 - 3 (i.e. 5.1, 6.7, and 6.5, respectively). At the fourth visit the rate dropped to 3.9, possibly due to characteristics of the women who were long-term participants in the study.

Determinants of Incident SIL

Availability of HPV results limited the number of events of interest, first occurrences of SIL, to twenty-nine (29). Figures 2- 8 show hazard curves according to HPV status, age, history of Pap screening, parity, and sexual activity variables (lifetime number of partners, number of partners in the past year and past five years). This information is also summarized by reporting the p-value for the log-rank test and the cumulative hazard rate by 6 months and 12 months of follow-up in Tables 6, 7 and 8. The cumulative rate represents the risk of lesion for a woman enrolled in the study according to the length of time for which she is followed and according to characteristics under study. Crude and adjusted Cox regressions are presented in Tables 9, 10, and 11.

The cumulative rate of SIL development in those who were HPV-negative at baseline was 0.5% by 6 months and 2% by 12 months; for those infected with low-risk HPV types, this rate was 4% by 6 months and 6% by 12 months; and for those infected with high-risk HPV types 6% by 6 months and 10% by 12 months.

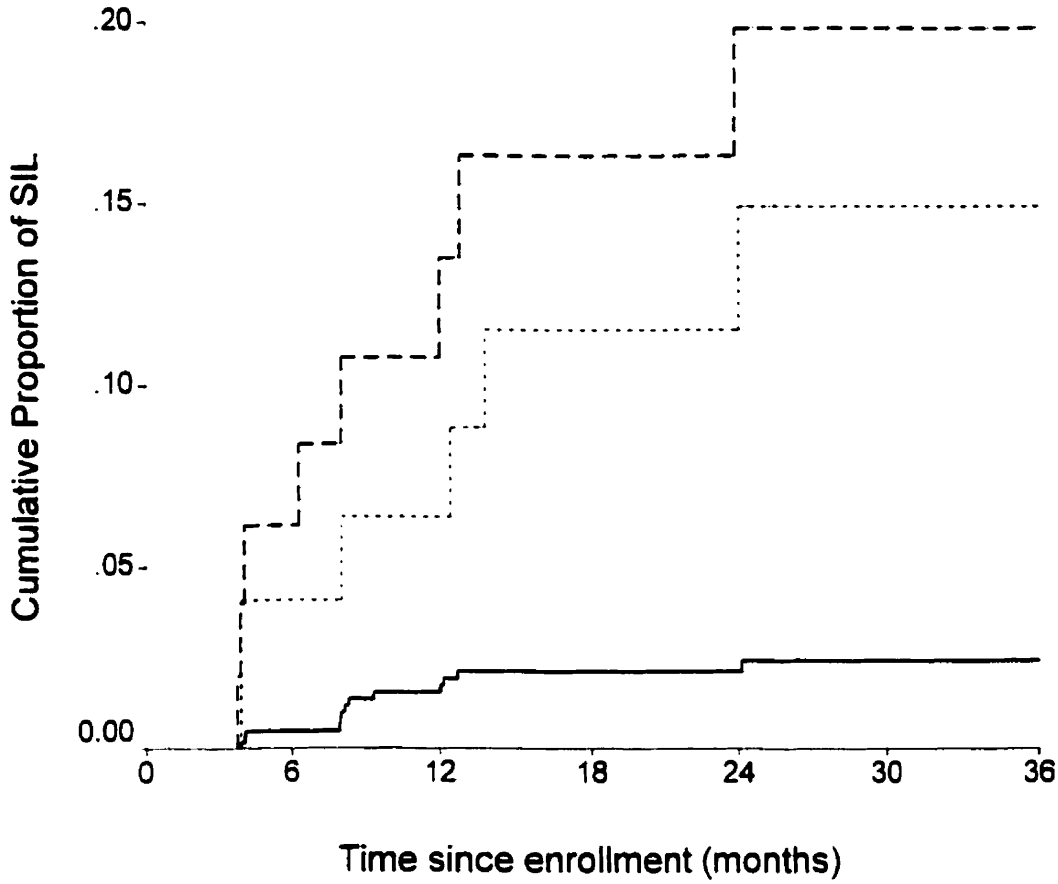


Figure 2: Cumulative Incidence of SIL among women with normal Pap smear at entry according to HPV Status. *Solid line*: HPV negative; *light broken line*: infected with low-risk HPV types; *heavy broken line*: infected with high-risk HPV types.

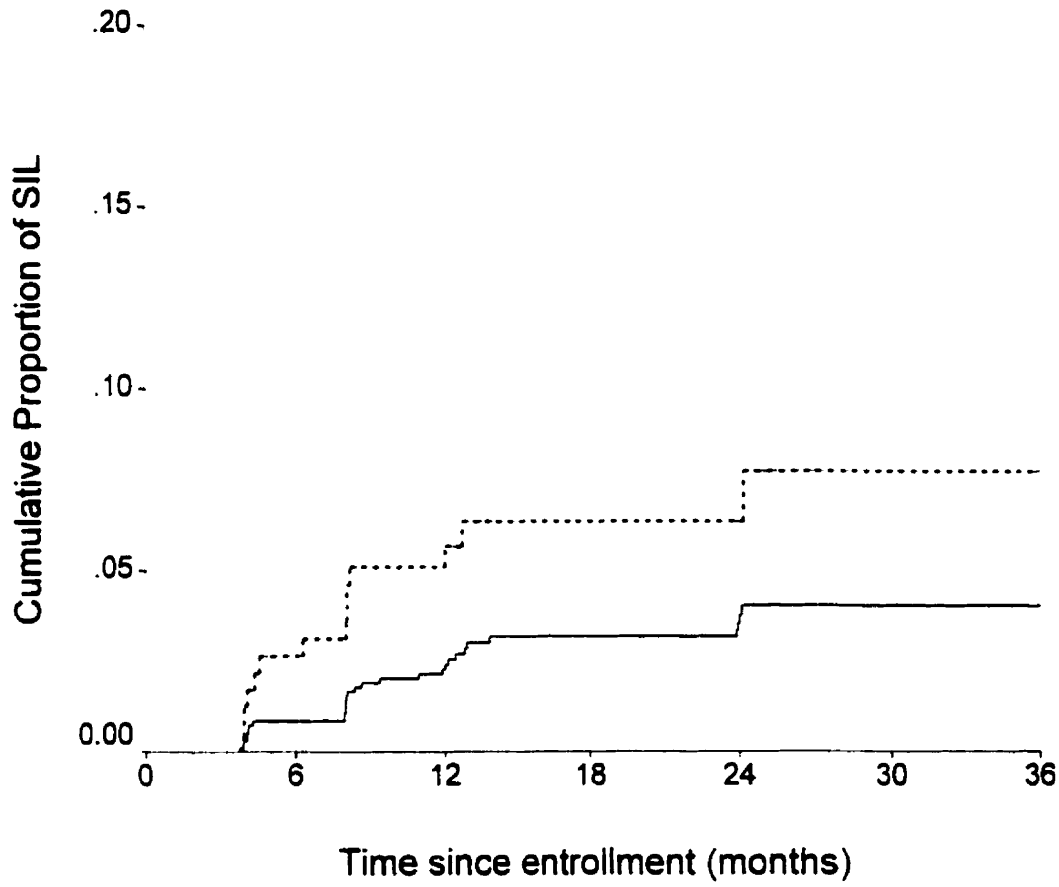


Figure 3: Cumulative incidence of SIL among women with normal Pap smear at entry according to Age at enrollment. *Solid line*:18-24 years; *broken line* 25+ years.

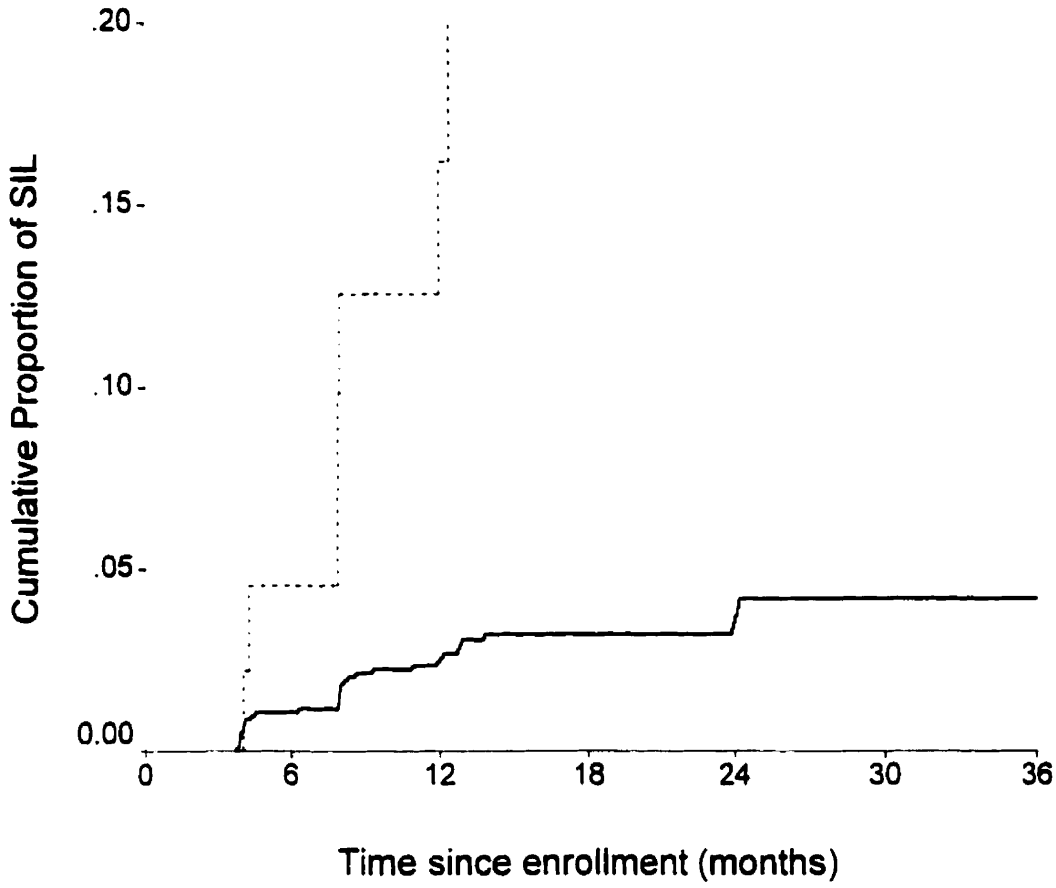


Figure 4: Cumulative incidence of SIL among women with normal Pap smear at entry according to previous Pap. *Solid line*: ever; *broken line*: never.

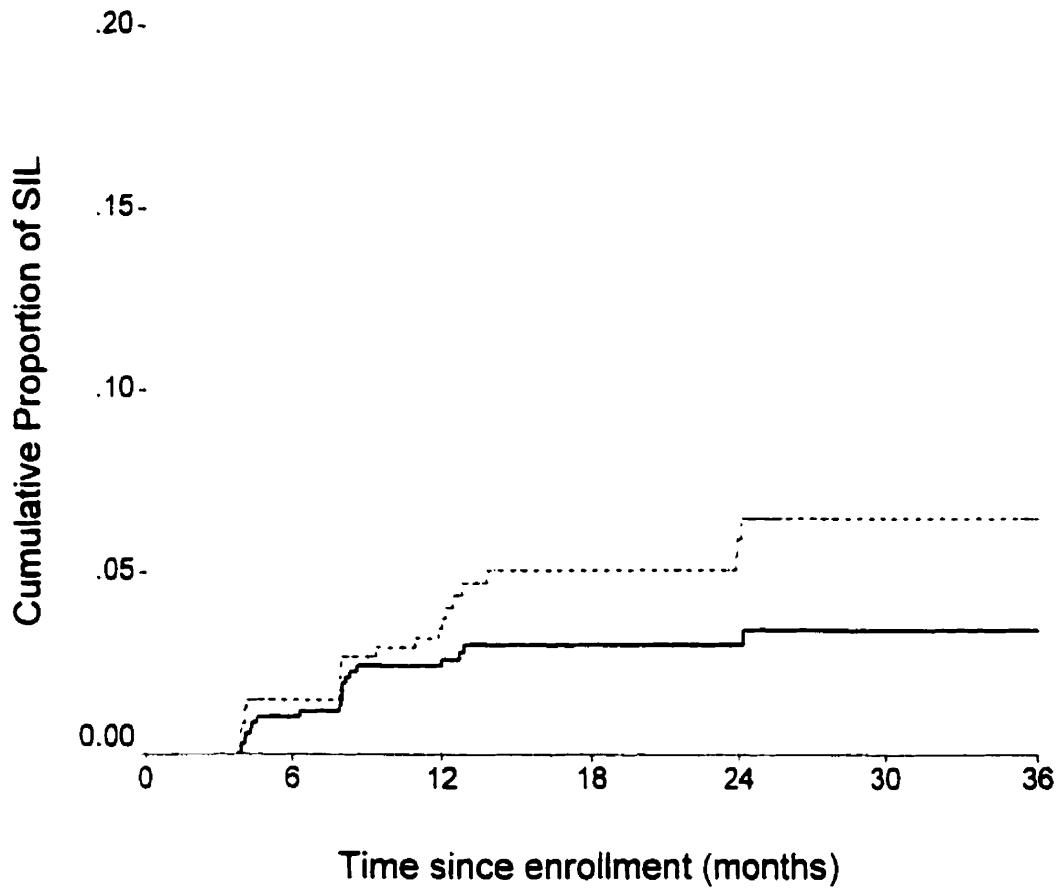


Figure 5: Cumulative incidence of SIL among women with normal Pap smear at entry according to parity. *Solid line*: less than 4 pregnancies; *broken line*: 4 or more pregnancies.

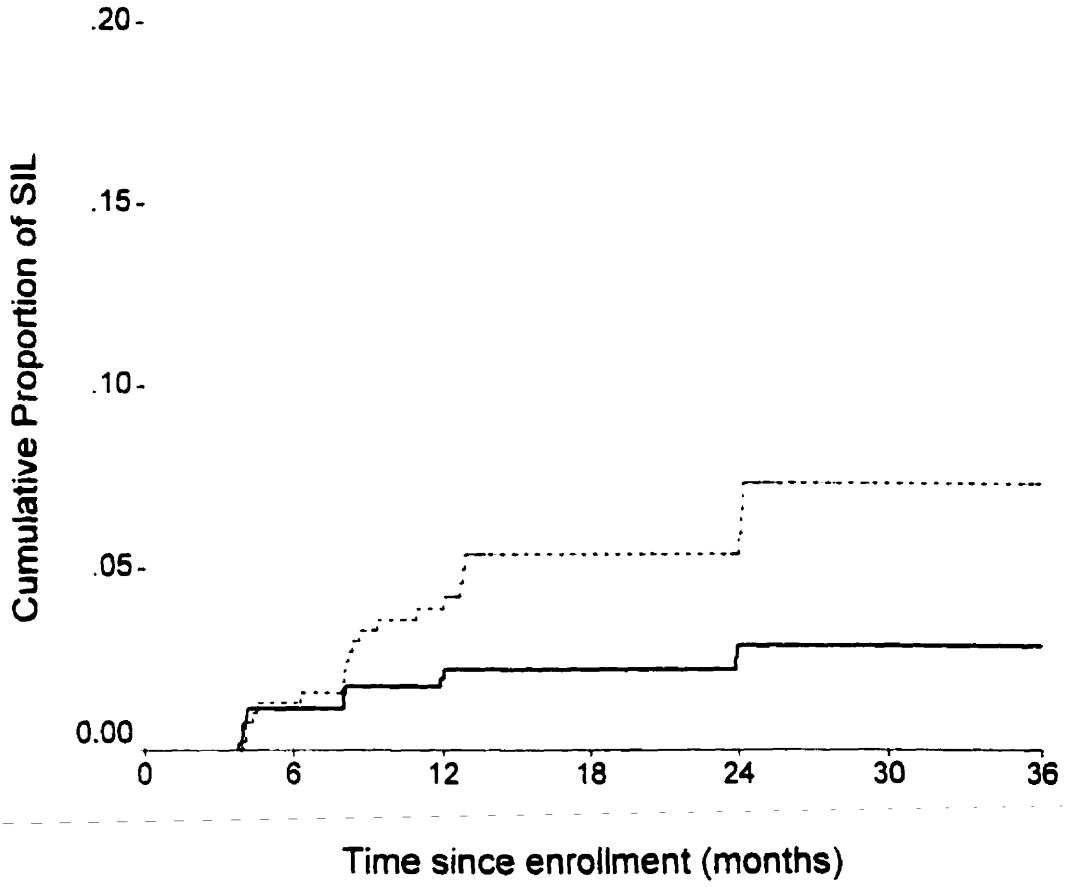


Figure 6: Cumulative incidence of SIL among women with normal Pap smear at entry according to lifetime number of sexual partners. *Solid line*: one or fewer lifetime sexual partners; *broken line*: 2 or more lifetime sexual partners.

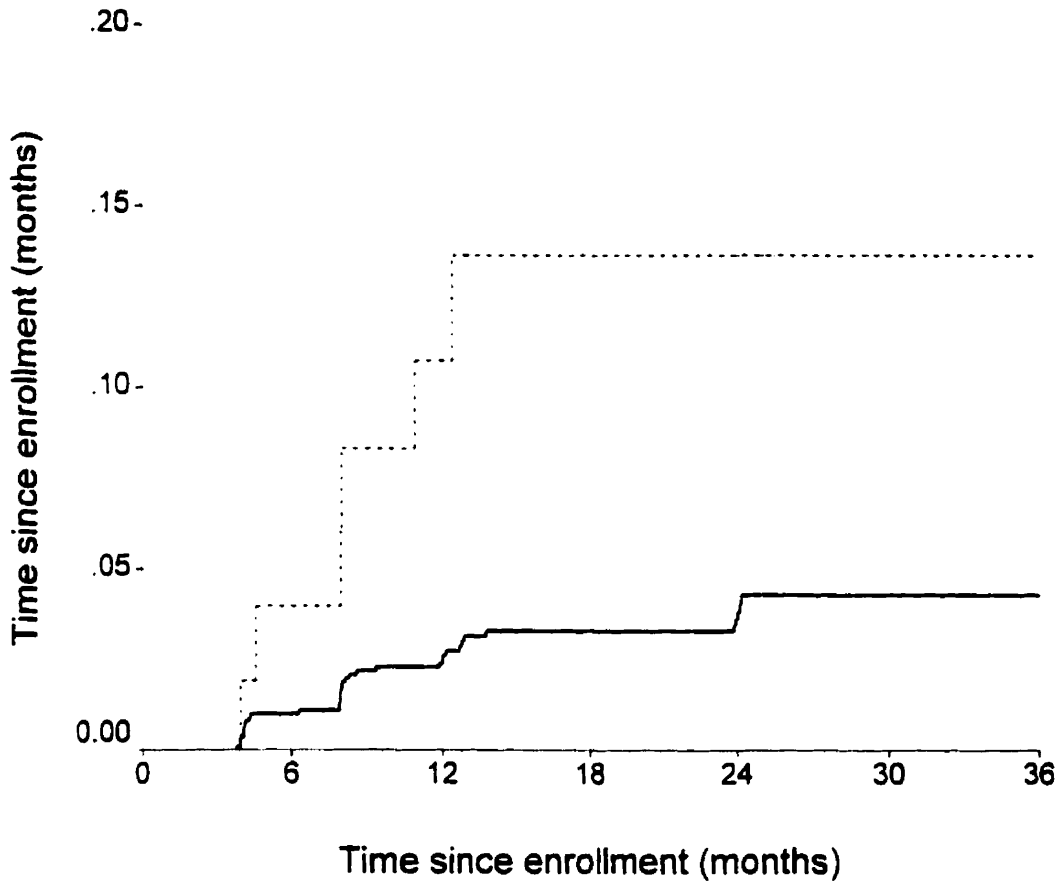


Figure 7: Cumulative incidence of SIL among women with normal Pap smear at entry according to number of sexual partners in the past 5 years. *Solid line*: one or fewer sexual partners in past 5 years; *broken line*: 2 or more sexual partners in past 5 years.

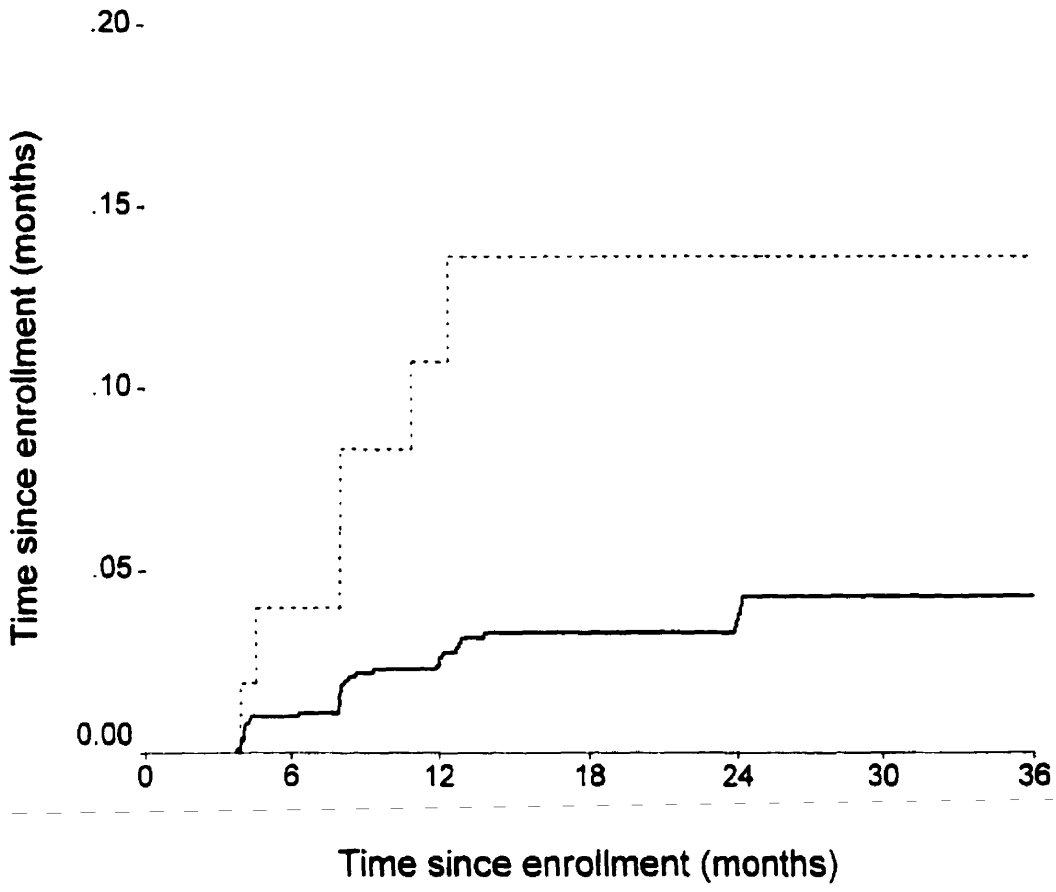


Figure 8: Cumulative incidence of SIL among women with normal Pap smear at entry according to number of sexual partners in the past year. *Solid line*: one or fewer sexual partners in past year; *broken line*: 2 or more sexual partners in past year.

Table 6: Cumulative Rates of SIL Development in the LMB Cohort According to Selected Sociodemographic Characteristics

Variable	Frequency n	Frequency First Instance of SIL	Cumulative Rate 6 Months (SE)	Cumulative Rate 12 months (SE)	p-value*
Age (Years)					
Continuous					0.6780
18-24	243	16	0.03 (0.1)	0.05 (0.015)	0.1000
25-34	486	13	0.004 (0.003)	0.018 (0.006)	
35-44	352	14	0.03 (0.009)	0.006 (0.004)	
45-70	140	4	0.03 (0.015)	0.03 (0.015)	
18 - 24	243	16	0.03 (.01)	0.05 (.015)	0.0200
25+	978	31	0.009 (.003)	0.022 (.005)	
Smoking					
Never	590	23	0.01 (0.005)	0.03 (0.007)	0.8800
Ever	631	24	0.011 (0.044)	0.03 (0.007)	
Never	590	23	0.01 (0.005)	0.03 (0.007)	0.7200
Former	419	18	0.01 (0.007)	0.03 (0.011)	
Current	212	6	0.013 (0.006)	0.03 (0.008)	
Ethnicity					
White	797	27	0.01 (.004)	0.023 (.006)	0.2100
Non-White	424	20	0.015 (.006)	0.004 (.01)	
Level of Education					
Some Elementary	301	14	0.027 (0.01)	0.05 (0.013)	0.5400
Elementary	709	23	0.005 (0.003)	0.016 (0.005)	
High School	178	8	0.02 (0.01	0.04 (0.01)	
Coll/Univ	32	2	0.07 (0.05)	0.07 (0.05)	
Elementary	1010	37	0.01 (0.003)	0.025 (0.005)	0.4300
High School+	210	10	0.015 (0.008)	0.004 (0.015)	
Income (US$/month)					
0-550	883	30	0.01 (0.004)	0.03 (0.005)	0.0900
550+	312	16	0.01 (0.007)	0.04 (0.012)	
0-200	316	0	0.013 (0.007)	0.03 (0.01)	0.3200
200-350	325	11	0.006 (0.005)	0.02 (0.008)	
350-550	242	6	0.013 (0.007)	0.023 (0.01)	
550+	312	16	0.01 (0.007)	0.04 (0.012)	

Table 7: Cumulative Rates of SIL Development in the LMB Cohort According to HPV Positivity, and Reproductive History

Variable	Frequency n	Frequency First Instance of SIL	Cumulative Rate 6 Months (SE)	Cumulative Rate 12 months (SE)	p-value*
HPV Result					
negative	646	15	0.005 (0.003)	0.020 (0.005)	0.0001
non-oncogenic	50	6	0.040 (0.028)	0.062 (0.034)	
oncogenic	51	8	0.060 (0.033)	0.100 (0.043)	
negative	646	15	0.005 (.003)	0.02 (.005)	0.0000
positive	101	14	0.050 (0.02)	0.080 (0.03)	
History of Pap Screening					
Continuous					0.0000
Never	40	5	0.030 (0.050)	0.090 (0.05)	0.0012
1,2	262	14	0.024 (0.010)	0.04 (0.013)	
3,4	258	10	0.020 (0.008)	0.025 (0.01)	
5 TO 9	253	6	0.004 (0.004)	0.17 (0.009)	
10+	366	7		0.015 (0.007)	
Never	51	7	0.05 (0.030)	0.12 (0.06)	0.0000
Ever	1168	40	0.01 (0.003)	0.024 (0.005)	
History of OC Use					
Never	191	10	0.030 (0.013)	0.05 (0.02)	0.3200
< 6	691	24	0.008 (0.003)	0.02 (0.006)	
6+	339	13	0.012 (0.006)	0.03 (0.01)	
Never	191	10	0.03 (0.013)	0.05 (0.017)	0.1400
Ever	1030	37	0.009 (0.003)	0.024 (0.005)	
Number of Pregnancies					
0-1	188	10	0.017 (0.01)	0.034 (0.014)	0.0200
2,3	532	13	0.008 (0.004)	0.02 (0.006)	
4,6	361	21	0.014 (0.06)	0.04 (0.01)	
7+	126	2	0.02 (0.011)	0.02 (0.011)	
less than 4	720	23	0.01 (0.003)	0.03 (0.005)	0.1000
4 or more	487	23	0.02 (0.006)	0.034 (0.009)	
Age at Menarche					
0 to 11	263	9	0.008 (0.006)	0.003 (0.012)	0.7700
12 to 19	956	38	0.013 (0.004)	0.03 (0.005)	

Table 8: Cumulative Rate of SIL Development in the LMB Cohort According to Sexual Activity Variables

	Frequency		Cumulative Rate		
Variable	n	First Instance of SIL	6 Months (SE)	12 months (SE)	p-value*
Age at First Intercourse					
Continuous					0.2200
<20	967	44	0.014 (.004)	0.103 (.006)	0.0100
20+	250	3	0.004 (.004)	0.009 (.006)	
Lifetime Number of Partners					
0 or 1	540	14	0.01 (0.005)	0.02 (0.006)	0.8700
2 to 3			0.01 (0.006)	0.04 (0.010)	
4+			0.01 (0.007)	0.03 (0.010)	
0,1	540	14	0.01 (.005)	0.02 (.006)	0.0180
2+	408	23	0.013 (.006)	0.04 (.010)	
Number of Partners (Past 5 Years)					
0 - 1	958	26	0.009 (.003)	0.021 (.005)	0.0002
2+	263	21	0.024 (0.01)	0.054 (.015)	
Number of Sexual Partners (Past Year)					
0 to 1	1152	40	0.01 (.003)	0.024 (.005)	0.0036
2+	53	6	0.04 (0.03)	0.100 (0.04)	

TABLE 9: Cox Proportional Hazard Regression Analysis for Selected Sociodemographic Characteristics

Variable	HAZARD RATIO (95%CI)			
	Crude	Age Adjusted	Adjusted for HPV	Adjusted for HPV & Age
Age (Years)				
Continuous	0.97 (0.93, 1.01)		0.99 (0.94, 1.03)	
18-24	1			
25-34	0.29 (0.11, 0.74)			
35-44	0.43 (0.17, 1.07)			
45-70	0.31 (0.07, 1.40)			
18 - 24	1		1	
25+	0.34 (0.16, 0.73)		0.46 (0.21, 0.99)	
Income (US$ per month)				
0-200	1	1	1	1
200-350	1.01 (0.39, 2.63)	0.98 (0.38, 2.57)	1.01 (0.38, 2.7)	0.98 (0.36, 2.63)
350-550	0.65 (0.17, 2.39)	0.66 (0.18, 2.44)	0.82 (0.22, 3.04)	0.75 (0.20, 2.81)
550+	2.3 (0.89, 5.98)	2.29 (0.88, 5.94)	2.32 (0.88, 6.1)	2.2 (0.84, 5.82)
0-550/mon	1	1	1	1
550+/mon	2.48 (1.09, 5.64)	2.48 (1.09, 5.63)	2.38(1.05, 5.42)	2.35 (1.03, 5.34)
History of Smoking				
Never	1	1	1	1
Former	0.72 (0.31, 1.68)	0.77 (0.33, 1.8)	0.57 (0.24, 1.35)	0.63 (0.27, 1.49)
Current	0.70 (0.23, 2.09)	0.75 (0.25, 2.23)	0.63 (0.21, 1.88)	0.68 (0.23, 2.04)
Never	1	1	1	1
Ever	0.71 (0.34, 1.5)	0.76 (0.36, 1.6)	0.59 (0.28, 1.26)	0.65 (0.3, 1.4)
Ethnicity				
White	1	1	1	1
Non-white	1.03 (0.08, 2.24)	1.06 (0.49, 2.3)	1.12 (.51, 2.44)	1.14 (.52, 2.49)
Level of Educational Attainment				
< Element	1	1	1	1
Element	0.49 (0.21, 1.17)	0.43 (0.18, 1.01)	0.37 (0.16, 0.90)	0.32 (0.13, 0.78)
High School	1.01 (0.37, 2.79)	0.85 (0.30, 2.36)	0.77 (0.28, 2.14)	0.70 (0.25, 1.96)
Coll/Univ	0.78 (0.1, 6.06)	0.72 (0.09, 5.67)	0.80 (0.10, 6.33)	0.68 (0.08, 5.41)
Elementary	1	1	1	1
High School+	1.49 (0.63, 3.5)	1.42 (0.60, 3.35)	1.44 (.61, 3.4)	1.45 (.62, 3.43)

Table 10: Cox Proportional Hazards Regression Analysis for HPV and Reproductive History Variables

Variable	HAZARD RATIO (95%CI) Crude	Age Adjusted	Adjusted for HPV	Adjusted for HPV & Age
HPV Results				
Negative	1	1		
Non-oncogenic	5.72 (2.19, 14.92)	5.19 (1.97, 13.64)		
Oncogenic	8.03 (3.37, 19.17)	6.93 (2.86, 16.79)		
Negative	1	1		
Positive	6.85 (3.26, 14.39)	6.06 (2.85, 12.88)		
Previous Pap Smears				
Continuous	0.89 (0.81, 0.98)	0.92 (0.83, 1.02)	0.91 (0.83, 1.0)	0.93 (0.84, 1.02)
Never	1	1	1	1
1,2	0.27 (0.08, 0.93)	0.53 (0.22, 1.24)	0.30 (0.09, 1.05)	0.31 (0.09, 1.09)
3,4	0.28 (0.08, 0.93)	0.31 (0.09, 1.05)	0.34 (0.10, 1.2)	0.39 (0.11, 1.37)
5 TO 9	0.19 (0.05, 0.70)	0.34 (0.10, 1.17)	0.23 (0.06, 0.90)	0.30 (0.07, 1.23)
10+	0.10 (0.03, 0.41)	0.15 (0.03, 0.70)	0.14 (0.03, 0.56)	0.18 (0.04,0 .82)
Ever	1	1	1	1
Never	5.4 (1.87, 15.63)	3.61 (1.18, 11.11)	4.33 (1.45, 12.98)	3.36 (1.08, 10.46)
Number of Pregnancies				
0-1	1	1	1	1
2,3	0.50 (0.17, 1.5)	0.83 (0.26, 2.63)	0.51 (0.17, 1.56)	0.78 (0.25, 2.45)
4,6	1.36 (0.48, 3.8)	2.99 (0.94, 9.44)	1.48 (0.53, 4.15)	3.5 (1.04, 12.19)
7+	0.29 (0.03, 2.49)	0.68 (0.07, 6.31)	0.25 (0.03, 2.2)	0.52 (0.06, 4.9)
0-1	1	1	1	1
2+	0.76 (0.29, 2.01)	1.28 (0.45, 3.6)	0.78 (0.29, 2.05)	1.14 (0.40, 3.2)
< 4	1	1	1	1
4+	1.72 (0.81, 3.67)	2.78 (1.2, 6.38)	1.77 (0.83, 3.78)	2.9 (1.23, 6.93)
Age at Menarche				
0 to 11	1	1	1	1
12 to 19	0.78 (0.33, 1.83)	0.90 (0.38, 2.15)	0.66 (0.25, 1.6)	0.73 (0.30, 1.75)
Use of Oral Contraceptive				
Never	1	1	1	1
<6 years	0.73 (0.27, 2.0)	0.85 (0.31, 2.37)	0.67 (0.22, 1.7)	0.74 (0.26, 2.05)
6+ years	0.74 (0.24, 2.27)	1.24 (0.37, 4.13)	0.77 (0.25, 2.37)	1.14 (0.34, 3.84)
Never	1	1	1	1
Ever	0.74 (0.28, 1.94)	0.94 (0.35, 2.52)	0.67 (0.25, 1.76)	0.82 (0.30, 2.2)

TABLE 11: Cox Proportional Hazards Regression Analysis for Sexual Activity Variables

	HAZARD RATIO (95%CI)			
Variable	**Crude**	**Age Adjusted**	**Adjusted for HPV**	**Adjusted for HPV & Age**
Age at First Intercourse				
Continuous	0.96 (0.86, 1.06)	0.98 (0.90, 1.1)	0.98 (0.88, 1.08)	1.00 (0.91, 1.11)
to 15	1	1	1	1
15 +	0.51 (0.24, 1.08)	0.60 (0.28, 1.3)	0.58 (0.27, 1.24)	.66 (.30, 1.45)
<20	1	1	1	1
20+	0.42 (0.13, 1.4)	0.56 (0.16, 1.93)	0.49 (0.15, 1.65)	0.59 (0.17, 2.08)
Lifetime Number of Sexual Partners				
0 to 1	1	1	1	
2 to 3	2.7 (1.14, 6.36)	2.7 (1.15, 6.42)	2.41 (1.02, 5.69)	2.48 (1.05, 5.86)
4+	1.34 (0.44, 4.08)	1.41 (0.46, 4.31)	1.06 (.35, 3.25)	1.08 (.35, 3.33)
<=1	1	1	1	1
2+	2.7 (1.14, 6.36)	2.73 (1.16, 6.45)	2.25 (1.03, 5.80)	2.51 (1.06, 5.96)
Number of Sexual Partners (past 5 years)				
0 - 1	1	1	1	1
2+	2.84 (1.34, 6.0)	2.39 (1.1, 5.15)	1.93 (0.89,4.18)	1.74 (0.79, 3.8)
Number of Sexual Partners (previous year)				
0 to 1	1	1	1	1
2+	3.11 (1.07, 8.99)	3.09 (1.07, 8.96)	1.86 (.62, 5.52)	1.92 (.65, 5.72)

Age

Of the 243 women who were 18-24 years old at enrollment, 16 (6.6%) developed a cervical precursor lesion during the course of the follow-up. Of the 978 women who were 25 years of age or older, only 3% developed such lesions.

I conducted univariate KM analysis using age of the woman at entry to the cohort against the time to development of lesion. The cumulative rate of SIL development was 3% (by 6 months) and 5% (by 12 months) in the 18-24 age group and 0.9% (by 6 months) and 2.2% (by 12 months) in the 25+ category.

Categorizing age in low age of 18-24 and high of 25+, I observed a strong protective effect (which achieved statistical significance) of older age. The cumulative hazard curve according to age is presented in Fig. 3. There was a statistically significant difference between these two groups at the 5% level.

Crude Cox regression analysis produced a HR of 0.34, (95% CI: 0.16, 0.73), meaning that a woman under 25 years of age had more than twice the risk of a woman 25 years of older of developing an incident precursor lesion before adjusting for other factors under study. The magnitude of effect and its CI were virtually unchanged by adjustment for HPV infection: HR 0.46 (95% CI: 0.21, 0.99).

HPV

On univariate KM analysis the main exposure variable, HPV, was strongly associated with time to development of SIL lesion; those who were HPV-positive with high-risk types at baseline visit were much more likely than those HPV-positive with low-risk types to develop lesions during the follow-up period of up to 3 years.

Of those who were HPV-negative (646) at baseline visit 2.3% (15) developed cervical precursor lesions within the time of follow-up. Of the 101 women who were HPV positive at baseline (with either low-risk HPV type or high-risk HPV type), 14%

developed these lesions during this follow-up period. Of those infected with low-risk HPV 12% developed an SIL; of those infected with high-risk HPV 14% developed SIL. The curves describing the difference in hazards according to HPV positivity are presented in Figure 2.

Infection with low-risk types of HPV carried a HR of SIL of 5.72 (95% CI: 2.19, 14.92), whereas (as expected) infection with high risk types had a HR of 8.03 (95% CI: 3.37, 19.17). Though slightly dampened, the strong association between HPV and SIL remained after adjustment for age.

Previous History of Pap Screening

Whether or not a women had previously attended Pap screening was found on KM actuarial analysis to be an important covariate for the outcome under study. Seven of the 51 (13.73%) women who had never had a Pap smear developed incident lesions during the follow-up period. In contrast, only 3.4% of those women who had previously had Pap screening developed cervical precursor lesions. The cumulative rate of SIL development for those who had never had a Pap smear was 5% by 6 months and 12% by 12 months; for a woman who had at least one previous Pap smear in her lifetime, the cumulative rate was only 1% by 6 months and 2%, by 12 months. On the basis of crude Cox regression analysis, the hazard ratio in these two groups was over five.

The best functional form for this variable was identified as the one in which I classified the women according to whether they had ever or never had a previous Pap test. When lifetime number of Pap smears was categorized into 5 groups, the confidence intervals overlapped extensively. Use of the Pap variable on a continuous basis was not appropriate, because its relation with risk was not linear. The crude HR obtained from Cox regression for never having had a previous Pap smear was 5.4 (95% CI: 2.29, 11.46). The youngest women who were also at highest risk of outcome by virtue of age, would be least likely to have ever attended Pap screening. Adjusting for age reduced the magnitude of this association HR: 3.61 (1.18, 11.11). The association between never having attended Pap screening and development of SIL thus persisted beyond the simple age effect.

History of Oral Contraceptive Use

Whether a woman had ever used oral contraceptives (OCs) or whether a woman had been a long-term or short-term user was available for analysis. When I classified women according to duration of OC use on the basis of three categories (never, < 6 years, >=6 years) KM analysis did not produce a more significant contrast than categorizing a woman's history of oral contraception use on an never-ever basis. In fact, the log-rank statistic was more significant using the latter classification. This was confirmed by Cox regression, though neither crude nor age-adjusted HRs were significant.

The cumulative rate of SIL development amongst the women who had never used OCs was 3% by 6 months and 5% by 12 months; for those who reported ever having used OCs, this rate was 0.9% by 6 months and 2.4% by 12 months. The p-value for log-rank was 0.14, but sufficiently significant contrast to be entered into the full multivariable model.

Smoking

I did not find an association between smoking and the risk of SIL on the basis of KM actuarial analysis. Neither categorization into ever-never or never-former-current showed any significant association with the rate of occurrence of cervical precursor lesions (see Table 6). The log-rank p-values under both approaches were non-significant. The crude Cox regression analysis confirmed that smoking was not a predictive variable (see Table 9). As described later, I did explore the ability of smoking to modify the HPV-SIL association.

Ethnicity

On KM analysis I found no evidence for a significant difference between the cumulative incidence of precursor lesions according to the whether a woman is white or non-white. The cumulative rates for white women were 1% by 6 months of follow-up and 2.3% by 12 months of follow-up; for non-white women this rate was 1.5% by 6 months and 0.4% by 12 months. The log-rank p-value was not significant (p = 0.21).

The univariate Cox analysis confirmed the lack of association between ethnicity and the development of SIL.

Education

I initially classified women into four categories according to educational attainment, namely having less than complete elementary education, having completed elementary schooling, having at least some high school completed and having at least some college or university education completed. KM actuarial analysis gave evidence of marginally significant differences between these groups with respect to their risk of outcome (log-rank p-value = 0.54). I subsequently re-classified the women according to their educational history, grouping those who had completed elementary schooling or less separately from those who had some high school or more, considering that this would be the relevant educational experience. Again, on the basis of KM analysis neither of these contrasts showed differences between the groups with respect to risks of occurrence of lesions.

The results from crude Cox regression analysis confirmed the lack of a significant difference in the risk of cervical precursor lesions between education groups; HR:1.49 (95% CI:0.63, 3.5). It does not appear that this result was confounded by age, as there was no evidence of a change in HR upon adjustment of the education covariate for age, HR:1.42 (95% CI: 0.60, 3.35).

Parity

When I classified women according to number of pregnancies (classified as <=1, 2 - 3, 4 - 6, and 7+), KM analysis indicated that this characteristic was significantly predictive of the risk of lesion development over time (p-value for log-rank .02).

When I classified the women into two groups (those who had been pregnant 3 times or fewer and those who had been pregnant 4 times or more), this provided an effective contrast as well. The cumulative rate for a woman in the low parity category was 1% by 6 months and 2.4% by 12 months; for a woman in the high parity category this rate was 2% by 6 months and 3.4% by 12 months.

Using five categories for parity resulted in HRs that indicated an inconsistent dose-response relationship. Comparing those who had been pregnant less than 4 times with those who had been pregnant 4 times or more, the relative hazard (HR) was 1.62, approaching significance (95% CI .90, 2.92). Once adjusted for HPV and age, the HR rose to 2.9 (95% CI:1.23, 6.93).

Age at First Intercourse

Using KM actuarial analysis, I found age at first intercourse to be associated with the risk of SIL development according to whether the woman had her first sexual experience before or after the age of 20. The p-value for the log-rank statistic comparing the two groups was 0.01. The cumulative rate for those who were younger than 20 years of age at first sexual intercourse was 1.4% by 6 months and 3% by 12 months; for those in the older age group the rate was 0.4% and 0.9% (by 6 and 12 months respectively).

Crude Cox regression showed a statistically non-significant protective effect HR: 0.42 (95% CI: 0.13, 1.4) of initiating sexual activity after the age of twenty. Neither adjustment for age nor adjustment for HPV resulted in statistical significance (see Table 10) .

Number of Sexual Partners

The form that I used for each of the three sexual activity variables (lifetime number of sexual partners, number of sexual partners in the past year and past 5 years) in all subsequent analyses is one that considers number of sexual partners dichotomized into low (none or one partner) or high (two or more partners). Figures 6, 7, and 8 graphically present the difference between each of the two groups of women according to each of these variables.

I found lifetime number of sexual partners to be a significant predictor of SIL on KM analysis (p-value for log-rank, p = 0.02). The risk of developing an SIL for a woman with none or one sexual partner in her lifetime was 1.1% by 6 months and 2% by 12

months. In contrast, a woman with two or more lifetime sexual partners had a risk of 1.3% by 6 months and 4% by 12 months.

The risk of cervical precursor lesions amongst women who reported a 'high' lifetime number of sexual partners (two or more) was more than twice that of a woman who reported a 'low' (none or one) number of lifetime sexual partners; HR:2.7 (95% CI: 1.14, 6.36). Upon adjustment for HPV the magnitude of the estimate was dampened; HR: 2.25 (95% CI: 1.03, 5.8).

I also measured sexual activity by the number of partners in the past five years. On KM analysis the difference in hazard between the two groups (low vs high, as above) was significant (p for log-rank <.0001). Those in the low category had a cumulative rate of 0.9% by 6 months and 2.1% by 12 months, while those in the high category rates of 2.4% by 6 months and 5.4% by 12 months.

The crude HR of 2.84 (95% CI 1.34, 6.0) was comparable in magnitude and precision upon adjustment for age; HR: 2.39, 95% CI (1.1, 5.15). Once this variable was further adjusted for HPV infection, however the effect of the association lost statistical significance; HR: 1.93, 95% CI (0.89, 4.18).

The number of reported sexual partners in the past year was highly predictive of the occurrence of incident lesions; those who had two or more sexual partners in the past year were significantly (log-rank p-value = 0.0036) more likely to develop an incident lesion during follow-up as compared with those who had none or a single sexual partner. Women who had two or more sexual partners in the past year had a 4% risk of incident lesions by 6 months of follow-up and a 10% risk of outcome by 12 months of follow-up. In comparison, women with 'few' sexual partners (none or one) had a 1% risk of developing a lesions by 6 months and 2.4% risk of developing a lesion by 12 months.

Upon crude Cox regression analysis, the hazard rate of women who had two or more sexual partners in the past year was 3.11 times (95% CI: 1.07, 8.99) that of

women with only one sexual partner. The magnitude and significance of the HR was retained through adjustment for age, but lost when also adjusted for HPV status at baseline.

Income

When I categorized income according to quartiles of US dollars per month, KM analysis showed a non-significant contrast (p for log-rank = 0.32) and on crude Cox regression produced HR with overlapping 95% CI (see Table 9). I therefore reclassified the women according to two levels of income distinguishing women whose monthly income was less than US $550 per month from those whose monthly income was greater than US $550 per month. The p value for log-rank using this approach to categorization was 0.09. The cumulative risk of SIL for a woman in both the low and high-income category was 1% by 6 months; by 12 months the high income category carried a risk of 10% while the low income category had a risk of 2.4%.

On crude Cox regression analysis income was found to be statistically significant showing an elevated hazard for those in the higher than US $550 per month category; HR: 2.45, 95% CI (1.09, 5.64). This association remained significant even after adjustment of income for age, for HPV and for both age and HPV (see Table 9).

Interactions

Two biologically plausible interactions were considered: the effect of OC use on the association between HPV and SIL and the effect of smoking on the association between HPV and SIL. Interaction implies differences in effects of a main exposure variable according to levels of a third factor. I created interaction terms for both these potential effect modifiers. Upon crude Cox regression neither of these approached statistical significance, even by a very liberal criterion (p for Wald statistic = 0.5). The low numbers of events precluded the ability to study the effect of these potential effect modifiers on the association between HPV and SIL.

Multivariable Model

I included the three covariates considered necessary *a priori* due to their previously established ability to predict risk of cervical precursor lesions in all the models. These three covariates were HPV status, age at baseline, and previous history of attending Pap screening. In the first model, as shown in Table 12, I also included history of oral contraceptive use (on the basis of potential biologic importance), number of pregnancies, age at first intercourse, lifetime number of sexual partners, number of sexual partners in the past year and past five years and income (on the basis of significance of KM analysis and crude Cox regression).

When I included the sexual activity variables in the model the HR for low-risk HPV was HR:1.7 (95%CI: 0.32, 8.82); and for high-risk HPV, HR:7.0 (95% CI: 2.53, 19.38). After removal of this group of variables, the HR for high-risk type was maintained HR:6.9 (95% CI: 2.85, 16.74) but the HR for low-risk types increased in magnitude.

The set of variables best able to predict the development of SIL was the one that included HPV positivity, age, previous history of Pap smear, income and number of pregnancies (i.e., final model in Table 12),. A woman infected with a high-risk HPV type had 3.85 times the risk (95% CI: 1.33, 11.13) of developing an SIL of a woman not so infected, controlling for all other variables in the model. This risk increased to a HR of 6.9 (95% CI: 2.85, 16.74) for those infected with high-risk HPVs. The two other variables that were included as risk factors on the basis of prior knowledge also appear to have an independent association with cervical precursor lesions. A woman never having had a Pap smear had 3.7 times the risk of someone who had had at least one Pap smear; and being of age over twenty five reduced a woman's risk of developing cervical precursor lesions by 72%.

Income and number of pregnancies also remained independently associated with the occurrence of cervical precursor lesions. A woman whose monthly household income put her in the high-income category (over US $550) was at 2.4 times the risk of developing cervical precursor lesions of a woman whose monthly income was below this

level, adjusting for all other characteristics under study. Women who had 4 or more pregnancies were at 3.3 times greater risk of developing cervical precursor lesions than women who had fewer than four pregnancies, again controlling for HPV status, history of Pap screening, age, and income.

Table 12: Multivariable Cox Regression Models

	Hazard Ratio (95% CI)	
	Full Model	Final Model
HPV		
Negative	1	1
Low-Risk	1.70 (0.32, 8.82)	3.85 (1.33, 11.13)
High-Risk	7.01 (2.53, 19.38)	6.90 (2.85, 16.74)
Pap (lifetime)		
Ever	1	1
Never	2.36 (0.58, 9.65)	3.68 (1.14, 11.85)
Age (years)		
18-24	1	1
25 +	0.18 (0.06, 0.55)	0.28 (0.11, 0.71)
Use of Oral Contraception		
Never	1	
Ever	0.75 (0.23, 2.40)	
Pregnancies		
<4	1	1
4+	2.73 (0.98, 7.60)	3.28 (1.30, 8.08)
Age at First Intercourse		
>20	1	
20+	0.80 (0.16, 3.96)	
Lifetime Number of Sexual Partners		
0 or 1	1	
2 or more	1.82 (0.64, 5.17)	
Number of Partners(Past 5 Years)		
0 or 1	1	
2 or more	0.62 (0.17, 2.32)	
Number of Partners (Past Year)		
0 or 1	1	
2 or more	9.93 (1.66, 59.16)	
Income		
< 550$ US	1	1
550$ US	3.73 (1.52, 9.16)	2.4 (1.02, 5.62)

Part V

DISCUSSION

Descriptive Analysis

Due to still limited numbers of outcome events available for analysis from the cohort, I used a combination of LSIL and HSIL as the dependent variable. This likely dilutes the strength of associations observed. A substantial proportion of LSIL are the manifestation of an HPV infection and since the outcome used in this analysis gives equal weight to LSIL and HSIL, the magnitude of effect is likely not as strong as would be observed were only HSIL considered as the outcome variable. Most natural history studies support the theory that LSIL is generally a benign lesion (Kiviat,1996).

The risk of any cancer increases with age, with this very often being the strongest determinant. In the case of cervical cancer it has been shown on an ecologic basis that the severity of lesions increases with age: LSILs are usually found in women in their 20's, HSILs in 25-35 year old women and ICC in women over 35 or 40 years of age (Schiffman and Brinton,1995). The ability to sample from the transformation zone decreases at age 35 and there is a dramatic decrease in prevalence of HPV infection beyond age 35.

The categorization of age into 18-24 and 25+ is coherent with a study of HPV and precursor lesions. The young age group is at greatest risk of both HPV and cervical precursor lesions, while the older group is protected with respect to both.

Very few of the study women had never had a Pap smear. Brazil's Ministry of Health recommends annual screening for sexually active women, reduced to every three years following two normal consecutive smears (Naud et al.,1997). Sao Paulo has a municipally funded screening program which provides women with Pap smears. Once histological results become available, the LMB cohort study will be able to report on the relative quality of the cytological screening available in Brazil.

Smoking remains a disputed risk factor for cervical cancer but may be involved at an early point in the causal pathway or through an interactive effect on the HPV-SIL

association. Though some have observed an association between smoking and cervical precursor lesions, no such effect has been observed in this study. The relevant exposure is most likely current smoking (i.e. concomitant with lesion development or HPV infection). This is an exposure that the LMB cohort will be able to report on in the near future, as each questionnaire asks about smoking history between visits.

History of OC did not appear to be associated with the development of SIL according to our data. Future analyses will also be able to include information on recent and current use of OC. Whether the effects are protective or put the woman at increased risk, it is likely that the relevant exposure is that which is coincident with HPV infection. The LMB Cohort Study is designed to accumulate information on use of OC and smoking between study visits. As information from Questionnaires 2-5 accumulates, the effects of these exposures can be more appropriately studied.

The high-income category in the study represents an annual household income of over US $6,600; 90% of the women have incomes below US $960 per month, or US $11,500 per year. Though on the basis of the data the income levels appear distinct with respect to development of SIL, it must be considered that these categories may not have "social" significance. Whatever those factors related to economic status that affect a woman's risk of developing SIL are, it may be that whether the woman's income is below or above US $550 per month does not make sufficient difference in her social reality to observe the expected association. However unlikely, based on previous evidence, it must be considered that there may be some unrecognized factor that is operating to increase the risk among women with higher income.

Ethnic origin is often used as an indicator of socio-economic status, but this population is known to be quite homogeneous in its poverty. Access to health care or health behaviors may differ with ethnicity, and there may be differences in history of attending Pap screening due differing cultural acceptance. Ethnicity was not a significant predictor of SIL development on crude analysis, nor was it significant upon adjustment for history of Pap screening.

Education is another measure that can serve as an indicator of socio-economic status and is also important with regard to screening behaviors. Women who lack understanding of the benefits of Pap smears are less likely to attend screening. Eighty-two percent of the study women have at most completed elementary schooling. The fact that these women report such a high attendance at Pap screening even prior to this study gives evidence that health care education can be achieved in the absence of formal schooling.

Early age at first intercourse is considered a classic risk factor for cervical cancer. Women whose sexual activity started at less than sixteen years of age have previously been shown to have twice the risk of developing cervical cancer of women who initiated sexual activity when they were over age 20 (Schiffman and Brinton,1995). This may be due either to the increased length of time during which exposure to HPV can occur or to the particularly vulnerable period of metaplasia. Along with the other sexual activity variables, early age at first intercourse appeared to be a marker of HPV infection,rather than an independent predictor of cervical precursor lesions.

The association between sexual activity and cervical cancer is well-established in other populations studied. The sexual activity of the women enrolled to the LMB cohort, as measured by number of sexual partners and age at first intercourse, appears to be relatively conservative. This is in keeping with the cultural norms for this population. Brazilian women are highly monogamous, as is the societal expectation.

Seventy-nine percent of the women in this study population had at most 3 lifetime partners; in contrast, cohort studies recruiting from among women attending STD clinics, almost 94% had 6 or more lifetime sexual partners (Koutsky et al.,1992).

Increasing number of pregnancies has previously been found to be associated with increased risk of invasive cervical cancer (Brinton et al.,1989). The mechanism of this risk factor is unknown, but it has been shown with a great deal of consistency. Some mechanisms postulated are nutritional, immunologic, traumatic or hormonal. In support of the trauma theory, researchers have found reduced risk in those who have

delivered their babies by c-section (Brinton et al.,1989). In this analysis parity of 4 or greater carries a risk of almost the same magnitude as does never having had a Pap smear.

Prevalence

The prevalence in this population of SIL at entry to the study (2.15%) appears to be quite similar to the rate reported by the study of American Planned Parenthood clinics (2.3%) (Sadeghi et al., 1988). This may be a reflection of the similarities of the clinic populations from which the studies recruit. Since the rate of CIN is known to decrease with increasing age, comparison of prevalence rates that have not been age-adjusted can only serve in broadest terms.

Persistence, Progression, and Regression

As expected, a majority (84%) of LSILs were found to regress by the next visit. This is an even higher rate of regression than reported by Ostar's meta-analysis (Ostar,1993), in which he found that 57% (n = 4,504) of low-grade lesions regressed. The studies that Ostar reviewed span many years, used varying systems of classification and lengths of follow-up and ascertained outcomes by either cytology or biopsy. Though not all differences between the studies can be overcome, when the data are restricted to those five studies that used cytological ascertainment of outcome, the average rate of regression of low-grade lesions falls to 45% (n = 971). The years of follow-up in these studies range from less than one year to a maximum of six years.

The LMB cohort women experienced persistence and progression rates much lower than the overall rates reported by Ostar. In our study 10% of women with LSIL had a similar grade of lesions by the next visit. Though not entirely comparable, particularly since lengths of follow-up times between visits are not consistent between the studies, the equivalent rate of persistors by cytological ascertainment in Ostar's review was 32%. Six percent (6%) of the LMB cohort study women who had LSIL at one visit were found to have lesions of higher cytological grade (HSIL) by the next visit; 25% of women in the studies reviewed by Ostar showed progression to CIS.

Such a relatively high rate of regression and low rate of persistence as experienced by the women of the LMB cohort, is somewhat surprising in the case of precursor lesions amongst women in whom the risk of the cancer so high. It is possible that a high incidence of precursor lesions in a population does not, in itself, lead to an elevated risk of cervical cancer. Rather the specific nature of those lesions or some other host, viral or environmental factors may predispose these women to cervical cancer.

An alternative explanation is that the natural history of precursor lesions is too ephemeral to be determined by cytological ascertainment. Previous authors have cautioned against conclusions based on short-term follow-up. Cytological results can show regression transiently either due to the limitations of the test itself or due to a true change in lesion status. Further, because the Bethesda system of cytological classification of LSIL includes HPV lesions, it is expected that the rate of regression of LSIL is affected in an important way by the ability of HPV to regress.

In future, an analysis that considers the evolving HPV status of the woman will improve the resolution of understanding in this area. It is possible, for example, that those regressors who also become HPV-negative on follow-up will turn out to be persistent regressors. Perhaps the long follow-up time requirement that researchers in the 80's found they needed in order to conclude that a given lesion had truly regressed to normalcy will be replaced by the short-term observation of regression of both the lesion and HPV status.

Measurement of Incidence Density

Incidence rates have been reported for only a very few populations. Most studies that were identified for this report used histological methods to ascertain incident lesions and so the rates are not directly comparable. Also, the studies reviewed span a period of almost 20 years, so there have been changes in clinical practice and ascertainment techniques. Finally, results from different studies reflect the peculiarities of the classification systems of the different moments, countries, and even regions. Of those populations that have reported incidence density rates of dysplasia and CIS

(combined), the range is between 140-500/100,000 woman-years (see Table 2). Using cytological ascertainment, the study conducted in the UK (1976-1977) reports an incidence rate of precursor lesions of 156/100,000 woman-years.

The incidence density rate of 2,316/100,000 woman-years found in this study gives evidence that these women are at very high risk of incident cervical precursor lesions. The high risk of cervical cancer in this population may be related to this high risk of precursor lesions. The proportion of lesions that progress to cervical cancer may or may not be similar to that of other populations, but having a large pool from which cancers arise might increase the incidence observed in the population. The women in this study are subject to more frequent Pap screening (every 4 months) than is routinely recommended in practice (annually) for healthy women. As with any special measures taken in a research context, there exists the risk that simply testing more frequently will cause over detection. Sensitivity analysis showed that this incidence-density rate is not an artifact of repeat testing.

On the basis of success of screening programs in developed countries, the adequacy or completeness of Pap screening in high-risk areas offers the first line of explanation for the observed rates. The elevated incidence rate in this population appears to be unrelated to the quality of cytological readings, as these are incident cases identified by review cytology.

It has been suggested (Brinton et al.,1989) that the high incidence of cervical cancer in Latin American countries may be related to male sexual relationships outside of marriage and visits to prostitutes. This has been termed the male factor and has been the object of study of only a few studies because of the difficulty of obtaining reliable information and the complicated logistics of including partners of female subjects in an epidemiological investigation. It is possible that the elevated rates of incident precursor lesions are the result of the same phenomenon.

Determinants of Incident SIL

Women selected for this study were those 848 who were initially found to be negative by cytology at first visit and for whom PCR results were available. Using a single cytological result to define a women as free of lesion is not as stringent a definition as some would require. Once the depth of information on each woman in the study is increased, use of two consecutive cytological results of 'within normal limits' (WNL) would provide more stringent entry criteria.

Women whose Pap test result was given a cytological classification of ASCUS were also included in the analysis. These women are at higher risk of developing the particular category of lesion of interest (LSIL or HSIL). To exclude these women would have reduced the number of incident lesions that were found during follow-up. Moreover, these women clearly adhere to the definition of those at risk of developing lesions.

The crude and age adjusted HRs for HPV positivity are in the range of 5.8 - 8.0, far lower in magnitude than the ORs reported from case-control studies. Risk estimates from prospective studies are typically lower than those from case-control or cross-sectional studies, because HPV positivity appears to be lost more often in controls than in cases. Under these designs those in whom HPV infection is detected may simply be more likely to harbour persistent infections.

Lifetime number of sexual partners has been shown to effect the risk of cervical cancer (Brinton et al.,1987). Questionaire 1 also provided information on the number of sexual partners in the past year and past five years. On crude analysis three sexual activity variables (lifetime number of sexual partners, number of sexual partners in the past five years and past year) were strongly associated with development of SIL. The crude HR of smallest magnitude among the history of sexual activity variables was associated with number of lifetime sexual partners. Next in magnitude was the HR for the number of sexual partners in the past five years, while the highest was the HR for number of sexual partners in the past year. This suggests that the occurrence of precursor lesions is associated with relatively recent sexual activity.

Upon adjustment for HPV, the association of partners in the past year and past five years disappeared. Further, upon inclusion of the sexual activity variable in the model the association of low-risk HPV types with SIL disappeared, while that of high-risk HPV types was maintained, furthering evidence that it is the sexual transmission of high-risk types that figures in the causal pathway of cervical cancer. Adjustment for sexual activity variables cannot explain away the effect of high-risk types, though it does explain the crude associations seen with low-risk types.

In spite of limited power to detect effects due to the small numbers of outcome events, two factors appear to maintain an independent association even after adjustment for such extremely strong explanatory variables as HPV, age and previous history of attending Pap screening. The variables that were retained through the modeling process and appear in the most predictive model, as well as those that were eliminated from the model, are of interest. According to these results all the sexual activity variables, including age at first intercourse, lifetime number of partners, number of partners in the past 5 years and number of partners in the past year appear to be correlates of HPV infection rather than independent predictors of cervical precursor lesions. This result is fully coherent with the presence of a sexually transmitted etiologic agent.

Those variables which remain in the model can themselves be considered explanatory variables whose effects are independent of HPV infection. In this analysis I found two characteristics to be independently associated with cervical precursor lesions: income and parity. That higher income is found to be associated with elevated risk of cervical precursor lesions is intriguing. Higher income (as a marker of socio-economic status) is expected to be protective on the basis of previously established risks of cervical cancer,. The first model which included all eligible variables (including sexual activity variables and income) showed an even greater estimate of association between income and development of cervical precursor lesions (i.e. HR:3.7 (95% CI 1.5, 9.2). The association between income and SIL was apparently not due to greater numbers of sexual partners among women with higher income.

From a data analysis point of view the categorization adopted for the income variable appeared to be predictive of development of SIL. It may be that this population was not characterized by sufficient heterogeneity in income level. It is possible that with respect to the mechanism involved in SES being protective, too few of these women achieved a level of income that would be 'socially' meaningful. A woman whose household income is as low as US $300, US $550, or even US $1000 per month may retain the characteristics that predispose her to the development of precursor lesions.

As discussed previously, increasing parity has consistently been shown to be associated with an elevated risk of cervical cancer. That this association with cervical precursors remains significant after adjustment for HPV status, age and previous Pap smear suggests that this factor may be involved at a very early stage in the causal pathway.

As always, it must be considered that the observed associations are attributable to bias. Biases in the study were minimized by enrolling all study subjects from the same clinic population, by having clinicians and cytologists blinded as to HPV status, by having review cytologists blinded to the results of Brazilian cytologists and by adopting uniform testing and standardized interview. Efforts to ensure that women return for follow-up according to the standardized schedule of return visits were, to the extent possible, equal for all women.

In spite of every attempt being made in the design to minimize its effect, selection bias must be suspected until it has been ruled out. This analysis was conducted on a set of women restricted by availability of cytological and PCR results. In order to assure myself and others that the effects of this restriction are random, I have carefully documented information on thirteen socio-demographic, sexual activity and reproductive history characteristics separately for women enrolled in the study, for women whose baseline cytology results were normal and for those for whom additionally, PCR results were available. Except for there being fewer women in the high income category in the restricted group (incidence study women) there appeared to

be no difference between these women and those enrolled to the study. There does remain the underlying concern that the women selected for this analysis were different in some unmeasured characteristic.

Also, not all women who were eligible to participate, accepted the invitation to enroll in the study. Records are being kept, to the extent that this is possible, so that eventually, differences between those who agreed to participate and those who didn't can be reported. Though, as already mentioned, the compliance rate has been high for the scheduled visits there are some women who become lost to follow up after the initial visit. Information gathered by the first questionnaire can eventually be used to compare these women with those who continue with the study. This analysis was conducted at an early moment in the history of the cohort. Information on losses to follow up was not sufficiently complete to produce a report on women who continued with the study and those who, sometime after the baseline visit, became non-participants.

Future Research Directions

Once sufficient data has been accumulated from the cohort, a similar analysis should be conducted using HSIL as the outcome of interest. HSIL is likely a later precursor lesion in the causal pathway leading to cervical cancer development. Analyses that consider the change in HPV status during the course of the follow-up are already underway.

Future study of the mechanism by which pregnancy increases the risk of cervical precursor lesions could lead to a further characterization of the causal pathway of this, and possibly even other, cancers. It would also be of interest to establish the temporal relationship between HPV infection, the moment of pregnancy and the development of cervical precursor lesions.

The LMB Cohort Study is designed to gather nutritional information using subsequent questionnaires. The area of nutrient deficit as a risk factor for cervical cancer will provide fascinating research topics in the study of cervical precursor lesions.

Further study of the effects of oral contraceptives and smoking will be possible as the study gathers information on exposure to these agents between visits.

Once histological results are available, the LMB Cohort will be able to provide Brazil's Ministry of Health with valuable information regarding the quality of the existing cytological screening program. Particularly in view of the hypothesis that poor quality and coverage of screening programs are responsible for the high incidence rates of invasive cancer in developing countries, such information could serve towards bettering conditions for this population of women.

Part VI

SUMMARY

It has been shown in this report that the women enrolled in the LMB Cohort are at high risk of incident cervical precursor lesions in comparison to women of other populations. As has been the experience in other populations, the majority of these women's SILs regress.

Cytology results according to the Bethesda system have been reported for the women selected into this study. A high percent had normal cytological results even at the first visit (95.8%) but at visit two, three and four, this percent of normal results plateaus at an even higher rate (97%). Due to the important effect of age, comparison of prevalence rates between studies that have not been age-adjusted cannot be compared. Information on the age, monthly income, ethnicity, education, smoking history, number of previous Pap smears, age at menarche, number of pregnancies, use of oral contraceptives, age at first intercourse, lifetime number of sexual partners, number sexual partners in the past 5 years, number of sexual partners in the past year and HPV status of those women who had normal cytology as gathered at baseline were selected as characteristics for analysis.

An individual woman's risk of developing an SIL has been reported for up to 6 and up to 12 months of follow-up and reported as the cumulative rate of SIL development according to each of the variables under study. In this analysis HPV results, history of Pap screening, age, number of pregnancies, income, age at first intercourse and number of sexual partners (lifetime, past 5 year, previous year) appeared to be significantly associated with a woman's risk of developing SIL; level of educational attainment, ethnicity, history of OC use and smoking, or age at menarche were not so associated.

Using Cox regression, history of use of OCs age at menarche, education, ethnicity, history of smoking and age at first intercourse were not significantly associated with SIL development upon crude analysis, nor upon Cox regression adjusted for age, HPV or both age and HPV.

On the basis of crude Cox regression among women who were cytologically negative at baseline, increasing age and even a single previous Pap smear were significantly protective; infection with either low-risk or high-risk HPV, having 2 or more sexual partners in her lifetime, in the past 5 years or in the past year put a woman at elevated risk of SIL, as did having a monthly income of more than US $550. No significant association was seen between number of pregnancies and development of SIL upon crude Cox regression.

Infection with either high- or low-risk HPV, never having had a Pap smear, more than two sexual partners (lifetime, past 5 years or previous year), or having a monthly income of US $550 or higher remained positively associated upon Cox regression adjusted for age. Upon adjustment for age, having had four or more pregnancies appeared to put women at significantly increased risk of developing an SIL, and this association continued to be observed after adjustment for both age and HPV.

Upon extensive adjustment for a priori confounders, the magnitude of the association between low-risk HPV and SIL was reduced. The magnitude of association between high-risk HPV and SIL was not altered upon this adjustment, furthering evidence that it is the high-risk HPVs that are oncogenic.

Multivariable modeling confirmed that four or more pregnancies and monthly income greater than US $550 are predictors of SIL, and their effects are independent of HPV infection, age or previous Pap screening. Others have noted an association between number of pregnancies and risk of cervical cancer, but contrary to these findings the literature has overwhelmingly reported the protective effect of higher income.

In addition to identifying these two independent risk factors for cervical precursors, this study found that the sexual activity variables, age at first intercourse, lifetime number of sexual partners, number of partners in the past year and past five years are all markers for low-risk HPV infection rather than independent risk factors for

SIL. The strong association between high-risk HPV types and cervical precursor lesions was maintained in both the presence and absence of these sexual activity variables further supporting the evidence that it is high-risk types that lead to cervical cancer.

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APPENDIX: Questionnaire 1

Data da entrevista: D: _____ M: _____ A: _____ Hora do início: _____

1. Nº no estudo: _____

2. Registro M.E.V.N.C: _____

3. Qual o seu nome? _____

4. Em que dia, mês e ano a Sra. nasceu? D: _____ M: _____ A: _____

   Portanto, quantos anos a Sra. tem? _____ anos

5. Grupo étnico (*Interpretação da entrevistadora*):
   ¦1¦ Branca ¦2¦ Mulata ¦3¦ Negra ¦4¦ Amarela ¦5¦ Índio ou descendente

6. Qual é o seu estado civil?
   ¦1¦ Solteira ¦2¦ Casada ¦3¦ Viúva
   ¦4¦ Separada ¦5¦ Vive maritalmente ("juntada" ou amigada)

7. Quais foram as suas ocupações/empregos nos últimos dez anos?

   _____

   _____

   _____

   _____

8. Até que grau escolar a Sra. estudou?
   ¦1¦ Analfabeta ¦2¦ Primário incompleto ¦3¦ Primário completo
   ¦4¦ Secundário incompleto ¦5¦ Secundário completo
   ¦6¦ Técnico-profissionalizante ¦7¦ Superior

9. Qual a sua religião?
   ¦1¦ Católica ¦2¦ Crente ¦3¦ Protestante ¦4¦ Judia (Israelita)

   ¦5¦ Espírita ¦6¦ Umbandista ¦7¦ outra (qual?) _____ ¦8¦ não tem

10. Incluindo a Sra., quantas pessoas vivem na sua casa? _____ pessoas

11. Qual é a sua renda familiar, ou seja, a da Sra. mais a dos que vivem em sua casa?

    CR$ _____ cruzeiros reais

12. Quais dos seguintes itens a Sra. tem em casa?

a) geladeira	¦1¦ Sim	¦2¦ Não
b) T.V. a cores	¦1¦ Sim	¦2¦ Não
c) telefone	¦1¦ Sim	¦2¦ Não
d) video-cassete	¦1¦ Sim	¦2¦ Não
e) carro	¦1¦ Sim	¦2¦ Não
f) carros adicionais	¦1¦ Sim	¦2¦ Não

13. Em que bairro a Sra. mora: _____

14. Há quantos anos a Sra. mora nesse local? _____ anos

15. Onde a Sra. nasceu?: Cidade:_____ Estado _____

16. Essa cidade era: ¦1¦ área rural ¦2¦ área urbana ¦3¦ subúrbio ¦8¦ não sabe

17. Onde a Sra. morou a maior parte de sua vida?
(*após os 12 anos de idade*) Cidade: _____ Estado _____

18. Esse local era: ¦1¦ área rural ¦2¦ área urbana ¦3¦ subúrbio ¦8¦ não sabe

19. A Sra. fuma ou já fumou?
¦1¦ Sim ¦2¦ Nunca (*se nunca, vá direto à questão 31*)

Se sim, fazer as perguntas referentes a cada tipo de tabaco:

Cigarros de papel industrializados

20. Quantos cigarros a Sra. fuma/fumava em média por dia, aproximadamente?
¦1¦ no máximo 1 ¦2¦ de 2 a 5 ¦3¦ de 6 a 10 ¦4¦ 11 a 20
¦5¦ mais que 20 ¦6¦ mais que 40 (2 maços)

21. Que tipos de cigarro a Sra. fuma/fumava?
¦1¦ somente com filtro ¦2¦ principalmente com filtro, as vezes sem filtro
¦3¦ principalmente sem filtro, as vezes com filtro ¦4¦ somente sem filtro

22. Com que idade a Sra. começou a fumar regularmente ? _____ anos

23. (*Se ainda fuma, perguntar*) Há quantos anos a Sra. fuma? _____ anos

24. (*Se parou, perguntar*) Durante quantos anos a Sra. fumou? _____ anos

Cigarros de fumo de corda, de palha ou papel

25. Quantos cigarros de fumo de corda ou palha a Sra. fuma/fumava em média por dia, aproximadamente?
¦1¦ no máximo 1 ¦2¦ de 2 a 5 ¦3¦ de 6 a 10 ¦4¦ 11 a 20
¦5¦ mais que 20 ¦6¦ mais que 40 (2 maços)

26. Com que idade a Sra. começou a fumar regularmente? _____ anos

27. (*Se ainda fuma, perguntar*) Há quantos anos a Sra. fuma? _____ anos

28. (*Se parou, perguntar*) Durante quantos anos a Sra. fumou? _____ anos

29. (*Se parou, perguntar*) Há quantos anos a Sra. parou? _____ anos

30. A Sra. fuma/fumou charuto ou cachimbo? ¦1¦ Sim ¦2¦ Não

Consumo de bebidas alcoólicas

31. A Sra. costuma/costumava consumir bebidas alcoólicas, mesmo que ocasionalmente?
¦1¦ Sim ¦2¦ Nunca *(se nunca, vá direto à q. 38)*

32. A Sra. costuma/costumava beber cerveja?
¦1¦ Não/ocasionalmente ¦2¦ no máximo um copo por semana
¦3¦ de 2 a 5 por semana ¦4¦ de 6 a 10 ¦5¦ 11 a 30 ¦6¦ mais que 30

33. A Sra. costuma/costumava beber vinho?
¦1¦ Não/ocasionalmente ¦2¦ no máximo um copo por semana
¦3¦ de 2 a 5 por semana ¦4¦ de 6 a 10 ¦5¦ 11 a 30 ¦6¦ mais que 30

34. A Sra. costuma/costumava beber pinga ou cachaça?
¦1¦ Não/ocasionalmente ¦2¦ no máximo um copo por semana
¦3¦ de 2 a 5 por semana ¦4¦ de 6 a 10 ¦5¦ 11 a 30 ¦6¦ mais que 30

35. A Sra. costuma/costumava uisque, gim, vodca ou outra bebida forte?
¦1¦ Não/ocasionalmente ¦2¦ no máximo um copo por semana
¦3¦ de 2 a 5 por semana ¦4¦ de 6 a 10 ¦5¦ 11 a 30 ¦6¦ mais que 30

36. Há quantos anos a Sra. bebe essas quantidades? _____ anos
(*as referidas acima?*)

37. Durante quantos anos a Sra. bebeu? _____ anos
(*Se parou de beber, perguntar*)

Eu gostaria agora de lhe fazer algumas perguntas sobre sua vida intima. Eu entendo que este é um assunto pessoal, mas conhecer estas informações será de grande auxilio na nossa pesquisa. Eu volto a lembrar a Sra. que todas as respostas serão mantidas em total segredo. Nunca estes dados serão revelados a alguém.

38. Que idade a Sra. tinha quando menstruou pela primeira vez? _____ anos
(se menopausada e passar a q. 11)

39. Quando a Sra. teve a sua última menstruação? D: ___ M: ___ A: ___

¦_¦ Puérpera ¦_¦ Lactante ¦_¦ Menopausada

40. Quando está/estava menstruada, o que a Sra. usa/usava como absorvente íntimo?

a) absorvente tipo "MODESS" comercial	¦1¦ Sim	¦2¦ Não
b) absorvente interno tipo OB/Tampax	¦1¦ Sim	¦2¦ Não
c) toalhinha de pano	¦1¦ Sim	¦2¦ Não
d) outro (*qual?*_____)	¦1¦ Sim	¦2¦ Não

41. Nos últimos cinco anos, quantas vezes a Sra. sentiu coceira na região genital?
¦1¦ Nenhuma vez ¦2¦ Algumas vezes (1-9) ¦3¦ Muitas vezes (10+)

42. Nos últimos cinco anos, quantas vezes a Sra. sentiu dor/ardor na região genital?
¦1¦ Nenhuma vez ¦2¦ Algumas vezes (1-9) ¦3¦ Muitas vezes (10+)

43. Nos últimos **cinco anos**, quantas vezes a Sra. teve corrimento vaginal?
¦1¦ Nenhuma vez ¦2¦ Algumas vezes (1-9) ¦3¦ Muitas vezes (10+)

44. Já fez ou faz uso de algum produto para tratamento ginecológico?
(mostrar ou ler a lista de nomes de medicamentos impressa no verso da página anterior)

_____

_____

45. A Sra. já utilizou algum produto que não seja de farmácia para tratamento ginecológico?
¦1¦ Sim (*qual:* _____) ¦2¦ Não

46. Nos últimos **dois dias**, a Sra. teve corrimento, coceira ou ardor na região genital? ¦1¦ Sim ¦2¦ Não

47a. A Sra. usa/já usou algum sistema que force água/líquidos para o interior da vagina tais como duchas, bidês, etc?
¦1¦ sim, sempre ¦2¦ sim, frequentemente
¦3¦ de vez em quando ¦4¦ nunca

47b. (*Se sim*) Com que produto? _____

48. Durante os períodos menstruais a Sra. costuma/costumava lavar seus órgãos genitais? (*Além do banho diário*)
¦1¦ Não ¦2¦ Sim, uma vez por dia ¦3¦ Sim, mais de uma vez por dia

49. A Sra. já teve alguma vez feridas na vagina ou vulva? ¦1¦ Sim ¦2¦ Não

50. Alguma vez a Sra. soube por um(a) médico(a) que tinha uma doença venérea ou sexualmente transmissível? (*se sim, qual?*)
¦1¦ Gonorreia ¦2¦ Cancro ¦3¦ Crista de galo ou condiloma ¦4¦ Sífilis
¦5¦ Herpes ¦6¦ Tricomoníase ¦7¦ Candidíase ¦8¦ Nunca teve

51. A Sra. já fez um exame de prevenção do câncer de colo de útero, também chamado Papanicolaou ou citológico ou citologia oncótica? ¦1¦ Sim ¦2¦ Não

52. (*Se sim*) Quantas vezes? _____ vezes

53. (*Se sim*) Quando foi a última vez que fez este exame?
¦1¦ No último ano ¦2¦ Há mais de um ano, mas menos que cinco
¦3¦ Há mais que cinco anos ¦8¦ Não lembra

Lembrete: Foro intimo e confidencialidade

54. Com que idade a Sra. teve a sua primeira relação sexual? _____ anos
(Se virgem, vá direto a q. 100, depois de certificar-se que ela nunca engravidou)

55. Quantas vezes a Sra. já engravidou? _____ vezes
(Se nunca, vá direto a q. 63)

56. Quantas destas gestações resultaram em partos normais? _____

57. Quantas foram por operação cesariana? _____

58. Quantas resultaram em aborto? _____

59. Em que ano foi a sua última gravidez? _____

60. Foi uma gestação completa? ¦1¦ Sim ¦2¦ Não

61. Enquanto grávida a Sra. continuava tendo relações sexuais com seu marido/parceiro? ¦1¦ Sim ¦2¦ Não

62. A Sra. costuma/costumava resguardar-se de relações sexuais após cada parto? ¦1¦ Sim ¦2¦ Não

63. Com que idade a Sra. começou a ter relações sexuais pelo menos uma vez por m

Aos _____ anos ->¦_¦ se nunca foi constante

64. Durante a sua vida inteira, com quantos homens a Sra. manteve relações sexuais

(*Insista para que ela dê uma resposta mesmo q. aproximada*) ___

65. Quantos destes parceiros foram regulares, isto é, com os quais a Sra. teve relaçõe sexuais regulares durante um periodo minimo de 6 meses, independentemente de morar na mesma casa?

_____

66. Que a Sra. saiba, quantos destes parceiros sexuais não foram fiéis, isto é, tiveram contato sexual com outras mulheres?

_____

67. No total, quantos parceiros sexuais a Sra. teve antes de 20 anos? _____

68. Quantos destes parceiros (*antes dos 20*) tinham menos que 20 anos? _____

69. Quantos destes parceiros (*antes dos 20*) tinham mais que 30 anos? _____

Se a paciente tiver menos que 20 anos, passar para a q. 7

70. No total, quantos parceiros sexuais a Sra. teve depois dos 20 anos? _____

71. Quantos destes parceiros (*depois dos 20*) tinham menos que 20 anos? _____

72. Quantos destes parceiros (*depois dos 20*) tinham mais que 30 anos? _____

73. Desde o inicio de sua vida sexual houve periodos em que a Sra. não teve relações por mais que um ano? Se sim, quantos periodos *(total em anos)*?

_____ anos

74. Em geral, considerando a maior parte de sua vida sexual, com que frequência a Sr tem mantido/manteve relações sexuais? *(Descrever a frequência e duração para cada periodo lembrado pela paciente)*

_____

_____

_____

75. Enquanto menstruada, a Sra. evita/evitava ter relações sexuais com seu marido/parceiro? (*Também considerar a hipótese do marido evitar)*
¦1¦ Sempre evitei ou marido evitou ¦2¦ De vez em quando
¦3¦ Só nos primeiros dias ¦4¦ Nunca

76. A Sra. costuma/costumava lavar seus genitais antes de ter relações sexuais?
¦1¦ Sempre ¦2¦ de vez em quando ¦3¦ Nunca

77. A Sra. costuma/costumava lavar seus genitais depois das relações sexuais?
¦1¦ Sempre ¦2¦ de vez em quando ¦3¦ Nunca

78. Durante os **últimos cinco anos**, com quantos homens a Sra. manteve relações sexuais? (*Insista para que ela dê uma resposta mesmo que aproximad*

_____

79. Que a Sra. saiba, quantos destes parceiros sexuais não foram fiéis, isto é, tiveram contato sexual com outras mulheres? _____

80. Quantos destes parceiros tinham menos que 20 anos? _____

81. Quantos destes parceiros tinham mais que 30 anos? _____

82. Durante este periodo dos **últimos 5 anos**, com que frequência a Sra. teve relações sexuais com seu marido ou parceiro(s)?

_____/semana _____/mes _____/ano

Dizer à paciente para se lembrar apenas dos últimos 12 meses

83. Durante os **últimos 12 meses**, com quantos homens a Sra. teve relações sexuais? (*Insista para que ela dê uma resposta mesmo que aproximada*)

_____

84. Que a Sra. saiba, quantos destes parceiros sexuais não foram fiéis, isto é, tiveram contato sexual com outras mulheres?

_____

85. Quantos destes parceiros tinham menos que 20 anos? _____

86. Quantos destes parceiros tinham mais que 30 anos? _____

87. Durante este periodo dos **últimos 12 meses**, com que frequência a Sra. teve relações sexuais com seu marido ou parceiro(s)?

_____/semana _____/mes _____/ano

Fim das perguntas ciclicas

Detalhes sobre os métodos anticoncepcionais usados

88. Quais são os métodos que a Sra. ou o seu marido/parceiro(s) tem usado/usaram para evitar filhos? (*assinalar todos os mencionados)*
¦1¦ pílula anticoncepcional ¦2¦ laqueadura ¦3¦ vasectomia
¦4¦ D.I.U. ¦5¦ condom ¦6¦ diafragma
¦7¦ Geléia espermicida ¦8¦ Coito interrompido/tabelinha/muco cervical
¦9¦ Outro: _____ ¦10¦ Não sabe

89. *(Se P.A., perguntar)* Com que idade a Sra. começou a usar P.A.? _____ anos

90. *(Se P.A.)* Durante quantos anos a Sra. tem tomado/tomou P.A.? _____ anos

91. *(Se P.A.)* Durante este(s) período(s) a Sra. obedeceu os intervalos regulares de descanso recomendados pelo médico? ¦1¦ Sim ¦2¦ Não

92. *(Se parou, perguntar)* Há quanto tempo parou de tomar P.A.? _____ anos

93. *(Se laqueadura, perguntar)*
Há quanto tempo foi a laqueadura que a Sra. fez? _____ anos

94. *(Se vasectomia do parceiro mais frequente, perguntar)*
Há quanto tempo foi a vasectomia do seu marido/parceiro? _____ anos

95. *(Se D.I.U., perguntar)*
Com que idade a Sra. usou D.I.U. pela primeira vez? _____ anos

96. *(Se D.I.U.)* A Sra. ainda usa D.I.U.? ¦1¦ Sim ¦2¦ Não

97. *(Se condom, perguntar)* Com que frequência seu marido/parceiro(s) usa(m) camisinha? ¦1¦ Muito raramente ¦2¦ As vezes ¦3¦ Sempre

98. *(Se diafragma, perguntar)* com que frequência a Sra. tem utilizado/utilizou diafragma? ¦1¦ Muito raramente ¦2¦ As vezes ¦3¦ Sempre

99. *(Se geléia, perguntar)* A Sra. tem usado a geléia espermicida de que maneira?
¦1¦ Principalmente como método único ¦2¦Principal/ associado ao diafragma
¦3¦ Principalmente associado à camisinha

Lembrete: Foro intimo e confidencialidade

100. A Sra. já praticou/pratica coito anal, isto é, relação com penetração pelo anus?
¦1¦ Sim, frequentemente ¦2¦ Sim, raramente ¦3¦ Não

Se não, vá direto à questão 105

101. (*Se sim, perguntar*)
Com quantos parceiros a Sra. já praticou/pratica coito anal? _____

Se mais de 1 na resposta anterior, iniciar as próximas perguntas enfatizando que a entrevistada deve se referir ao parceiro com q ela mais frequentemente praticou coito anal.

102. O seu marido/parceiro realizava/realiza penetração vaginal em seguida ao coito anal? ¦1¦ Sim ¦2¦ Não ¦3¦ As vezes

103. (*Se sim, perguntar*) Antes da penetração vaginal o seu marido/parceiro fazia/faz a higiene do pênis? ¦1¦ Sim ¦2¦ Não ¦3¦ As vezes

104. (*Alternativamente*) Se o seu marido/parceiro usava/usa camisinha para o coito anal, antes da penetração vaginal ele a retirava ou trocava/retira ou troca?
¦1¦ Sim ¦2¦ Não ¦3¦ As vezes
¦4¦ Não usava/usa camisinha

105. O seu marido/parceiro tinha/tem o hábito de praticar sexo oral na Sra., ou seja, contato da boca ou lingua dele nos seus genitais?
¦1¦ Sim, frequentemente ¦2¦ Sim, raramente ¦3¦ Não

106. (*Se sim, perguntar*) Com quantos parceiros a Sra. já praticou/pratica sexo oral desta maneira? _____

Se não, vá direto ao final

Se mais de 1 na resposta anterior, iniciar as próximas perguntas enfatizando que a entrevistada deve se referir ao parceiro com quem ela mais frequentemente praticou/pratica sexo oral.

107. (*Se sim, perguntar*) O seu marido/parceiro realizava/realiza penetração vaginal em seguida ao sexo oral?
¦1¦ Sim ¦2¦ Não ¦3¦ As vezes

Eu agradeco muito a sua colaboração com a nossa pesquisa. Se a Sra. tiver alguma pergunta, sinta-se a vontade em fazê-la. Caso queira comunicar-se comigo depois a Sra pode me procurar aqui durante a semana.

Horário de término da entrevista: ____:____

COMENTARIOS DA ENTREVISTADORA:

_____

_____

_____

_____

_____

Enfermeira: _____

Questionário codificado em ____/____/____ por _____

Dados digitados em ____/____/____ por _____

Dados conferidos em ____/____/____ por _____