CERVICAL CANCER PREVENTION AND THE ROLE OF HUMAN PAPILLOMAVIRUS

By

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This thesis is dedicated to my parents, Salvador Raphael de Lucas Lorenzato and Elaine Maria Barbosa Lorenzato, and family as a whole.
Abstract

Cervical cancer is the second most common cancer in women worldwide and the highest incidence is found in Recife, Brazil. This thesis investigates the relationship between various local risk factors in Recife, including the sexually transmitted infection of high-risk human papillomavirus (HR HPV) types, age of first sexual intercourse and first pregnancy, number of sexual partners, the male factor, effect of hormonal status, methods of contraception, smoking habits and education status. Two methods of HR HPV detection were evaluated. These were the Hybrid Capture I assay which was available commercially at the start of the PhD project in 1997 and a technique previously developed in UCL involving polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The association between individual risk factors and cervical lesions presenting with different cytological grades (ASCUS, mild dyskaryosis, moderate dyskaryosis, severe dyskaryosis and cancer) and histological grades (cervicitis, cervical intraepithelial neoplasia grades 1, 2 and 3 and cancer) was also investigated.

Infection with HR HPV was found to be the major risk factor for cervical cancer in Recife as is the case worldwide. Poor socio-economical conditions, poor education and an inadequate cytological screening system also contributed to the high incidence of cervical cancer. The results presented in this thesis show that, relative to cytological screening, HR HPV detection by PCR/RFLP provides greater accuracy in the identification of women with risk to have cervical neoplasia, simpler specimen manipulation, no requirement for subjective interpretative skills and lower unit cost. In addition, the test increases coverage for all eligible women as it can be carried out safely during pregnancy when most women will attend at least one antenatal visit. The potentials and the problems of self-sampling as a means of further increasing coverage of the at risk population were also investigated. A cost-effective protocol is proposed based on HR HPV detection for the early identification of women in Recife with or at increased risk of developing cervical precancer and cancer.
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List of abbreviations

ACIS = Adenocarcinoma in situ
AGCUS = AGUS = Atypical glandular cells of undetermined significance
AIDS = Acquired immunodeficiency syndrome
ALTS = ASCUS-LSIL Triage Study
ASCUS = Atypical squamous cells of undetermined significance
BPV = Bovine papillomavirus
BSCC = British Society for Clinical Cytology
C = Celsius
Ca = Cancer
CAPES = Brazilian Government Research Fund entitled Coordenaçâo de
Aperfeiçoamento de Pessoal de Ensino Superior
CDC = Centre for Disease Control
CIN = Cervical intra-epithelial neoplasia
C-section = Caesarean section
DNA = Deoxyribonucleic acid
EDTA = Ethylenediaminetetraacetic acid
EGD = Endocervical glandular dysplasia
FIGO = International Federation of Gynecology and Obstetrics
FUSAM = Fundaçâo Amaury de Medeiros (The State Health Authority)
G-CSF = Granulocyte colony stimulating factor
GM-CSF = Granulocyte/macrophage colony stimulating factor
GOG = Gynecologic Oncology Group
Gy = Gray (Radiation unit)
HCP = Hospital do Câncer de Pernambuco (The Cancer Hospital in Pernambuco)
HEC-18-I = HPV 18-immortalised ectocervical cell line
HG = High grade
HPV = Human papillomavirus
HR HPV = High-risk human papillomavirus
HRT = Hormone replacement therapy
HSIL = High grade squamous intra-epithelial lesion
HSV = Herpes simplex virus
IARC = International Agency for Research in Cancer
IFN = Interferon
IL = Interleukin
IMIP = Instituto Materno-Infantil de Pernambuco (The Institute of Mother and Child Health in Pernambuco)
INCA/PRO-ONCO = Brazilian National Cancer Institute
LCR = Long control region
LG = Low grade
LLETZ = Large loop excision of the transformation zone
LSIL = Low grade squamous intra-epithelial lesion
MCP-1 = Macrophage chemotactic protein 1
MDC = Mean daily cigarettes smoked
MHC = Major histocompatibility complex
MIP-1 = Macrophage inflammatory protein 1
MW = Minimal wage
NPV = Negative predictive value
OCP = Oral contraceptive pills
OR = Odds ratio
PBS = Phosphate buffered saline
PCR = Polymerase chain reaction
PPV = Positive predictive value
RFLP = Restriction fragment length polymorphism
SCC = Squamous cell carcinoma
SD = Standard deviation
SIL = Squamous intra-epithelial lesion
SUI = Stress urinary incontinence
TAE = Tris acetate EDTA
Taq = Thermus aquaticus
TBE = Tris borate EDTA
TCA = Trichloroacetic acid
TNF-α = Tumour necrosis factor-α
UVJ = Urethrovesical junction
VLPs = Virus-like particles
WHO = World Health Organization
Yr = Yrs = Years
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CERVICAL CANCER PREVENTION AND THE ROLE OF HUMAN PAPILLOMAVIRUS

“If the cellular abnormalities of cancer can be detected in biopsy sections, they can be detected just as well in smears from the lesions. What, in fact, is a smear such as we employ and describe below, if not a very limited type of biopsy?” “However, we do not at this time wish to claim that this method is perfect and capable of replacing the biopsy. It is, as all new methods, subject to criticism and liable to improvement.” “Considering that this procedure, applied to the diagnosis of cancer of the uterine cervix, is still in its beginnings, we must wait for future investigations to assign to it its precise role concerning the diagnosis of cancer of the uterine cervix.”

Aurel A. Babès, M.D.
(From the Department of Pathology, Faculty of Medicine of Bucharest, Romenia).

“The high percentage of false positives reported by Dr. Jones and her co-workers indicates that we have to learn more about the reliability and the pathognomonic value of some of our criteria. For this reason we strongly feel that this diagnostic procedure should be employed at present with great discretion, and that it should be entrusted to experienced pathologists.” “Smear reports should be conservatively worded ... No case should be reported as definitely positive unless the evidence is overwhelming.” “It has been noted that the rate of exfoliation in various types of carcinomas differs greatly ... If findings are negative, it is always advisable to repeat the smears.” “Although the preparation and staining of smears are relatively easy, their interpretation is rather difficult.”

George Nicholas Papanicolaou, M.D., Ph.D.

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1 Extracted from: Diagnostic du cancer du col utérin par les frottis. (1928) La Presse Médicale, Wednesday, the 11th of April; 29: 451-454.
“It has been admitted, however, that cytology was an arduous and time-consuming method of microscopic search for specifically malignant cells sometimes obscured by cellular desquamata, leucocytes, and blood.”

James Ernest Ayre, M.D.²

(From the Gyne-cytology Laboratory, Department of Gynecology and Obstetrics, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada. Later, from the Cancer Cytology Center, Dade County Cancer Institute, Miami, Florida, USA).

“The truth about the cervical smear must be recognised and told: it is one of the most difficult, if not the most difficult, laboratory test ever devised.” But it is also “… the most effective cancer prevention test known today.”

Leopold George Koss, M.D.³

(From the Department of Pathology, Montefiore Medical Center, Albert Einstein College of Medicine, New York, USA).


I. INTRODUCTION

Although the usefulness of cervical cytology as a mass screening tool has been under investigation for nearly three-quarters of a century, cervical cancer is still the major cause of cancer death in women in developing countries and a major public health problem worldwide. Over the years considerable improvements in the technique of cytological examination and in screening policies have been made but cytology is a subjective evaluation of what cells look like and human error is difficult to optimise. It has been some time now that researchers thought of a link between cancers and viruses, and the development of molecular techniques has facilitated the understanding of cancers and other chronic diseases.

The transformation zone is the area in the cervix and vaginal vault enclosed by the original squamous-columnar junction where transformation between columnar and squamous epithelium dynamically occurs (Singer & Monaghan, 2000). Arising from immature cells of the cervical transformation zone, embryonically originated from the Mullerian duct, which in turn is derived from the coelomic epithelium, cervical cancer is an abnormal tissue growth that invades the basement membrane of the cervical epithelium as a consequence of uncontrolled cell division. Neoplasia is the growth of cells that exceeds and is uncoordinated with the growth of normal tissue (Cotran et al., 1989). Cervical cancer is one of few malignant neoplasias that offer the possibility of being detected before they become invasive cancers. Direct access to cells where the malignant transformation occurs, both by scrapes of exfoliative cells (cytology, molecular biology tests) and by direct amplified visualisation (colposcopy, cervicography), makes this a unique disease, one that is theoretically 100% preventable. However, nowadays about half a million new cases of invasive cervical cancer are diagnosed every year worldwide. Most of these cases occur in developing countries and are detected in advanced stages. The incidence of invasive cervical cancer seems to have been increasing in women at younger ages during the last five decades.

Although a 10% false negative rate is considered to be acceptable for a screening test, for each woman who went in good faith for cervical cancer screening and ended up with invasive cervical cancer later on, the system failure is 100% and simply not acceptable. The losses are considerable – the screening programmes lose credibility, the patient's family loses temporarily or permanently its income (not to mention that more and more of
cervical cancers are affecting women at highly economically productive phases of life), and the costly cancer treatment and complications also poses a burden to health resources and patient's families.

This thesis attempts to add information on how modern laboratory techniques can be applied to clinical practice in the area of cervical cancer prevention. By bridging the gaps between clinical and basic sciences, the possibility of tackling problems more efficiently increases, thus increasing the chance of improvement in people's quality of life.

1. A historical problem
Evidence of neoplasms in ancient times is difficult to gather because fossilised skeletons are rarely discovered with soft tissue and the diagnosis is hardly ever unquestionable. Similar rationale applies to mummies of the early Egyptian dynasties. Just for illustration, a scientific paper reports an osteoma in a Cretaceous dinosaur bone fragment found in Garfield County, Montana (Sawyer & Erickson, 1985). A very good review of the historical aspects of cervical cancer is found in Shingleton & Orr Jr (1987). Some of the first reports about cervical cancer were one of Hippocrates (approximately 450 BC), who wrote that this disease had a poor prognosis, and one of the Hindus, who mastered surgical techniques in ancient times (5th century BC). According to the works of Galen and Celsus, cervical specula seem to have been used as early as the 1st century BC (Mann, 1887), giving an indication that the value of direct visualisation of the cervix for detection of abnormalities has been acknowledged since then. The factual evidence of this historical enlightenment is in the discovery of 2 specula found in Pompeii and Herculaneum, two neighbouring cities in the North of Italy, Province of Naples, that were devastated by the eruption of the Vesuvius volcano in 79 AD. Galen also described in his work entitled De Morbis Mulierum that cancer of the uterus had a poor prognosis (Mettler 1947). In the 6th century AD, Aetius of Amida, a physician from Alexandria wrote a chapter called "De cancris uteri" in his collection of four books entitled Medici Graeci Tetrabiblos (Jaméson, 1936). One of the first experts in diagnosis and treatment of cervical cancer described in Papanicolaou and Traut's book (1943) was Paul of Aegina about 600 A.D. Yet in the early 15th century, the Medieval Woman's Guide to Health reported that cervical cancer and vaginal discharges come from old cervical lesions that have not healed well (Rowland, 1981).
In 1673, Anthony van Leeuwenhoek (1632-1723), a Dutch linen merchant in Delft, created a primitive but effective instrument capable of magnifying images from specimens, the microscope, which first enabled him to count the threads in fabric for business purposes and later to observe and describe bacteria and protozoa. However, the scientific community at that time did not realise immediately the potential benefits of that advance in technology for our understanding of disease processes and their resulting implications for treatment of several diseases. It was not until nearly 200 years later that Louis Pasteur discovered the association of certain microbes with specific diseases (Keen & Jarrett, 1976).

About a century and a half after the invention of the microscope, Alfred Donne while in Paris studied the cellular components of fluids and tissues from different parts of the body such as blood, mucous, pus and vaginal secretions. He identified Trichomonas vaginalis in vaginal fluids, gave the first description of leukaemia and of the contents of colostrum (Donne, 1831, 1836, 1845).

At the beginning of the 19th century, cervical conisation was described and at that time it was indicated for the treatment of cervical cancer and infections (Lisfranc, 1815). In 1833, Boivin and Duges reported the amputation of the cervix as a modality of treatment for cervical chronic ulcerations (Boivin & Duges, 1833). In the 1840's, the actual cautery, as practised by the French school, was thought to be the only treatment, even though palliative, for cervical cancer. Before then, the teachings were that cervical cancer was always necessarily fatal. Chassaignac introduced the écraseur into surgery for the treatment of cervical cancer when the tumour was sufficiently pedunculated to be surrounded by the chain or wire loop, but not uncommonly the use of the écraseur would lead to iatrogenic lesions of neighbouring tissues. In 1860, J. Marion Sims treated a patient with cervical cancer using the écraseur; the patient had an accidental perforation of the peritoneal cavity, recovered from the surgery and died 8 to 10 months later of the cancer. After the écraseur, the electro-cautery was introduced by Middledorpff of Breslau and resulted in a relative improvement of disease-free survival. In the autumn of 1868, Noeggerath of New York successfully removed the cervix of a patient with cervical cancer using electro-cautery and the patient had a good recovery and became disease-free for 2 or 3 years, when she had recurrence and died. Later, J. Marion Sims added a blade to the écraseur adapting it to performing cervical amputation in the same way tonsils were
guillotined. The écraseur was an instrument with a snare device to place around the tumour, whereby tightening a screw, the snare cut through and amputated the snared part of the cervix. Sims then popularised the surgical cervical amputation by dissection and used cotton swabs soaked with Iron Sulphate and Zinc Chloride for haemostasis. Concomitant to the work of Sims, Reamy of Cincinnati used scissors to excise a conical plug from the cervix in one solid piece, and he operated on patients in the early stages of the disease using the Paquelin thermo-cautère (Sims, 1879).

On the 13th of April 1812, two surgeons from Milan, G. B. Paletta assisted by D. B. Monteggia, apparently attempting to amputate a cervix due to malignant involvement, ended up managing to remove the whole uterus through the vaginal approach, most likely performing the first hysterectomy ever, but the patient developed infection and succumbed. However, Koeberle of Strasbourg is the one usually credited as the first to have accomplished deliberately a successful hysterectomy, before Charles Clay who eventually made it in 1863 (O’Dowd & Philipp, 1994).

The Berlin physiologist, Johannes Muller, considered the father of clinical cytology and mentor of scientists like Virchow and Schwann among many others, mentioned the cellular structures of malignant tumours, described the fundamental microscopic differentiation between benign and malignant tissues, and was the first to prove that all malignant tumours were closely related physiologically (Muller, 1840). In 1843, Julius Vogel from Gottingen was the first to diagnose by exfoliative cytology a fistulated malignant tumour close to the mandibular angle and a benign ulcerative breast lesion (Vogel, 1843). In this same year, Gluge studied scrapings from several tissues including cancer and reported that cancer cells are double the ordinary size or more, and they not infrequently contain several nuclei (Gluge, 1843). In 1847, F. A. Pouchet while working in Paris on the theory of spontaneous ovulation and fecundation in humans, laid the early foundations of hormonal cytology after observing the characteristics of cells from vaginal secretions throughout the various phases of the menstrual cycle (Pouchet, 1847). In 1856, Lambl diagnosed a case of stage IV carcinoma of the cervix with bladder invasion by cytological examination of the urine (Lambl, 1856). In 1871, C. Friedlander, also applying the cytological method to study cervical cancer described that in women with carcinoma of the cervix cellular elements originating from the carcinomatous ulceration will be found suspended in the vaginal liquid. He also claimed that in cases of doubt the
excision of a small piece of tissue has to be performed for histological confirmation of the diagnosis (Friedlander, 1886).

R. Hooper in 1832 published the association between what John Clarke called in 1812 "the cauliflower excrescence of the cervix" and cervical cancer. In 1861, when it was generally accepted by the then expert physicians that cancer was originated somehow in relation to stress, F. von Scanzoni observed that city dwellers had an increased incidence of cervical cancer, thus suggesting a link between cervical cancer and sexual activity (Shingleton & Orr, 1987). In 1872, T. Gaillard Thomas was smart enough to take advantage of the advent of the light-microscopic examination of tissues and came out with the publication of a precise classification of cervical cancer (Ricci, 1945). As usual, when a new technology is developed, people not familiar with it tend not to appreciate its importance at first. This was the case during the implementation of light microscopy to the early diagnosis of cervical cancer; some physicians felt the technique was unreliable. In 1863, a Berlin medicine graduate (1843) and eminent professor, Rudolf Ludwig Karl Virchow (1821-1902), published 3 oncological volumes entitled *Malignant Neoplasms* where he gave the first histological and anatomical classification of benign and malignant neoplasms (Wullstein & Hellmer, 1970). Thus, a hallmark in the history of cancer diagnosis was achieved with the cellular definition introduced by Virchow who stated that cancer equals invasion of surrounding tissues.

Following the booming pattern of discoveries of the late 19th century some breakthroughs in the lines of cancer treatment were also occurring in other fields of knowledge such as Physics. In the early 1890's, the German scientist Wilhelm Conrad von Roentgen (1845-1923) while doing experiments with Sir William Crookes' cathode-ray tube observed that when electric current (electrons) was passed through the tube and struck its glass wall, there was production of an invisible light (irradiation) that made wood and other materials transparent, and metal and bones opaque on ordinary photographic plates. Roentgen also observed that this invisible light would make a piece of barium platinocyanide to fluoresce and proposed that a chemical reaction caused this phenomenon to happen. Since he did not know what that light was he called it X-ray and 6 years later he won the first Nobel Prize (Roentgen, 1895). About 5 to 10 years later X-rays were being used as treatment for cancer and gynaecological pruritis (Freund, 1904).
Antonie Henri Becquerel (1852-1908) based on Roentgen theory thought that stimulating crystals with ultraviolet light could produce X-rays. He therefore discovered the emission of radioactive substances similar to X-rays while doing some studies with uranium salts. Becquerel announced his discoveries to Pierre Curie (1859-1906) and Marie Curie (1867-1934) who observed that the radioactivity of pitchblende was 4 or 5 times greater than that of the uranium it contained, and they ended up isolating the radioactive metallic element radium (O'Dowd & Philipp, 1994).

In the first decade of the 20th century, cervical cancer was known as the *Morbus miseriae* for the association of this morbid entity with poverty, and the mortality associated with it accounted for about 20% of all cancers affecting women (Bonney, 1909).

In order to understand the endocrine physiologic changes in vaginal cells, an important study was performed in rodents (Stockard & Papanicolaou, 1917). As far back as 1923, Papanicolaou first began a systematic study of human vaginal smears at the Woman's Hospital of New York City (Papanicolaou, 1946). Since the spring of 1925, the study of cells taken from vaginal aspirates of women had been undertaken first in the clinic of Cornell Medical College and then in the Woman's Hospital in New York City. In the 3rd Race Betterment Conference at Battle Creek, Michigan (USA), Papanicolaou reported the results of the rodent study emphasising that he had discovered tumour cells of cervical cancer in vaginal aspirates (Papanicolaou, 1928). But at that time his paper appeared to have had little impact.

By 1927, the Romanian lecturer in pathology at the Faculty of Medicine of Bucharest, Aurel A. Babès (1886-1962), had been carrying out studies along with Professor C. Daniel of the Gynecologic Clinic of Bucharest on the cytological examination of cervical smears. He described that the material should be obtained with a platinum loop, with which several smears were made from suspicious lesions by rubbing it slightly at first and then more vigorously. Before taking the smear the person should carefully and lightly wipe the surface of the cervix with a pad of dry gauze. No cleansing of the vagina or use of antiseptic should be applied to the lesion prior to collecting the smears. The smears were allowed to dry in air before being sent to the laboratory where the cells were fixed with methyl alcohol and stained with the Giemsa method. This technique, the Romanian method, and its expectations were reported in Bucharest at the 23rd of January 1927
Conference of the Bucharest Gynaecological Society (Daniel & Babès, 1927a). Soon, the first results of what became known as the Babès Method was published in the scientific journal called Obstetrica si Ginecologica (Daniel & Babès, 1927b). Also, in 1928 Babès published in the French language a paper describing the use of exfoliative cytology for the detection of cervical cancer in Romania, stating that the cytological diagnosis of cervical cancer and what he called incipient lesions could be reliably made in 90% of cases studied (Babès, 1928). O. Viana, an Italian gynaecologist and obstetrician who published extensively on women’s health and had an special interest on gynaecological oncology, in November 1928 also published, in an Italian scientific journal from Rome, a work about the early diagnosis of cervical cancer by cytological smears (Viana, 1928), simultaneous to Papanicolaou’s monographs presented on the Third Batterment Conference.

In the United States of America, despite the methods for treatment, during the late 30’s the mortality rate from cervical cancer had only a scant decrease (Papanicolaou & Traut, 1943). This unsatisfactory reality was pivotal for the implementation of cervical cancer cytological screening at the Women’s Clinic of the New York Hospital, Cornell Medical College Association in the early 40’s.

Up to the mid 40’s, most cervical cytologic smears were made from vaginal aspirates. In 1946, Ayre published a study about the diagnostic accuracy for cervical neoplasia of smears prepared from vaginal aspirates compared with smears prepared from cervical scrapes, and found that smears from the cervix provided more accurate results yet costing $ 5.00 or less (Ayre, 1946). He then designed a wooden spatula specially shaped to collect cells from the cervical squamous columnar junction. After collection, the samples were spread onto microscope slides, fixed with 95% ethyl alcohol and stained with haematoxylin and eosin or the Papanicolaou staining method (Ayre, 1947). This forms the basis of the currently accepted cervical cancer screening test.

To date, more than half a century on, the false negative cytology rate is still a major problem in cervical screening programmes. Some authors report the false negative cytology rate in developed countries to vary from 13 to 36% (Wheelock & Kaminsky, 1989 and Kristensen et al., 1991). Others have reported false negative cytology rates showing even wider variation such as 20 to 70% (Fetherston, 1983; Bearman et al., 1987;
Giles et al., 1988; Tawa et al., 1988; Koss 1989a, 1989b, 1993; Szarewski et al., 1991; Reid et al., 1991; Cuzick et al., 1995). More worrying is that cytology false negative results often occur in young women (Rylander, 1976; Yule, 1978; Berkowitz et al., 1979; Clarke & Anderson, 1979; Adcock et al., 1982; Hall & Monaghan, 1983; Walker et al., 1983; Fetherston, 1983; Bain & Crocker, 1983; Elisman & Chamberlain, 1984; Paterson et al., 1984; Bearman et al., 1987; Robertson & Woodend, 1993). Furthermore, there is the problem of true false negative cytology (Martin, 1972; Rylander, 1976, 1977) that even after examination by expert pathologists the subjective interpretation of the slides is negative and the patients indeed have cervical neoplasia. Therefore, the problem cannot be solved only with improvement in cytology training and quality control. A recent evaluation of the Mexican national programme for early cervical cancer detection reports false negative cytology rates between 10 and 54% (Lazcano Ponce et al., 1998). A study assessing the quality of this programme recommends improvements in the sampling procedures and wider screening coverage. It reports that 60.1% of smears taken did not include endocervical cells and only 15.6% of women 25 years or older were having the Pap test for the first time (Salinas Martinez et al., 1997). Some authors claim that, due to the incomplete understanding of the natural history of cervical cancer, it is possible that cytological screening with its failures and difficulties may do more harm than good (Skrabanek, 1988; McCormick, 1989), others question how protected are screened women from developing invasive cervical cancer (Anonymous 1973; Rylander 1976; Berkeley et al., 1980). False assurance of safety may result in decreased credibility in cervical cancer screening programmes and liability to law suits. A negative cytology does not necessarily mean that the woman has a normal cervix and this misleads the understanding of the natural history of cervical neoplasia, which in turn leads to serious implications in gynaecological management and screening intervals. Rylander (1976) studying 177 women with invasive cervical cancer from the cytologically screened population of Stockholm observed that 48% had a false negative cytology, among which 35% had had a second false assurance that there was nothing wrong in their cervix. Forty-five percent of the population studied had had at least one negative Pap smear within 4.5 years prior to the detection of the malignancy, among these 82.8% had had a negative smear within the last 3 years (Rylander, 1976).

The natural history of cervical neoplasia continues to be studied. The idea of a continuous gradient of lesions from mild cervical intra-epithelial neoplasia (CIN) to invasive cervical
cancer has generated a lot of discussion, but now it has been accepted that cervical cancer and its precursors are indeed a continuum of histological lesions. The lowest histological grade of squamous intra-epithelial neoplasia is CIN 1 or mild dysplasia where abnormal cells with altered nuclear/cytoplasmic ratio are found throughout the epithelium and basaloid cells are present in up to the lower third of the cervical epithelium. In CIN 2 or moderate dysplasia, abnormal cellular proliferation occurs throughout the epithelium and basaloid cells are found in the middle third of the cervical epithelium. CIN 3 or severe dysplasia or carcinoma in situ is characterised by extension of the basaloid cell population throughout the whole thickness of the cervical epithelium without invasion of the basement membrane. Further discussion on the continuum model will be found under the heading of histology. In 1932, the histological pattern of carcinoma in situ was defined as a precursor lesion of cervical cancer (Broders, 1932). It was only in the 50's that moderate dysplasia was identified as a precursor of carcinoma in situ of the cervix (Fu et al., 1981).

2. Incidence and mortality
In Brazil and presumably in many other developing countries it is difficult to obtain accurate figures on incidence and prevalence of pre-malignant cervical lesions and invasive cervical cancer, as well as on mortality from cervical carcinoma. One of the reasons is that the database for cervical cancer precursors is still under construction in some cities and the mortality registry is made at a central department of the Ministry of Health in capital cities. The death certificates arrive irregularly to the Health State Authority where the data is gathered and entered into a computer database. For instance the mortality figures for the year 2000 are about to be published but there are still death certificates arriving from 1995 or any other year, and these will not be included in any statistics at all. It is too late to include them in previously closed statistics and they cannot be included in the current year.

The age-related incidence of cervical carcinoma increases steadily from an age as early as 16 years to reach a peak at 40-50 years and thereafter it remains level or declines slowly. It is important to notice that the incidence of invasive cervical cancer has been increasing in younger women, particularly those aged 20 to 29 years, in East Germany, Norway, some places in the United States and Israel (Munoz & Bosch, 1989). The fact of the increased incidence of cervical cancer in younger women is not only true for squamous
cell carcinoma, the same trend is being observed for adenocarcinomas of the cervix in the United States and Western countries as a whole (Peters et al., 1986; Schwartz & Weiss, 1986; Angel et al., 1992; Piura et al., 1996; Zreik et al., 1996). Also, it has been observed that the mortality trend from cervical cancer has been similarly increasing in younger women in the United Kingdom and Australia (Cook & Draper, 1984; Holman & Armstrong, 1987). After implementation of routine cervical cancer screening programmes in some countries with a coverage over 80%, there was a marked decrease in the incidence of cervical cancer. However, cervical cancer is still a major public health problem in developing countries – the main cause of cancer death in women. Its incidence among cancers affecting women is second only to breast cancer worldwide (Figure I).

According to an estimation of the Brazilian National Cancer Institute for 1998 based on the incidence of cancers diagnosed between 1980 and 1995 and from population census data from 1980 to 1991, the most incident cancers are as depicted in Figure II. However these numbers may be underestimated. Women are being affected by cancer more than men. Breast and cervical cancer incidences follow a pattern similar to the ones of developed countries where cervical cancer is only second to breast cancer.

About 500,000 new cases of invasive cervical cancers are diagnosed each year, 90% of them occur in developing countries where the disease is usually detected in advanced stages. In a study we have recently carried out in Recife (Lorenzato et al., 2000) it was observed that 76% of cervical cancers detected were stage II or above (Figure III).

The International Agency for Research in Cancer (IARC) publication No. 102 reports the highest incidence of cervical cancer worldwide in the city of Recife, located in Northeastern Brazil (83.2 per 100,000 women) (Whelan et al., 1990). The incidences of cervical cancer in Cali (Colombia) and Costa Rica followed the one in Recife (Figure IV).

Also based on IARC publications 102 and 143, the incidence of invasive cervical cancer in Brazilian cities where the data have been reported, the numbers are particularly high (Figure V) and 3 of them are among the 4 highest incidences of cervical cancer worldwide. These evidences pose a major challenge for testing cost-effectiveness of screening protocols and of new treatment protocols aiming to increase operability, the best treatment for malignant tumours, and improve survival and patient’s quality of life.
Figure I. Four most incident cancers worldwide (Adapted with permission from WHO/IARC publication no. 143, 1997).
Figure II. Four most incident cancers in Brazil (Adapted with permission from the Boletim do Ministério da Saúde, Brazilian National Cancer Institute - INCA/PRO-ONCO, 1998).
Figure III. Distribution of clinical stages of cervical cancers detected in a recent study carried out in Recife (Brazil), which details are described in the result section A.
Figure IV. Cervical cancer incidence in selected places worldwide (Adapted with permission from WHO/IARC publication no. 102, 1990).
Figure V. Cervical cancer incidence in selected cities in Brazil (Adapted with permission from WHO/IARC publication no. 102, 1990, and publication no. 143, 1997).
More recently, in 1997, the IARC publication No. 143 reports the new data on incidence of cervical cancer in the five continents (Parkin et al., 1997). As shown in Figure VI, the incidence in Cali (Colombia) and in Costa Rica have decreased, and the incidence in Belém (Northern Brazil), Trujillo (Peru), and São Paulo (Southeastern Brazil) have been reported as among the highest. The updated figures for Recife and Sao Paulo (Brazil) have not been reported in IARC publication No. 143, but for the purpose of comparison, the previously reported ones are depicted in Figure VI.

According to the figures above, the lowest incidences of cervical cancer are achieved in Israel (Non-Jews) and Finland. In the Nordic countries, national cancer registries have been effectively working for more than 30 years. In Finland, Iceland and Sweden nationwide organised population-based screening programmes were implemented in the 60’s to early 70’s. The screening intervals and age groups for the target population were as follows: every 2 to 3 years for women aged 25-69 years in Iceland, every 4 years for women aged 30-49 years in Sweden and every 5 years for women aged 30-55 years in Finland. In Denmark, these figures varied from county to county. In Norway, the organised screening programme had been implemented in only one county until recently which led to results of less pronounced reduction in the incidence of cervical cancer compared to the other Nordic countries (Pettersson, 1998; Hakama, 1992). The screening programme in Finland has resulted in the most pronounced reduction in the incidence and mortality from cervical cancer in the Nordic countries (Hakama et al., 1991). A recently published paper (Anttila et al., 1999) reports that since 1963, when a nation-wide organised screening programme was implemented, in Finland there has been a decrease of 80% both in the age-adjusted incidence of and mortality from cervical cancer. However, a striking increase of about 60% in the incidence of cervical cancer among women below 55 years of age during the last 4 years of the study period has been observed – up to 1995. The mortality rates though are still decreasing. It is important to notice that there has been a marked age shift in the incidence of both squamous cell carcinomas (SCC) and adenocarcinomas of the cervix.

A study analysing data from East Anglia, England and Wales showed that, although the overall incidence of cervical cancer is declining, mostly based on SCC surveillance, the incidence of cervical adenocarcinoma and SCC of the cervix showed an increase mainly in younger women aged 30-39 years. In East Anglia, the overall incidence of cervical
<table>
<thead>
<tr>
<th>Place</th>
<th>New cases per 100,000 per year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Israel (Non-Jews)</td>
<td>1.9</td>
</tr>
<tr>
<td>Finland</td>
<td>5.8</td>
</tr>
<tr>
<td>Canada</td>
<td>10.0</td>
</tr>
<tr>
<td>Porto Rico, EUA</td>
<td>11.2</td>
</tr>
<tr>
<td>Los Angeles/USA (Black)</td>
<td>13.2</td>
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<tr>
<td>Connecticut/USA (Black)</td>
<td>13.2</td>
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<tr>
<td>Scotland/UK</td>
<td>16.6</td>
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<td>England &amp; Wales/UK</td>
<td>16.7</td>
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<tr>
<td>Norway</td>
<td>16.8</td>
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<tr>
<td>Costa Rica</td>
<td>18.5</td>
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<tr>
<td>Denmark</td>
<td>21.2</td>
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<tr>
<td>Cali/Colombia</td>
<td>27.1</td>
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<tr>
<td>Poland</td>
<td>28.1</td>
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<tr>
<td>Concórdia, Argentina</td>
<td>30.4</td>
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<tr>
<td>São Paulo/Brazil</td>
<td>35.1</td>
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<tr>
<td>Trujillo, Peru</td>
<td>40.1</td>
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<tr>
<td>Belém/Brazil</td>
<td>44.4</td>
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<tr>
<td>Recife/Brazil</td>
<td>83.2</td>
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</tbody>
</table>

Figure VI. Cervical cancer incidence in selected places worldwide (Adapted with permission from WHO/IARC publications nos. 102, 1990, and 143, 1997).
adenocarcinoma increased more than 4-fold between 1971 and 1994 \( (P < 0.001) \). The increases in the incidence of adenocarcinoma in women aged 30 to 39 years were 2.5-fold and 3.5-fold for East Anglia and England/Wales, respectively (Stockton et al., 1997).

In Sweden, an age-cohort model indicated a stable 70-75% decrease in the incidence of cervical cancer for women born in 1940 and later compared with those born around 1923. An interesting observation was that, despite the well established reduction in the incidence of squamous cell carcinoma of the cervix with the combination of organised and opportunistic screening, the incidence of adenocarcinomas of the cervix has doubled (Bergstrom et al., 1999).

Another study, reporting the results of a national screening programme in the USA, emphasises the importance of reaching both younger and older women for cervical cancer screening. Among 312,858 women aged 18 years or older, those 40 years or older being more than half of the population studied, 3.8% had abnormal smears. The rate of CIN was highest among young women (going from 8.2% in 30 year-olds or younger to 1.0% in 65 year-olds or older). Women younger than 30 years had the highest rate of CIN 2 or worse lesions (19 per 1000 smears), and women 40 years or older had rates less than one-third that of the younger ones. The positive predictive value of first cytological screening cycle for high grade intra-epithelial neoplasia and cervical cancer were respectively 56.0% and 3.7% (Lawson et al., 1998).

3. The epidemic hypothesis

As described in the Oxford dictionary, epidemic is the occurrence of a large number of cases of a particular disease at the same time in a particular community. In spite of the evidence that cervical cancer is a preventable disease and that organised screening programmes can decrease its incidence and mortality, the cancer registry from the state of Pernambuco, of which Recife is the capital city, has been receiving increasing numbers of death certificates having cervical cancer as the basic cause of death since 1986 (Figure VII).

This data shows a practical example of a population without a well organised mass cervical cancer screening programme and may serve for the purpose of illustration and comparison to the so called "epidemic" hypothesis. A schematic illustration of what
Figure VII. Cervical cancer mortality in the state of Pernambuco. Figures may be underestimated (Adapted with permission from the State Health Authority, Brazilian Ministry of Health, 1998).
should happen to cervical cancer mortality rates over time in places with and without an epidemic situation in relation to a well-organised screening programme is shown in Figure VIII.

The curve of total annual mortality from cervical cancer in the United States in women aged 25 years and over between 1955 and 1975 (Ravenholt, 1989) seems to follow the pattern of the second line depicted on Figure VIII, most likely corresponding to what would occur as an example of a well organised screening programme implemented on a hypothetically epidemic situation. If this hypothesis is proven right in future work, it could at least partially explain some anxieties associated with what is expected from a well organised screening programme and the reality of what actually is achieved. Also, it would provide support for more effort to be invested in the development of a vaccine and the need for more clinical trials aiming to eradicate the spread of these sexually transmitted oncogenic viruses.

A study aimed at estimating the incidence of genital HPV infection in the general population in Rochester, Minnesota (USA), has shown that the annual age- and gender-adjusted incidence of genital warts increased from 13 to 106 per 100,000 between the 1950 to 1978 (Chuang et al., 1984). It has also been described in a chapter about the epidemiology of sexually transmitted diseases that there has been a dramatic increase in the incidence of sexually transmitted diseases after the 1950’s both in the USA and Europe (Aral & Holmes, 1995), particularly during the early days of sexual liberation, the introduction of oral contraceptives and when smoking became more common among women. Ravenholt (1989) claimed that the use of oral contraceptives in the United States of America since the 60’s has triggered the sexual revolution and with it there were epidemic increases in herpes simplex virus infection and other sexually transmitted diseases. He also reported a trend of rapidly increasing incidence of CIN 3 and cervical cancer among women aged 15 to 35 years in Connecticut (USA) from 1955 to 1975 (Ravenholt, 1989). The United States Centre for Disease Control (CDC) has also reported a considerable increase in the prevalence of HPV infection based on diagnosis of warty lesions in the US since the mid- 1960’s (CDC, 1983). Another study reported a 4.5-fold increase in the number of first visits for condyloma between 1966 and 1984 (Becker et al, 1987). From 1971 to 1982, there was an increase in the incidence of condylomas in the United Kingdom of 2.5 times.
Figure VIII. Schematic representation of cervical cancer mortality trends to illustrate the "epidemic" hypothesis.
Based on available data documenting a considerable increase in the incidence of genital warts, which are associated with low-risk HPV (types 6 and 11), and on the fact that in cells from cervical scrapes the prevalence of high-risk HPV types is higher than the low risk types, it is possible that there has also been a similar increase in high-risk HPV infection that has not been well investigated and/or documented yet. It has been estimated that in the 1980’s at least one third of all sexually active women were infected with HPV (Koutsky et al., 1988). It has also been estimated that at least 50% of sexually active adults had had a genital HPV infection (IARC, 1995). High-risk HPV types are not only associated with cervical neoplasias, including invasive cervical cancer, but also they contribute substantially to the worldwide occurrence of cancer of the upper aerodigestive tract, penis, vulva and anus (IARC, 1995).

Despite the efforts and investment so far on screening protocols based solely upon cytology, there is an urgent need to direct the strategies towards an effective and affordable combination of technologies to reduce the figures of about half a million new cases of invasive cervical cancer and, as a consequence, over 200,000 deaths a year that occur worldwide. Further research on the role of specific HPV types in relation to cervical cancer screening and vaccination policies should be undertaken.

4. Risk factors
Based on several studies investigating the epidemiology and natural history of cervical neoplasia, it is now understood that cervical cancer result from the combination of more than one risk factor.

4A. Age
Invasive cervical cancer, fortunately, occurs in very few young women under the age of 20 years, from the age of 20 to 50 or 60 years there is a sharp increase in the incidence (Muir et al., 1987). The fact that the incidence of cervical cancer stops rising or reaches a plateau after the menopause has been suggested to be due to the fall in female sex hormone production secondary to ovarian failure (Armstrong, 1982) or a consequence of decreased sexual activity.
4B. Race
It appears in general that race as a specific risk factor is usually confounded with the effect of socioeconomic status, where the women with lower income would be more prone to have lower level of health education and information regarding their rights or Government policies to prevent cancer.

4C. Socioeconomic status
Low socioeconomic status and low level of education, as just mentioned, are independent risk factors per se. Limited access for illegal immigrants to become included in the cervical cancer screening programme is another issue. The result being that the highest incidences of cervical cancer in developed countries are found in minority groups of the populations such as indigenous or migrants.

4D. Sexual behaviour
An important study reporting statistical analysis of death rates from cancer between 1760 and 1839 was carried out in the early 1840’s in Verona by an Italian physician who, while evaluating the variables related to cervical cancer, noted there was practically an absence of cervical cancer in Italian nuns and virgins and that it was quite common among married women and widows (Rigoni-Stern, 1842). The former Professor of George Washington University, Albert Freeman Africanus King (1841-1914) believed that spermatozoa was associated with the aetiology of cancer (Daniels, 1950).

In Israel, where the lowest worldwide incidence of cervical cancer is registered, both Jews and non-Jews women have very low incidence, most likely reflecting similar conservative sexual practices (Armstrong et al., 1992). The importance of sexual behaviour as a risk factor for cervical neoplasia is indirectly evident for it has been shown a strong correlation between increased number of lifetime sexual partners and higher prevalence of HPV infection (Schiffman, 1992b; Moscicki et al., 1990; Fisher et al., 1991). It has also been observed that single women and those with history of sexually transmitted diseases are more likely to have HPV infection and cervical specimen from virgins are unlikely to be HPV positive (Ley et al., 1991).

Other epidemiological variables, in fact really important ones, such as early age at first sexual intercourse, multiple male sexual partners or one high-risk male partner who have
many partners (Prindan & Lillienfeld, 1971; Singer, 1973; Singer et al, 1976; Buckley et al., 1981), and high number of pregnancies and vaginal deliveries have been reported to be associated with increased risk for development of cervical neoplasia including cancer (Munoz et al., 1992). The persistent association of multiparity with cervical neoplasia has been clearly demonstrated in well controlled studies for the confounding effects of other also important sexual, socioeconomical and educational variables (Brinton et al., 1989; Cuzick et al., 1989; Parazzini et al., 1989; Eluf-Neto et al., 1994). Women with poor genital hygiene themselves and those whose husbands had poor penile hygiene and low educational status have also been described as being at a higher risk for developing cervical cancer (Zhang et al., 1989; Brinton et al., 1989). An association between cancer of the penis and cancer of the cervix has been observed (Graham et al., 1980; Li et al., 1982; Smith et al, 1980; Vines & Ascunce, 1986). Wives of men with cancer of the penis had a 3-6 fold increased risk of developing cervical cancer compared to other women and wives of men whose previous wives had cervical cancer had a 2-fold increased risk of developing cervical cancer (Kessler, 1977).

4E. Sexually transmitted diseases

The strong association of cervical cancer and sexual activity soon raised the hypothesis of a sexually transmitted infection being related to it, later realised to be the human papillomavirus infection. Other sexually transmitted infectious agents such as herpes simplex virus type 2 (HSV-2), Chlamydia trachomatis, Trichomonas vaginalis, Candida Albicans, and gonorrhoea have been investigated as to a possible specific role in the oncogenic process of cervical neoplasia. It seems though that some of the association found with these other sexually transmitted diseases, when not a consequence of not very specific antibodies or lack of adjustment for number of sexual partners, may simply be related to the effect of chronic inflammation in the cervix. This may open the way for HPV to reach the immature basal and parabasal cells in the transformation zone, as an alternative to the mechanical effects of effacement, dilatation and laceration that may occur in the cervix during partition. Nevertheless, the transforming sequence Bgl II N fragment of HSV-2 was detected using nested PCR on 46 paraffin-embedded samples from patients with premalignant or malignant cervical lesions in 20 to 25% of the cases and in none of the samples from normal healthy controls tested (Lulitanond et al., 1994). Women with acquired immunodeficiency syndrome (AIDS) and the ones with immunesupression in general have increased risk for persistent high-risk HPV infection.
and development of cervical cancer compared to immune competent women (Maiman et al., 1993).

4F. Oral contraceptives

An increased risk of cervical cancer has also been reported in women using oral contraceptive pills (OCP) (Hildesheim et al., 1990; Kjaer et al., 1990; Munoz & Bosch, 1992). Long-term use of oral contraceptives and mutagenic metabolites, which are associated with ectropion formation, chronic cervical infections and metaplastic transformations have also been suggested as co-factors for the development of cervical cancer (Brinton et al., 1986, 1990). However, the majority of studies on oral contraceptives were not adjusted for sexual variables and frequency of Pap smear tests and among the ones that controlled for these confounders the results were controversial. The Oxford Family Planning Association contraceptive study (Zondervan et al., 1996) was a nested matched and randomised case-control study adjusted for social class, smoking, age at first birth and ever use of diaphragm or condom. It reported that the risk of cervical cancer (odds ratio - OR) associated with ever having used oral contraceptive was 4.44, the OR for carcinoma in situ was 1.73 and the OR for dysplasia was 1.07, suggesting a kind of crescendo gradient effect from CIN 1 to invasive cancer. On the other hand, it is also interesting to observe that, among current or recent oral contraceptive users, the risk (Odds Ratio) of having any type of cervical neoplasia was 3.34 for 49 to 72 months of use, 1.69 for 73 to 96 months of use, and 2.04 for 97 or more months of use, thus weakening the argument for a cumulative effect. A direct effect of oral contraceptive use in the doses prescribed in recent years upon the oncogenesis of cervical cancer requires further investigation.

4G. Chronic inflammation

It has been said long ago that cancer is related to chronic irritation (Dyas, 1928). In most cancers associated with viruses, the viral infection per se is not enough to induce a cell to invade adjacent tissues, evade the immune system, and establish distant metastasis. As hypothesised in the late 80's, viruses probably develop strategies to evade the immune system, thus promoting potential development of further uncontrollable malignant cell growth (Natali et al, 1989). In the case of HPV, from the beginning of viral infection to the appearance of invasive disease in an immune competent woman, it usually requires a persistent viral infection along with chronic cervicitis lasting about 10 years. This long
term infection or chronic inflammation appears to confuse the immune system as to what is self and non-self regarding the cells in the cervix. At the same time it is associated with signals for metaplastic transformation and intense DNA replication, hence increasing the possibility of mutations. These changes allow the cells to divide uncontrollably, mutate and become resistant to different drug therapies; they also allow for invasion of the basement membrane, the lymphatic system and other vessels, and for acquisition of the capability of producing distant metastasis. The exact molecular mechanisms involved in this pathological process are not fully understood as yet.

In the 50’s the theory that nucleic acid, viruses and genes were similar started to become popular. It was hypothesised that a virus could become integrated to the host cell genome, replicate its nucleic acid and trigger the neoplastic process. It has been proposed that a virus can act as a wild gene and integrate its nucleic acid material into the cell genome (Hornedo, 1958). In the early 70’s not much of a possible association of cervical cancer with a sexually transmitted viral infection was evident. It was only after a study was published reporting the link between cervical cancer and human papillomavirus (HPV) type 16 (zur Hausen et al., 1981) that research efforts and support started to be directed more objectively towards the natural history of cervical neoplasia. Nowadays, there is good epidemiological evidence to suggest that CIN and cervical cancer are associated with a sexually transmitted viral disease whose agents are high-risk HPV types (Munoz & Bosch, 1992; Bosch et al., 1995; Ngelelangel et al., 1998; Chichareon et al., 1998). High-risk HPV infection together with co-factors such as smoking and vitamin deficiencies seem to play a major role in the oncogenic process of cervical neoplasia development (Wright & Richard, 1990; Hellberg et al., 1986).

4H. Smoking

In the late 70’s, the first report on the contributory effect of cigarette smoking in the development of cervical cancer was published (Winkelstein, 1977). Tobacco smoking has been described as having immunosuppressive and mutagenic effects (Ferson et al., 1979; Sasson et al., 1985; Holly et al., 1986). Reductions in the numerical densities of Langerhans cells and helper/inducer T lymphocytes in the squamous epithelium of the cervical transformation zone have been observed in association with smoking, thus suggesting a local immunosuppressive effect on the cell mediated immunity by smoking (Poppe et al., 1995). It has been observed that women who did not smoke were not at
higher risk of having cervical HPV infection but the ones with HPV infection who smoked, including passive smokers, were indeed at higher risk of developing cervical cancer (Herrero et al., 1989; Slattery et al., 1989). At least one study though argues that the association of cigarette smoking and cervical cancer is epidemiologically difficult to be established independently from the correlation between cigarette smoking and sexual activity that exists in most cultures, and question whether the confounding effects have effectively been controlled (Phillips & Smith, 1994).

A study involving 40 women undergoing hysterectomy for reasons not described (22 smokers, 4 ex-smokers and 14 non-smokers), showed significantly increased levels of adducts in DNA from cervical tissue of smokers and ex-smokers compared to the non-smokers ($P = 0.0005$), using the method of $32P$-postlabelling after butanol extraction enrichment. When the same analysis was done using the nuclease P1 digestion enhancement method, the total adduct levels in cervical tissue from smokers were not significantly different ($P = 0.3$), but the level of a minor discrete adduct spot (adduct A alone) was significantly lower ($P = 0.02$) in smokers than in non-smokers. The difference was significant when using butanol extraction enrichment, which is suitable for detection of adducts formed by polycyclic aromatic hydrocarbons, aromatic amines, and nitroaromatic compounds. But there was no significant difference when the nuclease P1 digestion enhancement method, which is suitable for detection of only polycyclic hydrocarbon-DNA adducts and certain types of oxidative DNA damage, was used. The authors claimed that one explanation for these results is that aromatic amines and/or nitroaromatic components of tobacco smoke make a greater contribution to the overall DNA binding in cervical epithelium than in the respiratory tract and that not all adducts detected were related to smoking. They comment that there was no evident linear relationship between tobacco smoke exposure as measured by daily or lifetime cigarette consumption and adduct levels in cervical tissue. Whereas the contrary was true for lung cancer and this difference was most likely due to the fact that the increased risk of cervical cancer associated with smoking is much lower than the increase in risk of lung cancer. The authors concluded that some of the DNA adducts detected in cervical epithelium correlate with tobacco smoking and support the hypothesis that smoking-related cervical cancer results from exposure to genotoxic components of cigarette smoke that become activated to DNA-binding products in this tissue (Phillips & She, 1994). A similar study analysed DNA from 39 women undergoing hysterectomy due to benign
pathology (27 with normal smear, 2 with LSIL, 8 with HSIL, and 2 suggestive of cervical cancer) among whom 21 had never smoked and 18 had smoked cigarettes [11 were current smokers, 4 were recent (6 months) ex-smokers, and 3 long-term ex-smokers]. The authors found that the DNA samples from smokers had significantly higher adduct levels than the ones from non-smokers \((P = 0.024)\) and that the DNA samples from women with abnormal cervical smears also had significantly higher adduct levels than those from women with normal smears \((P = 0.015)\) (Simons et al., 1994).

The first direct evidence of the role played by cigarette smoking in the progression of HPV-initiated carcinogenesis using an in vitro model system was described in 1996. A study where a HPV 18-immortalised ectocervical cell line (HEC-18-1), which was non-tumorigenic and adapted to grow in serum and high calcium, was treated with cigarette smoke condensates until tumorigenic cells were produced. The moderate and late serum-adapted HEC-18-1 cell line in the control group that was not treated with cigarette smoke condensate remained non-tumorigenic, in contrast the group treated with smoke condensates produced a typical invasive squamous cell carcinoma from which a clonal line was established (Nakao et al., 1996).

4I. Diet

Low intake and blood levels of vitamin C and E, retinol and beta carotene have been observed in women with cervical neoplasia, including adenocarcinomas of the cervix (Romney et al., 1981; La Vecchia et al., 1984; Parazzini et al., 1988; Verreault et al., 1989).

4J. The role of Human Papillomavirus

Human papillomaviruses (HPV) are icosahedral non-enveloped DNA particles with a diameter of approximately 55 nm. They contain a double-stranded, circular and covalently closed DNA genome of 7500 to 8000 base pairs (Pfister & Fuchs, 1994). Their major capsid protein is L1, which is highly conserved among all types of papillomaviruses. HPVs are a subfamily of the Papovaviridae family along with the polyomaviruses (Shah & Howley, 1992), although given some differences in size and organisation of viral genome between these two groups of viruses, this classification is questionable (Bernard et al., 1994).
In the early 70's, the first attempts to find HPV in human malignant tumours using nucleic acid hybridisation were performed with complimentary RNA obtained from papillomavirus DNA isolated from plantar warts but, as it is clear to us now, the results were all negative (zur Hausen et al., 1974). The presence of a genital HPV DNA (HPV 6) in condyloma acuminatum was first reported in 1976 (Gissmann & zur Hausen, 1976), after that HPV 6 was cloned, characterised and sequenced (de Villiers et al., 1981; Schwarz et al., 1983). HPV 11 was then isolated from laryngeal papillomas and genital warts (Gissmann et al., 1983). At about the same time two other types of HPV were cloned directly from cervical cancer, these were HPV 16 and HPV 18 (Boshart et al., 1984; Durst et al., 1983). HPV 16 DNA was specifically prevalent in lesions with marked nuclear atypia and an aneuploid karyotype (Crum et al., 1984). It was observed that a relatively high percentage of individuals harbouring HPV infection escaped conventional screening methods for cytological and colposcopic alterations (Ferenczy et al., 1985; de Villiers et al., 1987). Transfection experiments have shown that two of the HPV early proteins, namely E6 and E7, are directly involved in both the establishment and the maintenance of cell immortalisation and transformation. It has been established that HPV 16 and 18 are able to induce malignant transformation and immortalisation of human cells (Tsunokawa et al., 1986; Yasumoto et al., 1986; Durst et al., 1987). There is therefore clear scientific evidence that HPV 16 and 18 are able to induce proliferative changes in infected cells. Although the replicative strategy of HPV has been characterised in vitro, the precise mechanism of interaction between the virus and its natural host under different disease conditions is not yet clearly understood. Another early protein, E5, may also play a role in the transformation process in human cells but it is not essential (Pim et al., 1992). In contrast, in bovine papillomavirus (BPV) infection of rodent cells, E5 has been identified as a major transforming gene (Bedell et al., 1987). There are cell lines that contain HPV 18 such as HeLa, SW 756, C4-I and C4-II, and other cell lines containing HPV 16 such as Caski and SiHa. These cell lines have been widely used for the understanding of the mechanisms by which HPV induces cervical neoplasia. These established cell lines derived from cervical cancers contain HPV DNA, which is integrated into the cellular DNA. The common features of the integrated DNA are that the upper regulatory region and E6/E7 genes are invariably intact and transcriptionally active, and the circular viral genome is disrupted and often deleted in the E1, E2 or L1 or L2 genes. The HPV proteins most readily demonstrated in transformed cell lines and cervical
cancer are the E7 and E6 proteins (Oltersdorf et al., 1987; Seedorf et al., 1987; Smotkin & Wettstein, 1986; Schneider-Gadicke & Schwarz, 1986).

Bear in mind that there are some problems with hybridisation techniques that require optimisation. The Southern blot hybridisation method is amenable to cross hybridisations when a large quantity of HPV DNA is present in a sample specimen since different HPV types contain varying degrees of homologous DNA sequences (Burk et al., 1986). The most commonly used probes for HPV detection by hybridisation techniques consist of full-length viral DNA cloned into vector pBR322 or related vectors (de Villiers et al., 1981; Gissmann et al., 1982; Durst et al., 1983; Boshart et al., 1984; Beaudenon et al., 1986; Boshart et al., 1986; Lorincz et al., 1986). However, probe cocktails containing pBR322 sequences may hybridise with homologous DNA sequences from other sources leading to false positive results (Ambinder et al., 1986; Hording et al., 1989). It is assumed that some of the positive signals not specific to HPV are due to plasmid or other DNA sequences borne by bacteria contaminating the samples (Ambinder et al., 1986; Hording et al., 1989; Lorincz et al., 1986; Tabrizi et al., 1991). The hybridisation of recombinant viral plasmids with pBR322 vector-homologous material often results in stronger hybridisation signals than those associated with the presence of viral DNA in clinical specimens (Ambinder et al., 1986). The other problem leading to false positive results in hybridisation techniques is probe-trapping, in which a clinical sample applied to a nylon membrane causes the probe to nonspecifically adhere to the membrane (Boshart et al., 1984). Also, the RNA probes may be trapped by blood or mucus secretions (Hording et al., 1990), which are not uncommon in scrapes from women with ectropion or cervicitis and are physiologically increased in the cervix throughout pregnancy.

It is generally agreed that methods for detection of HPV involving DNA amplification are more sensitive than the methods that do not amplify the amount of DNA which may be contained in cells collected from cervical scrapes (Schiffman et al., 1991; Schiffman, 1992a; and Gravitt & Manos, 1992). Based on these pieces of scientific evidence, it is important to use the accuracy of PCR as the gold standard of studies about HPV detection as a means of identifying women with cervical neoplasia and those at risk of developing it, the natural history of HPV infection and its transmission.
4J.a) In genital warts

Warts have been described by Cornelius A. Celsus in his book entitled *De Medicina* (Celsus, circa 25 AD) and by his Greek contemporary Galen. In Greece it was named condyloma and in Rome is was called verruca (little hill or eminence). The Romans considered genital warts to be sexually transmitted but it was a physician from London, Joseph Payne, who first recognised the infectious nature of warts (Payne, 1891). In 1907, it was suggested that common warts were caused by a viral infection (Ciuffo, 1907) but it was not until 1950 that the small spherical virus-like particles of about 50nm in diameter with surface capsomeres were demonstrated under electron microscopy (Strauss *et al.*, 1950). Later, one of Strauss associates, J. L. Melnick, recognised the similarities between this virus and other small double-stranded tumorigenic DNA viruses such as the polyomavirus of mice, the vacuolating virus of monkeys (SV40) and the Cottontail rabbit papillomavirus, thus classifying them as a type of papovaviruses (Melnick, 1962).

The distribution of HPV types in benign lesions in humans are as follows: cutaneous common warts (HPVs 2, 4 and 7), deep plantar or myrmecia (HPV 1), plane warts (HPVs 3 and 10), epidermodysplasia verruciformis-like macules (subgroup 1: HPVs 5, 8, 12, 14, 19, 20, 21, 24, 25, 36, and 47; and subgroup 2: HPVs 9, 15, 17, 22, 23, 37, 38, 46, 48 49, and 50), mucocutaneous genital warts (HPVs 6, 11, 16, 18 and 42), condyloma acuminata (HPVs 6 and 11), flat condyloma (HPVs 16 and 18), Buschke Lowenstein tumour (HPV 6), Bowenoid papulosis (HPVs 16 and 55), laryngeal papilloma (HPV 6 and 11), oral papillomas (HPVs 2, 6, 7, 11, 16, 18 and 57) and oral epithelial hyperplasia (HPVs 13 and 32).

The association of genital warts with venereal disease was corroborated by the observations of 22 women married to American service men working overseas and who acquired genital warts while abroad. The women developed genital warts themselves within 4 to 6 weeks of the return of their husbands (Barrett *et al.*, 1954). It has been reported that cutaneous warts spontaneously regress completely within 6 months in about 25 to 35% of patients (Massing & Epstein, 1963; Kirby *et al.*, 1988; Rader *et al.*, 1991; Eron *et al.*, 1986). This suggests the importance of the host's immune response in the pathogenesis of HPV associated lesions. Papillomaviruses induce both humoral and cell mediated immune response, however avoidance of persistent infection and regression of lesions is usually associated with the cell mediated immune response. These findings
suggest a scientific basis for the development of both prophylactic and therapeutic vaccines against HPV infection.

Cases of persistent wart virus infection have been observed in immunosuppressed recipients of renal allografts (Spencer & Andersen, 1970, 1979). Pathologies that suppress both the humoral and cell mediated response predispose the patient to HPV infection and persistence. This is in contrast to what happens in cases of myelomas, where only the humoral and not the cell mediated immunity is affected, and there is no increased risk of viral warts compared to immunocompetent patients (Morison et al., 1975). HPV types 6 and 11 are usually found in the episomal independent circular viral genome form as opposed to HPVs 16, 18 and other high-risk types that are usually found integrated into the cellular genome of patients with high grade SIL and cancers. HPV types 6 and 11 are mainly detected in benign cutaneous and mucocutaneous lesions or in lesions with low potential for malignant transformation (Munoz et al., 1988).

4J.b) In cervical neoplasia

Nearly all HSIL and cervical cancers have been found to be associated with HR HPV types. Some HR HPV types (HPV16, 18, 31 33) are known to be more oncogenic than others (HPV35, 45, 51, 52, 56) (Lorincz et al. 1992). The study by Franco and collaborators that has been undertaken in Sao Paulo (Brazil) since 1993 in collaboration with the McGill University (Canada) may provide valuable information with regard to what proportion of cervical neoplasias of each grade or which grades are true precursors of invasive cervical carcinoma (Franco et al., 1999). The ASCUS-LSIL Triage Study (ALTS) is a randomised prospective multicentric clinical trial that has been carried out in 4 centres in the United States since 1995 and involves 5066 women with either ASCUS/LSIL cytology who will be followed until 2001. It was designed to evaluate the most appropriate way to manage ASCUS cytology (prepared by the liquid-based method ThinPrep® Pap Test™) among immediate colposcopic referral, conservative management with cytological follow-up or secondary triage with HPV detection by Hybrid Capture II. These well designed studies will most likely bring more light into the natural history, the continuum model and management of cervical neoplasias. In fact, 2 papers have just been published on the ALTS study. One concludes that the Hybrid Capture II assay has limited potential to direct decisions about the clinical management of women with LSIL because a very high percentage (83%) of women with an LSIL cytology report are positive for
HPV DNA (ALTS Group, 2000). The other concludes that the Hybrid Capture II assay is a viable option in the management of women with ASCUS cytology. It has greater sensitivity to detect CIN 3 or cervical cancer and comparable specificity to a single additional cytology report of ASCUS or above (Solomon et al., 2001).

The current natural history of cervical neoplasia and policies for cervical cancer screening have been largely written based on cytological studies. Women with negative cytology reports did not usually have any further cervical investigation apart from routine cytological screening; therefore we can not be assured that that was indeed a true negative case, particularly in view of the varying reported rates of false negative cytology results. Another difficulty associated with cytology is the lack of prognostic value. As soundly stated by Burghardt and Ostor, at the present moment it is yet not possible to distinguish between the CINs that are truly pre-cancerous lesions and the ones that are nonspecific proliferations (Burghardt & Ostor, 1983).

Perhaps the use of HPV detection and typing by the accurate molecular biological techniques available today such as PCR and Hybrid Capture II can provide light to this essential field of knowledge, enabling improvements for future cervical cancer prevention and management guidelines.

5. Clinical aspects
Cervical cancer precursors are generally symptomless. When post-coital or spontaneous cervical bleeding occur in a non-pregnant, particularly menopausal, woman it is highly suggestive of the presence of abnormal blood vessels and cervical cancer.

The most recent cervical cancer staging system published by the International Federation of Gynecology and Obstetrics (FIGO) Cancer Committee (1995) is described in Table I.

A study evaluating the 5-year survival rate of women who had invasive cervical cancer detected during any trimester in pregnancy or in the puerperium has described no difference in survival comparing pregnant with non-pregnant women when the analysis of the data was adjusted for clinical stage of the malignancy (Creasman et al, 1970).
Stage I. The carcinoma is strictly confined to the cervix (extension to the corpus should be disregarded).

Stage Ia. Invasive cancer identified only microscopically. All gross lesions, even with superficial invasion, are stage Ib cancers.
   Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm.*

   Stage Ia1. Measured invasion of stroma no greater than 3 mm in depth and no wider than 7 mm.
   Stage Ia2. Measured invasion of stroma greater than 3 mm and no greater than 5 mm in depth and no wider than 7 mm.

Stage Ib. Clinical lesions confined to cervix or preclinical lesions greater than stage Ia lesions.
   Ib1. Clinical lesions no greater than 4 cm.
   Ib2. Clinical lesions greater than 4 cm.

Stage II. The carcinoma extends beyond the cervix, but has not extended to the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.

Stage IIa. No obvious parametrial involvement.
Stage IIb. Obvious parametrial involvement.

Stage III. The carcinoma has extended to the pelvic wall. On rectal examination there is no cancer-free space between the tumour and the pelvic wall. The tumour involves the lower third of the vagina. All patients with hydronephrosis or non-functioning kidney should be included, unless it is known to be due to other causes.

   Stage IIIa. No extension to the pelvic wall, but involvement of the lower third of the vagina.
   Stage IIIb. Extension to the pelvic wall or hydronephrosis or non-functioning kidney.

Stage IV. The carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum.

   Stage IVa. Spread of the growth to adjacent organs.
   Stage IVb. Spread to distant organs.

* The depth of invasion should not be more than 5 mm taken from the base of the epithelium, either surface or glandular, from which it originates. Vascular space involvement, either venous or lymphatic, should not alter the staging.

Table I. FIGO cervical cancer staging (1995).
6. Detection

Initially, the only way to detect cervical cancer was by direct visualisation of clinically invasive disease with the naked eye. After the advent of the microscope, a surgical sample of the suspected abnormal cervical tissue was excised (biopsy) and examined under the microscope to look for abnormal cells and invasion of surrounding tissue. Then Papanicolaou developed a method to study the cells exfoliated from the cervix, the Pap smears test. At about the same time, Hinselmann developed a method for amplified visualisation of cervical epithelium under powerful illumination, colposcopy. These techniques make up the tripod currently used in clinical practice for cervical cancer detection.

6A. Cytology

As explained below, under the heading of histology (since this technique was developed first), the ways of classifying cervical neoplastic lesions have been changing throughout time. The initial cytologic classification proposed by Papanicolaou based on numerical class designations from I to V, depending on the severity of cellular abnormalities, has been so modified that up to recently the terminology used in cytologic reports meant different things to different laboratories and clinicians. Some of the criticisms of Papanicolaou’s classification which was introduced 1948 were lack of correlation between cytological and histological terms, his class II classification included both benign and viral cytological abnormalities, and classes III and IV provided imprecise diagnosis of malignancy. Terms like suspicious, borderline, atypical, positive and code numbers or classes were thought not to be very specific and amenable to improvements. Up to 1988, the Papanicolaou classification was still fully used in some countries and there were some confusing cytopathologic terms such as dysplasias, cervical intraepithelial neoplasias, and carcinoma in situ being used interchangeably all over the world. It then became common sense that it was about time a standard terminology should be implemented for better scientific communications and improvement in the guidelines for treatment.

6A.a) Cytology reporting and colposcopic referral systems

Since the development and implementation of cervical cytology into clinical practice by Papanicolaou until recently, this first and only universally accepted cytology reporting system was based on five distinct classes of abnormalities. This widely used cytological classification has proven extremely useful not only for the screening and detection of
gynaecological malignancies but for other cytological samples as well. The original Papanicolaou classification was described as follows (Table II).

The objective of updating and correlating better cytologic and histologic nomenclatures for characterisation of the cervical epithelium is to come up with a simpler and more specific classification to improve guidelines for clinical management. With that in mind, the British Society for Clinical Cytology (BSCC) has created a well respected classification system for cytological reports that so far is intact and thoroughly tested. The BSCC classification was created in the late 80’s and uses the term dyskaryosis with the subdivisions of mild, moderate and severe to correlate with the histological grades of CIN 1, 2 and 3 (Evans et al., 1986). This classification seriously attempts to correlate the cytological abnormalities seen on the smears with an appropriate histological grade. The BSCC employs five grades for cytology reports (Table III).

In 1988, the National Cancer Institute Workshop resulted in the creation of a new system for reporting cervico-vaginal cytologic diagnosis, the so called Bethesda System, which was revised in 1991 (National Cancer Institute Workshop, 1989; The Revised Bethesda System, 1992). This new system (Table IV) is now widely used in the United States and in other countries.

The fact that the smears are evaluated for adequacy and that the findings are stated in descriptive diagnostic terms make up the two essential features of the Bethesda classification. Further subclassifications related to initial cytological changes induced by HPV infection, glandular abnormalities and squamous metaplasia are also described. The term koilocytosis is used in cytopathology to describe degenerated superficial or intermediate squamous epithelial cells showing both cytoplasmic vacuolisation and nuclear abnormalities characterised by enlargement, hyperchromasia and wrinkling. It is usually associated with dyskeratosis, parakeratosis and hyperkeratosis. The findings of koilocytosis in cytologic and histologic slides are suggestive of HPV infection. However, cytoplasmatic vacuolisation without the described nuclear alterations can be found in normally glycogenated squamous epithelium or in association with several types of inflammation, for instance as a consequence of trichomonal infection. The term atypical squamous cells of undetermined significance (ASCUS) is used to describe cellular changes that are not clearly reactive but lack the nuclear criteria required for a definite
Class I: Absence of atypical or abnormal cells;
Class II: Atypical cytology but no evidence of malignancy;
Class III: Cytology suggestive, but not conclusive, of malignancy;
Class IV: Cytology strongly suggestive of malignancy;
Class V: Cytology conclusive for malignancy.

Table II. George Papanicolaou’s classification for abnormal cells in cytological smears.
1. Unsatisfactory for assessment (with a reason stated);
2. Negative;
3. Nuclear changes bordering on mild dyskaryosis (= borderline);
4. Dyskaryotic cells: mild, moderate, and severe;
5. Malignant cells suggestive of invasive cancer: squamous cell and/or adenocarcinoma.

Table III. The BSCC cytological classification.
Adequacy of the specimen

- Satisfactory for evaluation
- Satisfactory for evaluation but limited by … (specify reason)
- Unsatisfactory for evaluation … (specify reason)

Descriptive diagnosis

Benign cellular changes

Epithelial cell abnormalities

Squamous cell
- Atypical squamous cells of undetermined significance (ASCUS): quantify
- Low-grade squamous intra-epithelial lesion (LSIL) encompassing: HPV, mild dysplasia and CIN I
- High-grade squamous intra-epithelial lesion (HSIL) encompassing: moderate and severe dysplasia, carcinoma in situ, CIN II and CIN III

Glandular cell
- Atypical glandular cells of undetermined significance (AGCUS): quantify
- Endocervical adenocarcinoma
- Endometrial carcinoma
- Extra-uterine adenocarcinoma

Table IV. The Bethesda System classification.
classification of squamous intra-epithelial lesion (SIL). The cellular features observed in ASCUS smears include a loss of the normal nuclear/cytoplasm ratio, associated with nuclear enlargement and the nuclei may appear hyperchromatic. However the criteria for ASCUS diagnosis is still controversial in different laboratories and in the opinion of different pathologists. In fact, the lack of a clear cut distinction between ASCUS and low grade squamous intra-epithelial lesion (LSIL) and the lack of inter-observer agreement have led to unspecific use and overuse of the term ASCUS (Sherman et al., 1994; Raffle et al., 1995). Condyloma acuminata lesions are associated with low risk HPVs, particularly HPV 6 and 11, but are included in the LSIL Bethesda classification. Studies evaluating atypical Papanicolaou smears, even the first result without a repeat Pap, have shown that 15% to 25% of women with such a report already have squamous intraepithelial lesions detected in their cervices (Jones et al., 1987; Lindheim & Smith-Nguyen, 1990; Himmelstein, 1989). A decade after the implementation of the Bethesda system into routine clinical practice, it seems that the classifications of ASCUS and AGCUS still represent a perplexing clinical problem reflected in the 20 to 25% of all smears in the United States being classified as such. Studies have shown that, despite the majority of ASCUS reports representing benign reactive cell changes, 5% to 10% of women with ASCUS smears actually harbour HSIL (Cox et al., 1995; Wright et al., 1995; Kinney et al., 1998). The result is that about 2 to 2.5 million women receive an ASCUS cytological diagnosis, among which up to 125,000 women will have CIN 3 or cervical cancer each year in the United States.

There is still controversy with regards to the management of women with ASCUS, AGCUS and LSIL cytology reports. Current recommendations in the United States for these mildly abnormal cytology reports are for the follow-up of ASCUS smears by repeat cytology every 4 to 6 months for 2 years, and colposcopy is recommended for cases of persistent ASCUS smears. Women with LSIL smears can be managed by either immediate colposcopic referral or cytological follow-up. However, a study involving 650 women with LSIL have shown that 18% of these women had HSIL on histologic examination of colposcopically guided biopsies, thus demonstrating the importance of immediate colposcopic referral of women with LSIL cytological reports to rule out high-grade cervical neoplasia (Montz, 1992).
A large prospective study was conducted in northern California, the Kaiser Permanente study, involving 46,009 participants from 12 clinics among which 973 had ASCUS cytology. The authors observed that secondary triage of liquid-based cytology samples from women with mildly abnormal smears using the Hybrid Capture II™ test (Digene Corporation) for detection of high-risk HPV types resulted in a sensitivity and specificity for identification of HSIL of respectively 89.2% and 64.1%, compared with a corresponding repeat cytology sensitivity of 76.2% (Manos et al., 1999). An earlier study showed that a considerable proportion of women with histologically proven HSIL (more than 33.3%) actually had ASCUS/LSIL smear results in a routine screening population (Kinney et al, 1998).

More prospective follow-up studies using HPV detection and typing should be supported to help establishing grounds upon clinicians can distinguish which of these mildly abnormal cytology have indeed potential for malignant progression from the ones that are basically a temporary benign cellular reaction.

Currently in the United Kingdom, the guidelines from the NHS cervical cancer screening programme recommend that women with cytologic report of moderate or severe dyskaryosis and those with cells suggestive of invasive carcinoma be referred immediately for colposcopic examination. In cases of mildly dyskaryotic or borderline smears, approximately 5% of all smears taken in the United Kingdom (250,000 smears a year), the women are followed up cytologically at 6 to 12 month intervals unless progression or persistence occurs: persistence in this setting means two mild or three borderline smears. Actually what happens is that about 250,000 British women are on cytological surveillance a year, among them 10 to 25% will be found to be harbouring high-grade underlying CIN lesions and another 10 to 25% will have low-grade intra-epithelial lesions. After two consecutive normal smears at least 6 months apart the woman is recommended to return to routine screening every 3 to 5 years (Duncan, 1993, 1997). However, the second cytological sample may be completely negative in about 60% of patients with significant neoplastic lesions (Koss, 1989b). The cumulative colposcopic referral rate in the United Kingdom after about four years of follow-up of mildly abnormal cytology was estimated to range from 14 to 64% and immediate colposcopic referral would reduce the risk of invasion in 54 to 84% (Soutter, 1994). Some authors claim that although cytological surveillance of women with mildly abnormal smears is
considered safe, it is not an efficient management strategy for it in some cases postpones the treatment of women with significant cervical neoplasia, and recommends immediate colposcopic referral of all women with any degree of dyskaryosis (Flannelly et al., 1994; Shafi et al., 1997). They later added that the failure of cervical cytology to detect all cases of CIN 3 and invasive cancer through this follow-up management protocol was a significant problem (Shafi et al., 1997).

The general media has reported a series of cases of cervical cancer screening misdiagnosis. On the 29th of November of 1999 at 20:00 h, the BBC2 television channel presented a documentary entitled “Disaster: A cancer in the system” publicly unveiling that cervical cancer screening programmes in Kent and Canterbury had to re-examine about 90,000 cervical smears due to considerable false-negative rates found in that region. At least 1,800 women were found to have been misdiagnosed, among which 8 had died from cervical cancer. In Warwickshire similar problems were also reported. The Times published on the 10th of September 1993 that the laboratory at the Inverclyde Royal Hospital in Scotland had released 70% of false-negative Pap smear results, which led to the re-examination of 18,000 smear tests. On the 11th of September 1993 it was published in The Times that over 1000 women in Birmingham had to be recalled for re-evaluation because their Pap smear test had been incorrectly diagnosed. Another 911 women in Liverpool had had the same problem as was described in the 27th of September of 1987 issue of The Sunday Times. Mismanagement of public funds directed towards preventive measures may lead to much higher expenses in treatment and to cover for law suits and damages to the patients or their families. As an example, on the 14th of December 1991 it was published in The Times that the husband of a women who died of cervical cancer in a case where medical malpractice or mismanagement was proven was awarded £87,000 in damages. Some facts such as the ones just described are not always to be found in the scientific literature but may serve for the purpose of example as to why the current practice of cervical cancer screening is sometimes questioned (Skrabanek, 1988; McCormick, 1989).

It is interesting to observe that at the early times of cervical cancer screening implementation, no randomised study was undertaken to demonstrate the efficacy of screening as opposed to not screening. Later, after the introduction of screening and proof that it does reduce cervical cancer mortality if coverage and frequency is appropriate,
such a study was considered unethical. In spite of that, well-organised cytologic screening for cervical neoplasia has reduced both the incidence of and mortality rate by cervical cancer, thus saving a large number of lives (Hakama et al., 1991). It is essential though that screening coverage reaches the highest possible fraction of the population at risk. However, there are some practical difficulties associated with conventional cytologic screening, some are related to appropriateness of cell sample collection, other are related to the preparation of the smear (spread of cells, fixation and staining) and other are related to human interpretation errors. Severe cervicitis may result in unsatisfactory cytology. The use of an Ayre spatula for the ectocervix and vaginal fornices, and a brush for the endocervical canal improve the quality of cell sampling when compared to vaginal aspirates or the use of cotton swabs for the endocervix. Limited number of cells collected, excessive presence of leukocytes and erythrocytes obscuring cellular morphological details, incomplete samples (e.g. lack of endocervical cells) and severe drying of the material collected are common reasons for unsatisfactory smears. The spread of cells onto the slides, its fixation and staining are extremely important to avoid clusters of cells or artefacts. Not less important is the expertise of those who read the smears; the number of slides read per day and the motivation of the cytotechnician or cytopathologist to thoroughly examine each cell on the slide. Another aspect that highlights the subjectivity of cytological and histological examinations is the body of evidence reported by experienced cytologists and histopathologists that shows marked disagreements both in intra-observer and inter-observer interpretations of smears and biopsies (Seybolt & Johnson, 1971; Bellina et al., 1982; Langley, 1984; Yobs et al., 1987; Ismail et al., 1989; Jenkins & Jarmulowicz, 1989; Robertson et al., 1989).

In Brazil, the majority of screening services offer the Pap smear test to any sexually active woman willing to attend. As a consequence of high incidence rates of cervicitis a significant number of these turn out to be inflammatory or unsatisfactory smears. In some health services, such as at IMIP hospital, when a woman comes to have a Pap smear done, she first sees a doctor who, after examining her, will either send her directly for smear collection or prescribe a course of antibiotic or hormonal therapy, when it is needed, before sending her for smear collection. This approach saves time and money since a considerable number of smears will not necessarily be repeated due to severe inflammation or insufficient number of cells. On the other hand, some women are determined to having the test done that day. When it is not done, they may not come back
at a later date due either to lack of education or misconception that it is not simple to have a Pap smear done in a single visit to the health facility. Careful counselling is important to assure compliance.

6A.b) Improvements in the cytological method
Aiming to optimise cytologic mass screening, automated systems have been developed. In order to facilitate and improve quality of cytology screening programmes intending to use automated reading systems, liquid-based methods for preparation of cervical cytology smears have been developed (Wheeless & Onderdonk, 1974; Garcia & Tolles, 1977; Hussain et al., 1978; Wooley et al., 1979). In the middle of the 70's, an automated cervical cancer screening system was developed to produce an optimally dispersed cell sample without cell debris. The cell-scanning machine used interactive systems for cell cluster disruption, cell dispersal and production of a monolayer of cells from fluid suspensions. A combination of 0.1% dithiothreitol and 40% alcohol in a balanced salt solution produced a self-limiting form of mucolysis, which was completed by a controlled syringe regime to result in an optimal sample. The microscope slides were coated with polylysine, a cationic polymer, and a positive charge was applied to the coated slides in order to attract the negatively charged cells, thus permitting satisfactory wet fixation and stain without loss (Hussain et al., 1978).

Several attempts have been made to decrease false negative cytology rates. Some were related to improving the collection of cervical scrapes and others focused on a better treatment of cells from the scrape aiming to reduce clusters and overlaying of cells. In 1986, a study carried out in Sweden described a new method for detection of cervical cancer whereby a pulse wash instrument was used for sampling of the cytologic material. Liquid jets with a high kinetic energy, such as with a diameter of 0.2 mm at a speed of 20m/s, are used to produce a successful rinsing of cervical epithelial cells. Additional benefits of this method were that, on top of the conventional smear, other diagnostic techniques like cytochemical, immunocytochemical and microbiological essays could be performed on the originally collected samples given that the cells were suspended in the flushing liquid. After performing both the conventional Pap smear and the pulse wash technique in 75 women attending cervical atypia clinics, the authors suggested that the pulse wash technique resulted in a more representative cellular sample (Naslund et al., 1986).
6A.c) Liquid-based cytology

In the early 90's, there were reports describing the variability in quality and results of cytological smears, in addition to the confusing terminology and less than optimal specimen adequacy where blood, mucus, debris and clusters obscured cells on the microscope slide (Joseph et al., 1991; Davey et al., 1992).

More recently, new commercially available automated liquid-based thin layer or monolayer cytology systems are being tested such as the ThinPrep® Pap Test™, AutoCyte Prep, the cytoscreen system CYTeasy and the PapSpin™ Test. Basically, the health worker collects the ecto- and endocervical samples of cells with a brush/broom-like device or with a spatula and a brush, and disperses the cells into a vial with preservative solution. The decision to collect samples with a brush and a spatula certainly increases the number of cells in the sample when compared with sample collections with an Ayre's spatula and a cotton swab, for instance. This simple but effective procedure substantially reduces the possibility of up to 80% of cells in the sample being discarded along with the spatula after conventional spread of cells on the slide (Hutchinson et al., 1994). The vials are taken to a laboratory where the automated processing occurs. The samples are shaken to detach cells from one another and from the walls of the vial making a homogenous concentration of free cells in the vial, which in the case of AutoCyte Prep the samples are strained with a Cyringe® and layered onto a density reagent and in the case of CYTeasy a photometric reader standardises the cellular density. Then the cells are spun by centrifugation onto a microscope slide to form a representative thin layer of clean cells from the sample, which is stained as usual and read either manually or by computerised imaging systems such as AutoPap or AutoCyte Screen. The remaining cells in the vial may be kept for preparation of additional equally distributed monolayers for extra morphological analysis or for additional tests like HPV detection.

The ThinPrep® 2000 processor (Cytic Corporation) is a device that automates the process of preparing cytology samples. The samples do not need to go on a shaker, a rotary drive mechanism using a filter (Gyn TransCyt® Filter) that rotates creating currents in the fluid of the vials which separates debris, disperses mucus, inflammatory exudates and blood, and spreads the cells in the sample. Another filter (Non-Gyn TransCyt® Filter) creates a gentle vacuum, which collects cells on the exterior surface of a membrane; this process of
cell collection is controlled by a microprocessor software that monitors the rate of cell flow through the filter that will end up on the membrane. The filter with the cells on the membrane is inverted and the cells collected on the membrane are gently pressed against a microscope slide where a slight positive air pressure and natural attraction cause the cells to adhere to the microscope slide, producing an even and thin distribution of cells in a defined circular area. A new fully automated batch processing version of the ThinPrep Processor, the ThinPrep 3000, has already been developed. It is capable of unattendedly processing 80 specimens from two trays loaded with 40 vials each and the bar coded slides are loaded into 4 staining racks of 20 slides.

The system mostly studied in clinical practice so far is the ThinPrep®. A study using the ThinPrep® processor to prepare cytological smears reported that this automated method produced randomised samples representative of the entire population of epithelial cells in the sampling device, thus reducing the possibility of false negative results compared to the conventional cytology method (Hutchinson et al., 1994). In addition to a significant reduction \( (P < 0.01) \) in the proportion of smears classified as ASCUS (3.64% in conventional cytology and 1.72% in ThinPrep), the better quality of sample adequacy in smears prepared by liquid-based cytology is clearly demonstrated in the study by Yeoh et al., 1999 (Table V).

An independent laboratory in New England tested the performance of the ThinPrep® method in a clinical setting and reported that it improves the quality of the specimen adequacy and increase the detection rate of SIL (Diaz-Rosario et al., 1999). Fourteen studies, amounting to a total of 275,129 participants from both low and high-risk populations, evaluating the efficacy of the ThinPrep® method in improving the preparation of cytological smears for detection of cervical neoplasia in clinical practice, comparing it to the conventional Pap smear test, have shown increases in LSIL detection varying from 6 to 341%, with a mean of \( 106.15 \pm 98.77\% \) and a median of 71%; and for HSIL detection the increase ranged from 16 to 233% with a mean of \( 87.75 \pm 74.98\% \) and a median of 54.5% (Hutchinson et al., 1994, 1999; Yeoh et al., 1999; Diaz-Rosario et al., 1999; Carpenter et al., 1999; Guidos et al., 1999; Dupree et al, 1998; Bolick et al., 1998; Papillo et al., 1998; Weintraub at el., 2000; Corkill et al., 1998; Roberts et al., 1997; Lee et al., 1997). If outlier figures are excluded from the analysis, the mean increase in
<table>
<thead>
<tr>
<th>Cytology</th>
<th>Unsatisfactory</th>
<th>Inflammatory</th>
<th>Scant cells</th>
<th>Endocervical cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>1.36%</td>
<td>10.94%</td>
<td>3.84%</td>
<td>51.23%</td>
</tr>
<tr>
<td>ThinPrep</td>
<td>0.56%</td>
<td>0.68%</td>
<td>1.08%</td>
<td>70.57%</td>
</tr>
<tr>
<td>P-Value</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table V. Summarised data on smear adequacy from the study by Yeoh et al., 1999.
detection of LSIL becomes $70.5 \pm 20.68\%$ and the median 70.5%; and the increase in the
detection of HSIL would have a mean of $74.55 \pm 62.31\%$ and a median of 54%.

However these are sophisticated devices which require an operating temperature of about
15 to 32 degrees Celsius and qualified technical support, thus most likely adding
considerable cost for mass cervical screening programmes.

6A.d) The sampling technique

Significant errors in cytological screening may occur due to poor sampling techniques.
Sampling errors during the act of collecting cells for cytological smears are reported to be
2 to 3 times more frequent than errors by smear reader or screening errors (Gay et al.,
1985).

A study was carried out in New York to evaluate the efficacy of Papanicolaou smear test
preparation using cervical scrapes of both ecto- and endocervix collected with a saline-
moistened cotton-tipped swab compared to the collection of cervical scrapes with a
plastic spatula for the ectocervix and with a saline-moistened cotton-tipped swab only for
the endocervix (Shen et al., 1984). The authors showed that in the 408 samples collected
with the saline-moistened cotton-tipped swab the quality of smears was less adequate. It
produced 6% smears with a scanty cellular yield compared to 3% of inadequate smears
produced by the 361 scrapes collected with a plastic spatula for the ectocervix and a
saline-moistened cotton-tipped swab for the endocervix. In addition, they showed that the
smears collected exclusively with saline-moistened cotton-tipped swabs resulted in higher
false negative rates compared to the smears collected with plastic spatula (Table VI).
Based on their findings, the authors recommend that cervical smears be taken with a
spatula rather than using a cotton swab exclusively (Shen et al., 1984).

If the aim of a screening programme is to prevent malignant lesions of the cervix and
vaginal fornix, cells for identification of precursor lesions are best collected by the use of
a device that effectively samples them at that particular anatomical site. Papanicolaou
himself recognised that vaginal aspirates were less optimal a sample than cervicovaginal
scrapes.
<table>
<thead>
<tr>
<th>Collection Devices</th>
<th>CIN 1</th>
<th>CIN 2</th>
<th>CIN 3</th>
<th>Cancer</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>False negative Cotton swab</td>
<td>60%</td>
<td>42%</td>
<td>16%</td>
<td>20%</td>
<td>32%</td>
</tr>
<tr>
<td>rates Plastic spatula</td>
<td>27%</td>
<td>29%</td>
<td>14%</td>
<td>0%</td>
<td>18%</td>
</tr>
</tbody>
</table>

Table VI. False negative cytology rates related to how smears were collected (Shen et al., 1984).
6A.e) Cervical cytology standards

It is generally accepted that a negative smear test does not guarantee that there is no cervical neoplasia, and this argument is used in court for lawsuits related to false negative cytology results. Also, it is important to emphasise the value of specular examination of all sexually active women in search of cervical pathology prevention or treatment, particularly because the smear test is not a substitute for visual inspection of the cervix.

Other crucial and yet controversial issue related to cervical cancer screening is the need to establish a standard protocol that determines the best combination of screening tools and proper intervals for cervical cancer prevention. Of prime importance is the definition and ultimate goal of cancer screening. Organised screening programmes are defined as programmes run by official health authorities with the clear goal and responsibility of covering all women at risk of developing a specific disease within a circumscribed jurisdiction. Women at risk for developing cervical neoplasia include all women who have ever been sexually active. The ultimate goal is thus a reduction in the occurrence of new cases of invasive cervical cancer and avoidance of mortality related to it. However, if one considers screening women for cervical cancer as a method of selecting those with invasive disease for treatment and perhaps cure one cannot reduce the incidence of cervical cancer although it may affect its mortality rate. It is generally agreed that it is rather important to identify women at risk of developing cervical neoplasia. This can be achieved by either detecting cervical precursor lesions that increase the risk of cervical cancer development and excising them or by evaluating risks at a much earlier stage in women harbouring the major risk factor for the development of cervical neoplasia (HR-HPV infection) and following them more regularly.

It is extremely important that the definition of screening and its main objective be internationally standardised. The interpretation of results achieved on a standard basis in different parts of the world can have essential implications for the improvement of screening policies. The guidelines currently recommended for cervical cancer screening are based on cytologic surveillance only, which is known to have problems and the protocols vary from country to country.

Based on the evidence that about 5% of deaths from cervical cancer in the United Kingdom occur in women aged less than 35 years, some authors advocate that cervical
cancer screening should begin in early adolescence, particularly to educate girls under the age of 16 years about the need for regular cervical smears (Robinson et al., 1988).

In the United States of America the guidelines for cervical cancer screening recommend that all women who have ever been sexually active or are 18 years of age or older have an annual Pap smear test and pelvic examination (USA Pap test guidelines, 1988). The dynamics of society have been leading to some changes in sexual habits. There was an increase in sexual liberation in the 60’s. There are several epidemiological studies showing that HPV infections occur more frequently in younger than in older women (Bauer et al., 1991; Munoz et al., 1992; Melkert et al., 1993; Nishikawa et al., 1991; Schiffman, 1992b; Bauer et al., 1993; de Villiers et al., 1992). The new generations are tending to have sexual intercourse at an earlier age and without proper contraception, hence England registers the highest rate of adolescent pregnancy in Western Europe, an important public health and socioeconomic problem.

Among 403 sexually active adolescents aged 12 to 16 years, 14 had evidence of CIN I (3.5%) detected in a study carried out in the late 70’s. It was important to note that in 11 of these 14 girls whose age at first intercourse was known, 5 had had first intercourse within the previous 2 years and 6 had had it between 2 and 4 years previously. The authors, therefore, recommended routine screening of sexually active adolescents (Hein et al., 1977). A study enrolling 271 sexually active adolescents aged 13 to 22 years undertaken at a clinic for sexually transmitted disease at the Montefiore Medical Center in New York, observed that these adolescents were at significantly higher risk of developing cytological abnormality, particularly LSIL, compared with all adult females (Edelman et al., 1999). Another recent study involving adolescents from an outpatient clinic in Yugoslavia, reported that among 235 sexually active teenagers histologically confirmed CIN 1 and CIN 2/3 were found in 7.23% and 0.85% respectively of all cases studied, and again concluded that it is necessary to routinely screen all sexually active adolescents (Ravic et al., 1999). In the United Kingdom cervical cancer screening is managed through a computerised database and the guidelines recommend to local health authorities that all women aged 20 to 64 be invited for screening with a recall appointment at least every five years (National Audit Office, 1992). In the Netherlands the guidelines recommend screening women aged 34 to 64 years every 3 years. In Sweden women aged 30 to 49 are screened every 4 years, in Finland women aged 30 to
55 years are screened every 5 years, and in Iceland women 29 to 69 years of age are screened every 2 to 3 years. In 1986, an IARC working group of experts recommended as a consensus that women aged 30 to 60 years should be screened at least every 3 years (IARC Working Group, 1986). It has been argued that since 40% of all deaths from cervical cancers occur in women over 65 years of age, screening programmes should not exclude this population of women (Fletcher, 1990).

What does screening mean after all? Should populational screening be aimed at identifying cervical cancer, its precursors or women at risk to develop it? Given that the natural history of the disease should not change in relation to political boundaries, what is the most appropriate age to begin and the right interval for cervical cancer screening? More research on the natural history of cervical neoplasia using molecular techniques will help on the improvement of guidelines for better screening protocols and properly answer questions like who is at risk to develop cervical neoplasia, what is the age group that should be screened and the best screening interval, among others.

For example, if one considers cervical cancer screening as a means of detecting cervical cancer then recommending screening only women over 35 years of age is reasonable, given that most cervical cancers occur after this particular age. However, if one aims to screening a population for cervical neoplasia, having in mind that women with HSIL are at increased risk of developing cervical cancer and knowing that CIN 2 and 3 more frequently occur in younger ages, in order to prevent cervical cancer the best should be to try to detect all or most young women with HSIL so that they can be treated. By doing so, more women at economically productive phases of life will be protected from developing this devastating disease.

6B. Colposcopy
In 1924, von Hinselmann in Germany utilised intense light source and image magnification to identify early cancer lesions of the cervix. In 1925, Hinselmann reported the construction of the first colposcope by combining von Eicken’s frontal light source to a Leitz binocular dissecting microscope (magnifying lens). The invention of the colposcope was a landmark for a new era in cervical cancer detection (Hinselmann, 1925). Colposcopy became a very important tool for the detection of cervical cancer; it links the primary screening tool (cytology) to the gold standard diagnostic technique (histology).
The colposcope should provide a magnifying power of 10 to 40 times. During a colposcopic examination it is usual to use normal saline, acetic acid and iodine solution. The use of normal saline on the cervix applied with a soaked cotton-wool swab removes some secretions and facilitates the examination of the subepithelial vessels through a green filter (Kolstad & Stafl, 1982). By applying to the cervix strengths of acetic acid varying from 3 to 5% the tissues, especially columnar and abnormal epithelia, become slightly swollen. The abnormal epithelium assumes a whitish or opaque appearance described as being due to coagulation of epithelial and stromal cytokeratins that lasts for about 50 to 60 seconds (Maddox et al., 1994). As the last staining procedure in colposcopy, a 5% iodine solution (Lugol’s solution) is applied to the cervix, the so called Schiller’s test. The superficial fully differentiated epithelial cells from the cervix and vagina stain dark brown because of the presence of glycogen. Columnar and immature metaplastic epithelia lack glycogen and remain unstained. In areas of epithelium infected with HPV, including areas of neoplastic epithelium, the glycogen content is reduced or absent, thus the iodine test results in a yellowish discoloration and sharp demarcation.

Today colposcopy is widely used all around the world. There are different protocols for colposcopic referral. In the United Kingdom, colposcopy is indicated for women with high grade (HG) squamous intra-epithelial lesions (SIL), in other words moderate and/or severe dyskaryosis, or repeated low grade (LG) SIL (mild dyskaryosis) (Singer & Monaghan, 1994). In the United States, all women with a Pap smear result of low grade SIL and above are referred to colposcopy. The question as to how best to manage ASCUS cytology is still debatable. However, in some developing countries like Brazil and Argentina, where colposcopy has become a relatively cheap tool, there is an attempt by some services to use it as a screening method in combination with cytology in order to compensate for unacceptable rates of inaccuracy in cytological reports. On the other hand, the cost and massive organisational requirements, the impossibility of assessing lesions high up in the endocervical canal and the possibility of considerable false positive rates, prevent colposcopy from being used as a single primary screening method. Indeed, colposcopy is the best method for evaluation of atypical smears for it enables the identification of the worst area of a cervical lesion, thus guiding where the biopsy should be performed, although it is an expensive and time consuming method and it also requires many years experience for the practitioner.
Given that mass screening using routine colposcopy in addition to cytology is impractical and uneconomical, an optical instrument that allows permanent objective documentation of cervical visual findings called the cervicograph was developed and tested in the early 80's. The cervicogram, which produces images comparable to the ones seen at direct visual colposcopic magnification and resolution, can be obtained by a technician and sent to an expert for evaluation. In a study comparing the diagnostic accuracy of cytology, cervicography and colposcopy for the detection of cervical neoplasia, 296 among a total of 700 participants had abnormal cytologic findings. Of the 136 histologic proven cervical neoplasia, including invasive carcinoma, 2.9% were detected solely by cervicography and 5.9% were detected solely by colposcopy. The concordance between cervicographic and colposcopic suspicious findings was of 91.1%, and among the 404 participants routinely screened with no cytologic abnormality, nine cases (2.2%) had cervical neoplasia detected solely by means of cervicographic findings (Stafl, 1981a, 1981b).

6C. Histology

A uniform nomenclature for cytologic and histologic diagnosis is essential for clinicians to understand the relevance of findings at the microscopic level and as a basis for patient management. In 1886, Williams observed that in areas surrounding invasive tumours of the cervix there were some lesions that were neither invasive cancer nor normal epithelium, and created the concept of premalignant lesions or cervical cancer precursors (Williams, 1888). Josef Albert Amann's book of gynaecological histology shows an early illustration, most likely the first, of what is classified today as CIN 3 with extension to endocervical glands, however the lesion was originally classified at the end of the 19th century as carcinoma of the cervix originating from squamous epithelium (Amann, 1897). Later, Cullen arrived at a better definition for these non-invasive lesions that resembled cervical cancer and the concept of these cancer precursor lesions became more accepted (Cullen, 1900). In a study in the early 20th century comparing the histologic similarities between invasive cervical cancer and the so called "surface carcinoma", Schauenstein proposed that malignant cellular changes confined to the cervical epithelium represented precursor lesions of invasive cervical carcinomas (Schauenstein, 1908). But the term carcinoma in situ appears to have been first suggested by Julius Schottländer and Fritz Kermanner in 1912 (Schottländer & Kermanner, 1912). Schiller has described that
cervical cancer could be diagnosed histologically in its pre-invasive stages (Schiller, 1927). Broders then introduced the term carcinoma in situ for lesions in which the full thickness of the epithelium was replaced by undifferentiated neoplastic cells (Broders, 1932). The term carcinoma in situ was initially only used by pathologists, but it soon became a common term in clinical practice. In a retrospective review of biopsy specimens from women who subsequently developed invasive cervical cancer, it was observed that the characteristic cell changes in the epithelium compatible with carcinoma in situ was present in them (Smith & Pemberton, 1934). Thus, a relationship between carcinoma in situ and invasive cervical cancer had been established where the precursor lesion had a high likelihood of developing into invasive cervical cancer if not treated properly and if the patient lived for long enough. This important concept was confirmed by other studies (Kottmeier, 1962; Koss et al., 1963; McIndoe et al., 1984).

Koss and his assistant Durfee, while examining both histological and cytological slides from cervix of New York patients in the mid 50’s, observed a specific and unusual cell pattern. It was characterised by the presence of large cells with relatively small but irregular and hyperchromatic nuclei surrounded by clear and transparent cytoplasm as if the nucleus were suspended in an empty space. The term koilocytic atypia was then first coined based on the Greek word “koilos” which means hollow or cavity (Koss & Durfee, 1956). The fact that koilocytes were found in both condylomas and in intra-epithelial neoplastic lesions of the uterine cervix such as mild, moderate, and severe dysplasias led to the suggestion that these intra-epithelial lesions were flat mucosal equivalents of wartlike condylomas (Meisels & Fortin, 1976; Purola & Savia, 1977).

The spectrum of lesions with cytologic and histologic features between normal cervical epithelium and carcinoma in situ was then classified as dysplasias (Reagan & Hamonic, 1956) and subclassified into either mild, moderate or severe depending on the degree of thickness of the cervical epithelium replaced by undifferentiated neoplastic cells (Reagan et al., 1969). In the mid 50’s, it was also reported that inflammation was present in 32% of dysplasias, 68% of carcinomas in situ and 98% of invasive cervical cancers (Reagan & Hamonic, 1956). With the purpose of emphasising a single continuous disease entity, a new terminology was proposed and replaced the initial classification of premalignant lesions of the cervix. The term cervical intra-epithelial neoplasia (CIN) replaced dysplasia.
and the subclassification CIN 1, CIN 2 and CIN 3 replaced respectively the terms mild, moderate and severe dysplasia along with carcinoma in situ (Richart, 1968).

Based on at least one cytological follow-up study, the model of a continuum progression of neoplastic lesions from cervical dysplasia to carcinoma in situ and invasive cancer has become generally accepted (Richard & Barron, 1969). However, other studies failed to confirm the claim (Jordan et al., 1969; Coppleson & Brown, 1975; Ishiguro et al., 1983, Burghardt, 1985). The continuum model or steady progression of mild to moderate to severe dyskaryosis was also rejected by other authors (Miller, 1985; Koss, 1986; Kirby et al., 1992).

As described later in this thesis in the section of the results involving patients from Recife (Brazil), one case of CIN 1 was diagnosed in a 13 year-old girl, one case of CIN 2 was diagnosed in a 14 year-old girl, and one case of invasive cervical cancer stage IB was detected in a young girl aged 16 years. These findings raise serious concerns with regard to public health service policies for dealing with the management of cervical neoplasia. A similar case occurred in 1999 at the Cancer Hospital in Recife where a histologically proven CIN 3 was diagnosed in a 15 year-old girl. This led to a thorough debate on the subject because the Brazilian National Health Service could not refund the money for treatment under the premise that this particular pathological entity does not occur at this age, according to the guidelines for cervical neoplasia (Porciuncula, 1999, personal communication). The technical guidelines reported by the World Health Organisation based on cytological screening suggest that there is a typical sequence of events from CIN 1 to invasive cervical cancer, which takes 23 to 38 years (World Health Organisation, 1988). The Canadian Task Force postulated that it requires a 10-year interval for cervical dysplasias to become carcinoma in situ and 35 years more for the carcinoma in situ to become invasive cervical cancer (Walton’s Report, 1976). This postulate is clearly a simplification otherwise the former Argentinean first lady, Eva Duarte Peron, would not have died at age 33 years of cervical cancer. And so many other young women who have been diagnosed with cervical cancer, including the case of the 16 year-old patient (Lorenzato et al., 2001), would not have had time to develop it. Mathematical models have been devised to reduce the frequency of screening tests aiming to increase cost-benefit without detriment to the efficacy of the screening protocol (Brown & Wells, 1986). However, these mathematical models are based on assumptions about progression.
and regression rates of different grades of CINs primarily assessed by cytological interpretations of cervical smears which have some limitations (Bearman et al., 1987). Furthermore, some of these mathematical models are based on screening histories of women aged 35 to 64 years during the 60's and 70's (IARC working group, 1986), which may not be typical in the present day situation.

A report on the natural history of cervical neoplasia described that among women with mildly abnormal cytology lost to follow-up, about 50% of them later presented with progression of the grade of cervical neoplasia (Spriggs, 1981). It has been estimated that the relative risk for women with cytological abnormalities corresponding to subclinical HPV infection to develop CIN 3 over the following 6 years was 15.6 compared to women without HPV infection by cytology. In women younger than 35 years this risk was raised to 38.7 (Mitchell et al., 1986). Based on histological assessment of cervical HPV infection, approximately 33.3% of women with HPV in the cervix develop CIN within a year (Nash et al., 1987). Another study described that 16% of women with histological diagnosis of cervical HPV infection developed HSIL and microinvasive carcinoma of the cervix within one year (Walker et al., 1986). A prospective follow-up study involving 513 women with cytological abnormalities suggestive of cervical HPV infection, showed that 15% of the abnormalities regressed, 60% persisted and 25% progressed to CIN within 2 years (Syrjänen et al., 1987). Similar results were described among 100 women with mild cervical atypia who were followed up to 2 years with cytology, colposcopy and viral tests: 16% regression, 58% persistence and 26% progression (Campion et al., 1986). An interesting study showed that among women initially HPV negative and with normal cytology, 28% of those who developed persistent HPV infection after 2 years ended up with CIN 2 or 3 (Koutsky et al., 1992). A critical review of the literature on the natural history of cervical neoplasia described a progression rate from CIN 1 to CIN 3 of 11% and a regression rate of CIN 1 lesions of about 57% (Ostor, 1993). In a meticulously carried out follow-up study from 6 months to 10 years, Koss et al. (1963) have shown beyond doubt that single-point biopsies (usually 4-point punches) are perfectly capable of locally eradicating areas of in situ cervical carcinoma. Particularly in cases with long-term follow-up and with several sets of biopsies at intervals of several months or years. Even so, it did not prevent its regrowth in other areas of the cervix. The fact that 3 borderline lesions progressed to carcinoma in situ of the cervix within 1, 2, and 6½ years, respectively, clearly points out the necessity for very close long-term follow-up of minor
cell abnormalities. Among 25 cases of in situ cervical carcinoma that regressed after biopsy, 13 (52%) did not present regrowth of a lesion (Koss et al., 1963).

The definitive diagnosis of cervical cancer is given by histopathologic examination of satisfactory biopsy specimens. The most common histologic types of cervical cancer are squamous cell carcinoma, adenocarcinomas and mixed cell carcinomas like adenosquamous carcinomas among others, although other types such as verrucous carcinoma, sarcomas, lymphomas, transitional cell carcinoma and, rarely, metastatic melanomas also occur in the cervix.

The revised classification of pathology of the uterine cervix according to recommendations of the International Society of Gynecologic Pathologists is shown in Table VII (Anonymous, 1990):

After the introduction of a mucin stain, preferably PAS/Alcian blue, as part of the routine histopathological examination, many tumours initially diagnosed as poorly differentiated squamous cell carcinomas based on haematoxylin and eosin stain were actually found to be poorly differentiated adenocarcinomas or adenosquamous carcinomas (Benda et al., 1985; Ireland et al., 1987; Buckley et al., 1988). This improvement in the staining of histological slides led to a change in the reported incidence of cervical cancer of the squamous cell carcinoma type that used to be reported as 85 to 90%. It is now estimated that about 25% of cervical cancers are adenocarcinomas and a roughly similar proportion are adenosquamous carcinomas. Squamous cell carcinomas are, therefore, responsible for 50 to 70%, at most, of all cervical carcinomas (Buckley & Fox, 1989). This may at least partially explain the epidemiological observations that the incidence of adenocarcinomas has been increasing.

A multicentric Gynecologic Oncology Group (GOG) study undertaken in the United States of America involved a total of 813 patients with cervical cancer stage Ib (> 3-mm invasion) without paraaortic nodes involvement or gross extra-cervical disease was primarily aimed at evaluating the association of cell type with disease-free interval and survival after radical hysterectomy (Look et al., 1996). This study included 645 women with squamous cell carcinomas (79%), 104 with adenocarcinomas (13%) and 64 with adenosquamous carcinomas (8%). The authors described no statistically significant
<table>
<thead>
<tr>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinizing</td>
</tr>
<tr>
<td>Non-keratinizing</td>
</tr>
<tr>
<td>Verrucous</td>
</tr>
<tr>
<td>Papillary transitional</td>
</tr>
<tr>
<td>Lymphoepithelioma-like</td>
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<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>Endometrioid</td>
</tr>
<tr>
<td>Clear cell</td>
</tr>
<tr>
<td>Minimal deviation</td>
</tr>
<tr>
<td>Well-differentiated villoglandular</td>
</tr>
<tr>
<td>Serous</td>
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<tr>
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<table>
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<tr>
<th>Other epithelial</th>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Adenoid basal</td>
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<tr>
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<tr>
<td>Small cell</td>
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<tr>
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Table VII. Cervical cancer histopathologic classification by the International Society of Gynecologic Pathologists, 1990.
differences ($P = 0.29$) in disease-free interval among cell types, but the survival of patients with adenosquamous carcinomas, even after adjustment for capillary-lymphatic involvement, GOG performance status, depth of invasion and clinical tumour size, was significantly worse than the survival of patients with squamous cell carcinomas ($P = 0.02$) or adenocarcinomas ($P = 0.007$).

The endocervical type and the minimal deviation type account respectively for about 70% and 10% of cervical adenocarcinomas (Young & Scully, 1990; Hurt et al., 1977). There is a clear-cut association between minimal deviation adenocarcinomas and the Peutz-Jeghers syndrome (McGowan et al., 1980; Kaku et al., 1985; Chen, 1986). The Peutz-Jeghers syndrome is a rare autosomal-dominant disorder characterised by hamartomatous polyposis of the gastrointestinal tract and melanin pigmentation of the skin and mucous membranes and it is frequently associated with some rare malignancies such as ovarian sex cord tumours with annular tubes, cervical adenoma malignum and testicular sertoli cell tumours. The gene LKB1/STK11 located on chromosome 19p13.3 and encoding a serine threonine kinase is thought to act as a tumour-suppressor gene; it is reported to be defective in patients with Peutz-Jeghers syndrome and some cancers (Westerman & Wilson, 1999; Westerman et al., 1999). The majority of authors agree that the prognosis for minimal deviation adenocarcinomas is unduly poor (Kaku et al., 1985; Gilks et al., 1989; Rahilly et al., 1992). The villoglandular adenocarcinomas are reported to present as friable papillary or polypoid tumours, affecting relatively young women and having a good prognosis (Young & Scully, 1989; Jones et al., 1993). The clear cell adenocarcinomas of the cervix are histologically identical to clear cell adenocarcinomas of the ovary, endometrium and vagina, and a number of them but not all occur in young women who have been exposed to diethylstilboestrol during prenatal life (Puri et al., 1977; Kaminski & Maier, 1983; Anderson & Robboy, 1996). Glassy cell carcinomas of the cervix are uncommon but aggressive tumours that affect young women (Lotocki et al., 1992).

The small cell tumours are usually clinically and macroscopically indistinguishable from squamous cell carcinomas, mostly affect women in the 5th decade of life, behave very aggressively and are associated with a high risk of early recurrence (Field et al., 1964; Mackay et al., 1979). They are part of a heterogeneous group of malignant neoplasms, the true small cell squamous cell cervical carcinomas are a minority of misdiagnosed
neuroendocrine tumours; the neuroendocrine small cell carcinomas account for about 2% of all cervical cancers (Clement, 1990). The neuroendocrine cervical cancers are highly malignant and associated with extremely poor prognosis, and in the majority of cases they are associated with HPV 18 (Perrin & Ward, 1995; Stoler et al., 1991). Another reason for the specific identification of small cell cervical carcinomas and their exclusion from or isolated analysis in trials evaluating squamous cell carcinomas or adenocarcinomas is the fact that surgery or radiotherapy alone for the treatment of these peculiar cancers, even at early stages, has been shown to be inadequate. A study showed a recurrence rate of 100% and a mortality rate within 8 to 31 months of 86% for patients with neuroendocrine small cell carcinoma of the cervix stage Ib or IIa treated with surgery or radiotherapy (Sheets et al., 1988).

Table VIII showns the updated histologic classification of neoplasia of the cervix by Jo Ann Benda and Richard Zaino (1996).

**Comments on cervical carcinoma specific cell types:**
The following comments are details and part of the histologic classification of cervical neoplasia by Benda and Zaino (1996):

**Keratinizing:**  Well formed keratin pearls are necessary, and a single pearl is sufficient.

**Large cell non-keratinizing:**  Single cell keratinization may be present. Interacellular bridges present. (Mucicarmine stain may be used to exclude poorly differentiated adenosquamous carcinoma).

**Verrucous carcinoma:**  A distinct acanthotic, papillary growth pattern with minimal cellular atypia invades by large, bulbous masses, without distinct nest infiltration.

**Warty carcinoma:**  A papillomatous surface growth with keratinization is present, but typical invasion by nests of cells with desmoplasia.

**Papillary transitional:**  Basaloid cell population growing in papillary configuration resembles papillary transitional cell carcinoma of the bladder.

**Small cell carcinoma:**  Tumours resemble oat cell or intermediate cell type lung carcinoma. With special techniques, subsets of squamous, glandular or neuroendocrine types may be identified. All small cell carcinomas should be excluded from cervix protocols for squamous carcinoma or adenocarcinoma. It
I. Epithelial Neoplasia

A. Squamous

1. Squamous intraepithelial lesions
   a) Cervical intraepithelial neoplasia (CIN) 1, mild dysplasia
   b) CIN 2, moderate dysplasia
   c) CIN 3, severe dysplasia, carcinoma in situ

2. Invasive squamous cell carcinoma (see comments on specific cell types)
   a) Keratinizing
   b) Nonkeratinizing
   c) Verrucous
   d) Warty (condylomatous)
   e) Papillary (transitional)
   f) Lymphoepithelioma-like

B. Glandular lesions

1. In situ lesions
   a) Endocervical glandular dysplasia (EGD)
   b) Adenocarcinoma in situ (ACIS)

2. Invasive adenocarcinoma
   a) Mucinous (endocervical, intestinal, signet ring cell)
   b) Endometrioid (may have squamous differentiation)
   c) Clear cell
   d) Minimal deviation (adenoma malignum)
   e) Serous
   f) Mesonephric
   g) Villoglandular

C. Other invasive tumours (see comments on specific cell types)

1. Adenosquamous
   a) Gland forming
   b) Large cell carcinomas with intracellular mucin (see comments)

2. Glassy cell

3. Adenoid cystic

4. Adenoid basal
5. Carcinoid
6. Small cell (see comments)
7. Undifferentiated

D. Collision tumours

II. Mesenchymal Malignancies

A. Leiomyosarcoma
B. Endocervical stromal sarcoma
C. Sarcoma botryoides (embryonal rhabdomyosarcoma)
D. Alveolar soft part sarcoma
E. Endometrioid stromal sarcoma
F. Other

III. Mixed Epithelial and Mesenchymal Malignancies

A. Adenosarcoma
B. Malignant mixed mesodermal (mullerian) tumours (carcinosarcoma)
   1. Atypical polypoid adenomyoma
C. Wilm's tumour

IV. Miscellaneous Malignancies

A. Malignant melanoma
B. Lymphoma and Leukaemia
C. Germ cell
   1. Yolk sac tumour
   2. Mature cystic teratoma

V. Secondary tumours

Table VIII. Histologic classification of cervical neoplasia by Benda & Zaino (1996).
must be specifically stated that small cell carcinomas are being included in a protocol.

**Adenosquamous:** Includes tumours with glandular and squamous growth patterns. Those large cell carcinomas with squamous differentiation and intracellular mucin have been called poorly differentiated adenosquamous (preferred terminology), mixed tumours, or mucoepidermoid. Without special stains, most of these would be designated large cell non-keratinizing. These tumours should be specifically identified as different from the clearly gland forming types of the adenosquamous carcinomas. Endometrioid carcinoma with squamous differentiation should be so designated, and not called adenosquamous.

**Glassy cell carcinoma:** For purposes of Gynecologic Oncology Group (GOG) protocols, glassy cell carcinoma will be considered a subset of adenosquamous carcinoma and eligible for any protocols that include adenosquamous carcinomas.

**Collision tumours:** Definite co-existing squamous cell carcinomas and adenocarcinomas that grow into each other should be noted, and not called adenosquamous.

These details in histologic reporting are important for comparisons of treatment outcomes and for a better understanding of why cervical cancers acquire such variable tissue differentiation. There also exists a grading system for squamous and adenocarcinomas but it seems to be of little predictive value.

**7. Treatment**

Taking into account the rationale used by the Bethesda system to simplify the reading of cytological smears, thus decreasing the margin of cytological errors compared to histological assessments, improvements in the management of premalignant cervical lesions should be possible if HPV detection and typing can be proven to differentiate women more likely to have progression of a precursor lesion from those whose lesions will spontaneously regress. Therefore the possible overtreatment of lesions that would regress could be avoided yet providing scientific basis for reassurance of the patients to overcome the anxiety associated with having a premalignant lesion being managed conservatively.
At the other extremity of the spectrum, it has been demonstrated that patients with advanced cervical cancer stages IIb and III have metastatic involvement of peri-aortic lymph nodes in 21% and 31.5% of cases, respectively (Heller et al., 1990). It is generally accepted that metastatic involvement of peri-aortic lymph node considerably affects negatively the clinical outcome of patients with cervical cancer since the possibility of widespread disease is higher. Given that radiotherapy attempts to control locoregional malignant disease, the 3-year survival rate for cervical cancer patients without either pelvic or peri-aortic lymph nodes involvement was reported to be 90% compared to 45% in patients with pelvic and peri-aortic lymph nodes involvement (Twiggs et al., 1984). It therefore is at least philosophically plausible that an effective yet tolerable chemotherapeutic scheme, if used as a primary line of therapy for patients with advanced cervical cancer not eligible for surgical treatment, should improve clinical outcome, particularly if followed by either surgery or radiotherapy or both.

7A. Premalignant lesions

CIN 1 is usually managed conservatively since it shows a high rate (approximately 70%) of spontaneous regression or no progression (Nasiell et al., 1986; Campion et al., 1987; Jones et al., 1992; Kurman et al., 1992; Syrjänen et al., 1992; Ostor, 1993; Flannelly et al., 1994; Holowaty et al., 1999) and there is no method available as yet to predict which will progress and which will not. In a follow-up study with a mean period of 18.7 months, 343 women with cytological evidence of HPV infection were followed by colposcopy with or without biopsies among which 25% of lesions regressed, 61% persisted and 14% progressed to carcinoma in situ (Syrjänen et al., 1985). Treatment for CIN 1/LSIL is still a controversial issue. Cases of HPV-associated cervical lesions have been recommended to be treated just as other dysplastic lesions (Kaufman et al., 1983). The American Center for Disease Control (CDC) guidelines reported in 1998 also recommended that HPV-associated infections be treated to prevent widespread of these sexually transmitted oncogenic viruses. However some trials are on the way to evaluate possible strategies that can provide information on prediction of lesion outcome, thus decreasing the chances of overtreatment and at the same time reassuring doctors and patients that the chosen approach is the best management option. It is generally agreed that all CIN 2 and CIN 3 should be treated by complete excision of the lesion using methods such as the classical knife cone biopsy, laser excisional cone biopsy or diathermal large loop excision of the transformation zone (LLETZ). The use of low voltage diathermy loop was initially introduced for the purpose of diagnosis and treatment of cervical neoplasia in France and
it was presented at the World Congress of Cervical Pathology and Colposcopy in 1981. The original method used a small loop of 0.5 cm to excise the transformation zone in strips (Cartier et al., 1981). Later, Prendiville reported the use of low voltage diathermy loop for taking cervical biopsies and introduced the term LLETZ where by a large diathermy loop was used to remove the transformation zone in one or two fragments (Prendiville et al., 1986, 1989).

Excisional methods are preferred as opposed to destructive treatment modalities such cautérisation or laser vaporisation since a much better assessment of the lesion can be performed by histological examination of the excised tissue, particularly to determine if the margins of resection are clear of disease, and to rule out the presence of microinvasive or fully invasive cervical carcinoma. In order to avoid excessive bleeding, burning or excision of unnecessary vaginal tissue, and cervical stenosis, especially in women of childbearing age, it is important to carry out the excisional procedure with caution. In some cases, for example when a patient in addition to the HSIL has fibroids and defined parity or in older women whose transformation zone is inside the endocervical canal and can not be seen adequately, hysterectomy is performed.

Electrocoagulation, cryotherapy and laser vaporisation for tissue destruction of histologically proven cervical lesions and the transformation zone have also been used, but these methods do not provide material for histologic documentation of what has been removed and may be associated with higher rates of recurrence as described later (Townsend et al., 1981). Electrocoagulation (also called cautery or electrodiathermy) and sharp shallow conisation were reported in the late 40's to treat, successfully and without resulting in sterilisation, approximately 85% of 135 young patients with cervical carcinoma in situ (Younge et al., 1949). Eight years later, Younge reported at the Third National Cancer Conference that the conservative treatment of carcinoma in situ with cautérisation resulted in a 70% cure rate (Younge, 1957). Other studies evaluating the success rate of cautery in the treatment of carcinoma in situ showed that it varies from 90% to 97.3% (Richart & Sciarra, 1968; Chanen & Hollyock, 1971, 1974; Chanen & Rome, 1983). It is extremely important though that invasive disease be definitely ruled out and that the patient be followed for a long period. In at least one case of an initially histologically reported CIN 2 treated with cautery at the Cancer Hospital in Recife (Brazil) the patient was 19 years later diagnosed with a
stage IIIB squamous cell carcinoma of the cervix, presenting in the mean time with negative cytology reports.

Cryotherapy was one of the first destructive modalities clinically used for the treatment of premalignant cervical disease (Crisp et al., 1970). But it leads to a profuse and long-term discharge and, since the depth of destruction (usually 5 to 6mm) cannot be closely controlled (Crisp, 1971), unsatisfactory cure rates ranging from 25 to 75% may result as early reported by Creasman et al., (1973). A decade later, the use of an effective double freeze cryotherapy technique based on the depth of tissue destruction rather than on time of freeze produced better results (Figge & Creasman, 1983). Laser vaporisation has been used extensively. It enables more precise tissue destruction, including vaporisation of lateral vaginal and vaginal cuff lesions, it also leaves less necrotic tissue, heals faster than and has similar success rates as cryotherapy (Wetchler, 1984).

It was in the mid 50's that cold knife biopsy became the indication for excision of carcinoma in situ and a method of diagnostic cervical biopsy for study of early cervical cancer (Thornton et al., 1954; Scott & Reagan, 1956). And nowadays knife cone biopsy may be preferable to loop excision when a deep cone with intact margins is required or when there is suspicion of microinvasion or adenocarcinoma.

Below are the results of some studies on treatment of premalignant lesions. Complete removal of CIN 3 by conisation was reported to have been the adequate treatment, achieving cure of all 634 women in whom no recurrences or progression of disease were observed (Burghardt & Holzer, 1980). On the other hand, other authors report recurrences of carcinoma in situ (ranging from 0.8 to 2.4% within 2 to 11 years) and progression to invasive cervical carcinoma (ranging from 0.9 to 2.1% within 4 to 11 years) of adequately treated cases of CIN 3 either by conization, cervical amputation, simple hysterectomy, radiotherapy or a combination of surgery plus radiotherapy (McIndoe & Green, 1969; Boyes et al., 1970; Kolstad, 1970; Creasman & Rutledge, 1972; Kolstad & Klem, 1976; McIndoe et al., 1984). The study by Creasman & Rutledge (1972) involving 861 women with carcinoma in situ treated as just mentioned reported a 2.9% recurrence rate (25 patients and 27 recurrences) for carcinoma in situ (20 cases) and the development of invasive cervical carcinoma (7 cases), among which 48% occurred within 2 years after the primary treatment and 20% occurred 5 years or more after treatment (Creasman &
Rutledge, 1972). McIndoe et al (1984) observed that 1.5% of women presenting with negative smears as opposed to 22% of women presenting with abnormal smears developed invasive cervical cancer after adequate treatment for CIN 3 during a median follow-up of 4 years. The risk of developing invasive cervical cancer after adequate excision of CIN 3 and negative follow-up cytology compared to negative cytology in women who had never had CIN 3 was calculated as being 3.2. The risk of women presenting with abnormal cells on follow-up cytology after proper management of CIN 3 to develop invasive cervical disease compared to women with same age and normal cytology was increased 24.8 times. Five of 12 patients who underwent complete excision of a CIN 3 and who subsequently presented with negative Pap smears ended up developing invasive cervical carcinoma (41.7%), compared to only 5 of 139 patients who had incomplete excision of a CIN 3 and ended up with invasive disease (3.5%). Thus raising the hypothesis that, irrespective of whether or not a CIN 3 is completely excised, free margins do not seem to be the major variable influencing subsequent recurrence of the lesion or the possibility of development of invasive cervical cancer (McIndoe et al., 1984). In addition, it highlights that post-treatment cytological follow-up is not a good predictor of clinical outcome. It is important to remember that surgical tissue aggression is considered to activate the immune system. Another important factor may be the possible presence of untreated HR-HPV lesion or infection in the partner. This form of reservoir may act as a source of re-infection and persistent infection that is known to increase both the risk of development and progression of cervical neoplasia, and deserves further investigation in this setting – post-treatment follow-up.

A follow-up study of up to 8 years involving 2,116 women who underwent treatment for CIN in different institutions in the United Kingdom reported that women who had been treated for CIN were still at a 5-fold greater risk of developing invasive cervical cancer than women who never had CIN (Soutter et al., 1997). Another study described that 53 out of 66 patients (80%) who underwent ablative treatment for CIN had recurrence within one year (Townsend et al., 1981). Here again, information regarding persistence of HPV infection may help in the clinical guidelines for post-treatment follow-up.

7B. Invasive cervical cancer
Surviving documents registering attempts to treat cervical cancer describe that the Greeks used local fumigation therapy for hygienic purposes and symptomatic relief. In the 6th
century AD, Aetius of Amida used vaginal irrigation with herbal solutions and baths or poultices to minimise discomfort. Later, it was attempted to inject the tumours with extract of nightshade (Astruc, 1762). In the 19th century, leeches, red-hot cautery and caustics were tried. It was observed that cervical cancer was chemosensitive and remission could be induced in patients with advanced disease. It was believed that chemotherapeutic drugs had a role to play in the treatment of cervical cancer before surgery and radiotherapy were considered (O’Dowd & Philipp, 1994).

After all advances in surgery and radiotherapy for the treatment of invasive cervical neoplasia, the data still show that there is room for improvement in the currently available treatment protocols. Table IX summarises some studies on patient survival after surgery and/or radiotherapy.

It has been reported that from 1945 to 1954 the treatment modality currently available in four United Kingdom centres resulted in an overall 5-year survival rate ranging between 40 and 41.3%, the rate in seven centres was under 38%, and in one centre it was only 26.1% (Lederman, 1964). The reported figures of 5-year survival rates for the Royal Marsden, Chelsea, Hammersmith, University College and Middlesex Hospitals in London from 1944 to 1972 were as follows: Stage I (71%), stage II (48%), and stage III (21%) (Mould & Staffurth, 1979). Even after a period of 20 years from 1949, cervical cancer survival rates following the treatment of choice did not improve, particularly for stage II and above (Jolles, 1980). The percentage of patients alive after 5 years of treatment actually decreased over time.

The choice of treatment for each patient with cervical cancer will depend on a number of factors such as patient’s determination to preserve reproductive and sexual functions, age, tumour volume, depth of invasion, lymphatic or vascular involvement, parametrial extension, and extrapelvic involvement. A report involving a large number of women with stage Ib squamous cell carcinoma of the cervix showed that the 5-year survival rate among 2,600 patients treated with radical surgery was 83.4% compared to a similar survival of 85.5% among 1,995 patients treated with radiotherapy (Delgado, 1978). However, among other advantages, surgery is a shorter treatment, may preserve the functional ovaries by repositioning them away from the radiation field, and offers the
<table>
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<th>References</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Mean</th>
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<tr>
<td>Blaikley et al., 1962</td>
<td>71%</td>
<td>45%</td>
<td>19%</td>
<td>4%</td>
<td>35%</td>
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<td>Kottmeier, 1976</td>
<td>80%</td>
<td>59%</td>
<td>32%</td>
<td>7%</td>
<td>45%</td>
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<tr>
<td>Jolles, 1980 (1949-1953)</td>
<td>67%</td>
<td>59%</td>
<td>33%</td>
<td>6%</td>
<td>41%</td>
</tr>
<tr>
<td>Jolles, 1980 (1964-1968)</td>
<td>70%</td>
<td>58%</td>
<td>25%</td>
<td>8%</td>
<td>40%</td>
</tr>
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Mean survival by stage  
72%  55%  27%  6%  40%

Table IX. Five-year survival rates after surgery and/or radiotherapy as the treatment modality for cervical cancer.
possibility of removing as much as possible of the malignant tissue, thus giving a better assessment of the disease extent and prognosis. An analogy with regards to the latter surgical advantage mentioned over radiotherapy could be made in relation to treating HSIL with loop excision or knife cone biopsy as opposed to laser vaporisation. But surgery has its limitations. Cervical cancer stages IIb and beyond are currently best not treated with surgery since a safe tumour-free margin of resection is often not feasible and the risks of surgical complications are increased, instead radiotherapy is the treatment of choice for advanced cases of the disease. Also, patients over 70 years, considerably obese, with serious cardiovascular or pulmonary disease, or with decompensated or complicated diabetes do not usually make a suitable candidate for surgical treatment (Nordqvist et al., 1979) and are primarily treated with radiotherapy.

Some authors do not recommend lymphadenectomy for patients with verrucous carcinoma of the cervix as they are reported to rarely metastasise, and radiotherapy is thought to be detrimental for patients with this particular histologic type of cervical carcinoma (Partridge et al, 1980; Kawagoe et al., 1984).

7B.a) Surgery

The evolution of surgical treatment for cervical cancer has flourished concomitantly with the improvements in diagnosis and prevention. In 1575, Ambrose Pare recommended the amputation of the cancerous cervix as a form of treatment. Attempts to surgically remove the cervix are reported to have begun in the 16th century. The first successfully performed cervical amputation for the treatment of cervical cancer was out by Tulpius of Amsterdam in 1652. However the survival rate remained very poor. In 1813, Conrad J. M. Langenbeck performed the first intentional simple vaginal hysterectomy in patients with cervical cancer. Sauter performed the same procedure in 1821. As a consequence of the high mortality rate related to operative complications such as shock, haemorrhage and peritonitis, vaginal hysterectomies for the treatment of cervical cancer temporarily fell into disfavour (Ricci, 1945).

In 1895, C. Rumpf in Germany and J. G. Clark, a then resident physician at the John Hopkins Hospital in the United States, were the first to begin performing total abdominal hysterectomies combined with removal of the iliac lymph nodes and part of the broad ligament as the primary treatment of cervical cancer. Friedrich Schauta from Vienna has
developed a vaginal approach for radical hysterectomies where the uterus and parametrium were radically removed, yet resulting in an operative mortality rate of 3 to 4% in 1901, considerably lower than the abdominal approach (Schauta, 1890, 1902). Radical vaginal hysterectomy became popular in Europe, but the inability to dissect the pelvic lymph nodes, through which there is early spread of the malignancy, made it difficult for widespread acceptance of the procedure in the United States.

In 1912, Ernest Wertheim (1864-1920) reported his vast experience with radical hysterectomies where he removed the uterus, the medium portion of the parametrium and paracolpos, and performed a selective lymphadenectomy, removing only enlarged nodes or nodes suspicious for metastasis. The first radical hysterectomy performed by Wertheim was on the 16th of November 1898 and it took about two and a half hours. Among his 500 cases operated as described above from basically 1899 to 1912, the procedure resulted in complication and mortality rates of 31.5% and 18.6%, respectively (Wertheim, 1912).

By the beginning of the 20th century, the surgical procedure of choice was aimed at removing as much as possible of the parametrium, the vaginal cuff was widely excised in some cases but the lymph nodes were not routinely removed. J. C. Clark after analysing the results of the different surgical procedures under investigation at that time, including Wertheim’s abdominal hysterectomy, simple vaginal hysterectomy and radical vaginal hysterectomy, acknowledged that Wertheim should receive the credit for developing and simplifying the radical abdominal operation for cervical cancer treatment (Clark, 1913).

In 1913, there was a scientific meeting in Halle an der Saale at which Wertheim was supposed to present his extensive experience with treatment of cervical cancer by radical abdominal surgery (he had been operating on 1 case every 10 days for over 8 years and it would then take him only 60 to 75 minutes). However, after hearing the enthusiastic and less morbid therapeutic results for the use of radiotherapy, Wertheim withdrew his paper. Yet again, due to high complication rates by urinary fistulas and the mortality associated with this radical surgery in its early days, the procedure was temporarily abandoned in the United States.

Fred J. Taussig during the period of 1930 to 1942 did a trial using the combination of radiotherapy and iliac lymphadenectomy in 175 women with stage IIb cervical
carcinoma, which resulted in an operative mortality rate of 1.7% and a 5-year survival rate of 38.6% (Taussig, 1943). Joe Vincent Meigs modified Wertheim’s radical abdominal hysterectomy by adding to it the routine bilateral pelvic lymphadenectomy, which Taussig demonstrated to him, and the removal of as much paracervical and paravaginal tissue as possible, as suggested by C. Kauffmann of Marburg. The results were that in 25 patients with FIGO stage II cervical carcinoma and 57 patients with stage I disease, the 5-year survival rates were respectively 60.7% and 80.7%, and there were no operative deaths (Meigs, 1951).

It has been observed that 3.9% to 9.4% of patients with microinvasive cervical carcinoma with stromal invasion between 3 and 5 mm and with a width not exceeding 10 mm (Lohe’s classification, 1978), which is close to the current stage Ia2, already have lymph nodal metastasis, and for this reason the authors recommended a total abdominal hysterectomy and routine pelvic lymphadenectomy as the adequate treatment in these cases (van Nagell et al., 1983; Simon et al., 1986). The detailed histologic investigation by Nancy Simon and collaborators involved 91 cases of cervical cancer patients with invasion up to 3 mm and 34 cases with invasion between 3.1 to 5 mm in depth and 0.08 to 20 mm (median = 4.8 mm) in width, which is close to stages Ia1 and Ia2, and who were treated with either conization (7%), vaginal (23%) or abdominal hysterectomy (15%), or radical (42%) or modified radical hysterectomy (14%) with pelvic lymphadenectomy. They reported a total of 5.6% of cases with lymphvascular space involvement, among which none had lymph node metastasis and only one had single focal parametrium involvement. Of the 69 patients treated with radical or modified radical hysterectomy with pelvic lymphadenectomy, including 43 (62%) who had invasion up to 3 mm, only 1 patient (1.4%) had lymph nodal metastases, based on these findings and review of the literature they proposed that cervical cancer with depth of invasion up to 3 mm and no lymphvascular space involvement may be treated with either total abdominal or vaginal hysterectomy. If lymphvascular space involvement is present it is reasonable to perform a modified radical hysterectomy with pelvic lymphadenectomy. It is also noted that in a compiled analysis of their 69 cases with 670 others previously reported (van Nagell et al., 1983; Hasumi et al., 1980; Seski et al., 1977; Leman et al., 1976; Yajima & Noda, 1979; Taki et al., 1979; Boyce et al., 1981; Bohm et al., 1976), one can conclude that there is a highly significant difference between cervical cancers with stromal invasion up to 3 mm, whose lymph node metastatic rate was ≤ 1% and those with stromal invasion ranging
from 3.1 to 5 mm in depth, whose lymph node metastatic rate was 8%. And adding their cases to others previously reported and also long-term follow-up studies (van Nagell et al., 1983; Hasumi et al., 1980; Seski et al., 1977; Leman et al., 1976; Roche & Norris, 1975) made a total of 378 patients with early invasive cervical cancer with up to 5 mm of stromal involvement, from which 54 (14.3%) had lymphvascular space involvement and only of these had lymph node metastasis (1.9%) (Simon et al, 1986).

Nowadays, the procedure widely used as the surgical treatment of choice for early invasive cervical cancer, usually FIGO stages 1a2 to IIA, is a combination of the radical abdominal hysterectomy of Wertheim and the routine bilateral pelvic lymphadenectomy that Meigs proved could result in satisfactory 5-year survival and operative mortality rates. Cervical cancers arising from a residual stump from a previous subtotal hysterectomy may either be treated with surgery or radiotherapy, the choice of treatment should follow the same principles applied to the intact uterus. In case of surgery, vesical and intestinal adhesions may be found on the previous surgical site. Surgery is also the treatment of choice for operable invasive cervical cancers diagnosed during pregnancy (Barber & Brunschwig, 1963; Barber, 1989).

In a series involving 628 patients with cervical cancer stage Ib to IIb who underwent radical hysterectomy and pelvic lymphadenectomy, parametrial involvement was found in 7% of stage Ib cases, 23% of stage IIa, 34% of stage IIb. When comparing lymph node involvement with presence or absence of parametrial involvement, the authors observed a considerable increase in rates of lymph node metastasis from 12 to 32% in stages Ib cases and from 9 to 70% in stages IIa cases (Inoue & Okumura, 1984). In cervical adenocarcinomas, the prevalence of lymph node involvement among 102 women who underwent radical hysterectomy and lymphadenectomy has been reported as 14.6% for stage I and 40% for stage II (Berek et al., 1985). At least 2 studies reported that bilateral lymph nodal involvement leads to a considerable decrease in 5-year survival rates when compared to unilateral lymph nodal involvement. The mean 5-year survival rate reported in these studies was 58.1% for unilateral lymph nodal involvement and 28.5% for bilateral involvement (Hsu et al., 1972; Pilleron et al., 1974). Another study has reported that in patients with negative parametrial margins on the radical hysterectomy specimen the 5-year survival rate was 94.4%, in contrast with 40.8% in patients with involved parametrial margins. The 5-year survival was 82% for patients without endometrial cavity
involvement compared to 45% for patients with tumour extension into the lower segment of the uterus. They have also observed 5-year survival rates of 47.1%, 33.3% and 0% when the tumour involved the inner third, middle third and outer third of the pelvic side wall, respectively (Noguchi et al., 1983).

The presence of histologically proven metastasis in common iliac nodes is associated with a 5-year survival rate of 23%, and if lymph nodal involvement is limited to the level below the common iliac arteries, then the cure rate is reported to be 60% (Martimbeau et al., 1982). It is interesting to observe that tumour volume is the most important factor for evaluation of clinical outcome of patients with carcinoma of the cervix and yet it is not specifically accounted for in the FIGO cervical cancer staging system.

The postoperative incidence of complications associated with radical hysterectomy is reported as follows: vesical atony, defined as residual urine of over 100 ml 3 months postoperatively, occurs in about 3% of cases (Green & Morse, 1969; Langley et al, 1980). Stress urinary incontinence (SUI) is reported to occur in 10 to 50% of cases (Thornton, 1954; Forney, 1980; Kadar et al, 1983; Petri, 1984; Kadar & Nelson, 1984; Kristensen et al., 1984), however it has been argued that some of these patients with SUI diagnosed after radical hysterectomy already had it before the procedure or had an increased predisposition to develop SUI due to previous anatomophysiologic changes, and this morbid entity may have just not been properly investigated (Christ et al., 1983; Farquharson et al., 1987a, 1987b). Perineal ultrasonographic evaluation of the urethrovessical junction (UVJ) mobility and opening status is a simple and objective method of identifying and following up women with UVJ hypermobility and those most likely to develop SUI (Vierhout & Hol, 1998; Brandt et al., 2000). A study has shown that 33% of women with what they called preoperative bladder neck support weakness developed SUI postoperatively, compared to none who had normal bladder neck support (Farquharson et al., 1987a). A prospective study involving 90 patients, among which 15% were diagnosed as having SUI prior to surgery, showed that 23% of the SUI cases got worse after radiotherapy, 26% worsened after radical hysterectomy, and 63% worsened after a treatment combination of surgery and radiotherapy. Bladder compliance was reduced after radiotherapy and highly significantly reduced in patients who underwent radical surgery followed by radiotherapy (Farquharson et al., 1987b). Others (Kadar &
Nelson, 1984) have also observed the fact of increased SUI severity after the combination of radical surgery with radiotherapy as a treatment modality for cervical cancer.

The incidence of ureteral fistulae after radical hysterectomy is reported to range from 0 to 5.6% (Park et al., 1973; Langley et al., 1980). Most authors describe the incidence of lymphocyst formation, which is usually asymptomatic, after radical hysterectomy with lymphadenectomy as ranging from 2 to 9% (Lagasse et al., 1974; Benedet et al., 1980).

Averette and collaborators from the Division of Gynecologic Oncology at the University of Miami School of Medicine have reported their 25-year experience with the Miami technique, a modified Wertheim-Meigs radical hysterectomy, including 978 patients with stage IB (88.5%) and IIA cervical carcinoma. After 1970, the Miami technique incorporated routine paraaortic lymphadenectomy as well as pelvic lymphadenectomy, whereas before paraaortic lymphadenectomy was selectively done on suspicious lymph nodes and pelvic lymphadenectomy began at the level of the aortic bifurcation (Averette et al., 1993). The Miami modifications included: vaginal closure and reconstruction using bladder and rectosigmoid serosa, retroperitoneal drainage through abdominal suction catheters, and suspension of the denuded ureters with ipsilateral obliterated hypogastric artery (Hoskins et al., 1976). They reported an overall corrected 5-year survival rate of 90.1% and for the 61 patients whose surgical margins showed involvement this rate decreased to 50.8%, with a surgical mortality rate of 1.4%. Observed rates of ileus and small bowel obstruction, chronic lymphoedema, and urinary fistulae after surgery and after radiotherapy were 6%, 2.6%, and 0.8% and 0.6%, respectively. The cumulative 5-year survival rates for squamous cell carcinomas, adenocarcinomas and adenosquamous carcinomas were respectively 90.7%, 80.5% and 63.5%, showing a highly significant difference \( P = 0.0001 \) between survival rates of patients with squamous cell as opposed to adenosquamous carcinomas. The difference in survival between patients with adenocarcinomas and adenosquamous carcinomas was also significant \( P = 0.01 \), but the difference between patients with squamous cell carcinomas and adenocarcinomas was not \( P = 0.70 \). The overall incidence rates of pelvic and paraaortic lymph nodal metastasis were respectively 17.7% and 6.3%, and these were associated with highly significant decreases in survival, respectively of 63.5% and 40.8%; if the patient had a combination of pelvic and paraaortic lymph nodal involvement the 5-year survival rate was 18.4% \( P \)
< 0.000001). The recurrence rate for stage IB was 13.7% and for stage IIA it was 41.4% (Averette et al., 1993).

A study reporting a 14-year experience with cervical cancer treatment by radical hysterectomy involved 397 patients with stage Ia2 associated with lymph node involvement to stage IIA. They described that 73.1% of the tumours were squamous cell carcinoma, among which 54.5% were large cell non-keratinizing and 2% were small cell carcinomas; 26.9% were adenocarcinoma including 8.6% which were adenosquamous carcinomas and 0.5% were clear cell adenocarcinomas. The complication rates were as follows: 2.7% of patients had deep venous thrombosis, 1% had severe lymphoedema and 0.5% had vesicovaginal fistulas. Of all patients, 16.6% had lymphnode metastasis and the 5-year survival rate was 68% for patients with more than 2 lymph nodes involved by metastatic disease. Recurrences occurred in 11.8% and 9.1% of patients had died due to the malignancy. The overall 5-year survival rate was 92.2% (Sivanesaratnam et al., 1993).

7B.b) Radiotherapy
At the beginning of the 20th century, Margaret Cleaves in 1903 and also Danysz were among the first to use radiotherapy for cervical cancer treatment (Shingleton & Orr, 1987). Robert Knox from King’s College London wrote that radium was used to treat cervical cancer but he acknowledged the tendency to tumour recurrence (Knox, 1915). Since 1920 a dispute has continued among those who advocate surgery for treatment of cervical cancer and those who advocate radiotherapy.

Nowadays, the standard treatment modality for cervical cancer stages IIb to IVa is exclusive radiotherapy with a combined approach encompassing external pelvic radiation and brachytherapy, the latter being the most important component. The Patterns of Care Study undertaken in the United States of America reported that, in patients with cervical carcinoma stages I to III, the 4-year survival and pelvic control of disease rates were respectively 36% and 46% with external radiation only, and 67% and 78% with the addition of brachytherapy (Coia et al., 1990). A study undertaken at the M.D. Anderson Hospital involving patients with bulky (> 6 cm) cervical carcinomas stages Ib to IIb observed pelvic recurrence rates of 33% and 16%, respectively for patients treated with lower doses of radiotherapy to point A compared to the ones receiving higher doses (Eifel
et al., 1994). In a multivariate analysis, Lanciano observed that younger patients with cervical cancer stages I and II had a decreased rate of pelvic control of the disease, in other words an increased recurrence rate (Lanciano et al., 1991). Her findings have been similar to what was found by other authors (Kapp et al., 1983; Lowrey et al., 1992).

Radiotherapy markedly alters the vagina and is associated with significant sexual dysfunction. More specifically, radiotherapy makes sexual intercourse impossible or less pleasurable in up to 78% of patients who underwent this modality of therapy compared to 6% rate of sexual dysfunction in patients treated with surgery (Abitbol & Davenport, 1974; Bruner et al., 1993; Cull et al, 1993). The rate of major radiotherapy complications requiring hospitalisation, surgery or causing death is reported to vary from 5 to 15% (Eifel et al., 1995). A study evaluating urinary symptoms and urodynamic tests before, during, 1 to 2 months after and 5 to 6 months after radiotherapy has shown a significant ($P < 0.001$) increase in urinary residual volume and reductions in peak urinary flow rate, volume at first desire to void, cystometric capacity and bladder compliance. However, these parameters of bladder function returned to normal after 5 to 6 months post radiotherapy, except for bladder compliance that remained significantly reduced ($P = 0.05$) in patients treated with a total bladder dose over 3,000 rads (Farquharson et al, 1987c).

In patients with cervical cancer involving peri-aortic lymph nodes, the treatment of choice is extended field radiation encompassing the retroperitoneum with a dose to the peri-aortic region not exceeding 45 to 60 Gy. The 5-year survival rate for patients with low volume locoregional cervical cancer undergoing extended field radiation ranges from 20% to 40% (Rotman et al., 1994). A review on the use of radiotherapy in the management of cervical cancer reported that despite the refinements in the delivery of external radiation and brachytherapy applied in current protocols, there has been little improvement in survival and, instead, an increase in both locoregional and distant metastasis in recent years, and suggested that efforts should be directed towards new combinations of treatment modalities (Kim, 1993).

Further research is taking place to determine the optimum radiotherapy regimen. The hope for improvements in radiotherapy results rests in the new treatment planning expected from three-dimensional dose distribution, which may bring more accurate dose
delivery thus decreasing complications and increasing cure rates. Alternative combinations of treatment modalities such as neoadjuvant chemotherapy, surgery and radiotherapy protocols have also been undertaken and will certainly broaden the understanding of how best to treat advanced cervical cancer.

7B.c) Chemotherapy
Undoubtedly, surgical removal of all malignant tissue is the best treatment for cancer. Unfortunately, the majority of cervical cancer cases diagnosed in developing countries are not eligible for surgery as a primary treatment modality for reasons of technical impracticability. Neoadjuvant chemotherapy, in other words pre-operative chemotherapy, may offer potential improvements to treatment outcome for patients with locally advanced cervical cancer by reducing the number of lymph nodes with metastatic involvement but mainly by decreasing the volume of hitherto inoperable tumours.

The potential benefits associated with using chemotherapy as a primary modality of treatment for advanced cervical cancer as opposed to radiotherapy include avoidance of the poor tumour vascularization secondary to fibrosis resulting in impaired access of cytotoxic drugs to the tumour cells after radiotherapy. In addition, the radiation toxic effects on pluripotent cells, which should differentiate into blood cells, often compromise bone marrow function. Thus the primary use of radiotherapy imposes serious limitations on the level of efficacy of chemotherapy due to the negative effects of radiation on having to reduce the dosages of antineoplastic agents. It has also been reported that radioresistance due to increased DNA repair capability, enhanced free radical quenching or raised threshold for apoptosis may induce cross-resistance to the antineoplastic activity of chemotherapeutic drugs (Louie et al., 1985; Eastman & Schulte, 1988; Masuda et al., 1988; Carmichael & Hickson, 1991; Lane, 1992).

It is usually stated in the literature that chemotherapy is not effective against cervical cancer. Much of the evidence comes from non-randomised studies that show a range of cumulative responses from 10 to 58% (Piver et al., 1978; Behnam et al., 1981; Sorbe & Frankendal, 1984; Kumar & Bhagarva, 1991) and randomised studies (Wallace et al., 1978; Greenberg et al., 1977; Alberts et al., 1987) showing ranges of complete response of 0 to 22%, of partial response of 0 to 55% and of median survival of 4 to 17 months. These studies involved patients with locoregional and/or distant metastatic cervical
cancer, including those with recurrent disease after surgery and/or radiotherapy. However, chemotherapy schemes provide highest antineoplastic activity in tumours without previous surgery or radiotherapy for reasons mentioned above; its potential advantages rely on a first line treatment approach. Also, studies evaluating monotherapies and some combination schemes using neoadjuvant cisplatin alone or in combination with epirubicin in locally advanced cervical cancer followed by radiotherapy did not show significant difference when compared to exclusive radiotherapy (Tattersall et al., 1992, 1995). It is important though to try and combine drugs with the highest proven monotherapy results with the least toxic effect and follow a logical sequence of treatment modalities that may result in the best clinical outcome and quality of life for the patients.

There are some interesting prospective and randomised clinical trials using triple chemotherapy schemes as neoadjuvant treatment for locally advanced cervical cancer, followed by surgery and/or radiotherapy. A prospective randomised clinical trial involving 151 patients with squamous cell carcinoma of the cervix stage Ib bulky tumour (diameter > 2 cm or volume > 27 cm³) has been undertaken in Buenos Aires. On one arm of the study, 76 patients were treated with neoadjuvant chemotherapy, and on the other 75 patients were treated with radical hysterectomy including pelvic and paraaortic lymphadenectomy from the inferior mesenteric artery downwards plus whole pelvis radiotherapy (50 Gy). The neoadjuvant chemotherapy scheme included 3 cycles of cisplatin 50 mg/m² in day 1 (push 15 minutes), vincristine 1 mg/m² in day 1 (push), and bleomycin 25 mg/m² in days 1, 2 and 3 (continuous infusion for 6 hours), with a 10-day interval between cycles (Sardi et al., 1990a). They have observed a significant difference (P = 0.008) in disease free survival when comparing patients with tumour volume > 60 cm³: in the neoadjuvant chemotherapy group the disease free survival rate was 87.1% compared to 67.5% in the surgery plus radiotherapy group. For tumours with volume ≤ 60 cm³ or diameter < 3-4 cm, the authors did not find a significant difference in disease free survival and do not recommend neoadjuvant chemotherapy. The 4-year survival rate was also significantly better in the neoadjuvant chemotherapy arm (88%) as opposed to 60% in the surgery plus radiotherapy arm (P = 0.05).

Sardi and collaborators have undertaken another study using the same neoadjuvant chemotherapy scheme as described above, but with a different design. This time, it was a phase II clinical trial involving 151 women with advanced cervical cancer who underwent
neoadjuvant chemotherapy as above and were followed up for at least 2 years (107 with stage IIb and 44 with stage IIIb). It also included 101 non-randomised patients (51 with stage IIb and 50 with stage IIIb) who had undergone surgery and/or radiotherapy in the same Institution for the purpose of comparing the results. They have reported a complete tumour regression (almost reepithelized cervix on colposcopy and marked volume reduction on ecography and physical examination) rate of 26% for stage IIb and 11% for stage IIIb. It was also reported that on histopathologic findings, among the 33 patients with tumours included in the group with complete tumour regression, 60% had cervical lesion < 0.5 cm and 4% had parametrial residual lesion, but none had lymph node metastasis. The operability rate after neoadjuvant chemotherapy in patients initially with IIb was 71% and in patients initially with stage IIIb it was 44% (overall, 62.2%). When comparing the results from using 3 cycles of neoadjuvant chemotherapy with cisplatin, vincristine and bleomycin with a 10-day interval to the results obtained with exclusive radiotherapy, they have observed a significant difference in 2-year disease free survival \( (P = 0.01) \) both in patients with stage IIb (79% versus 47%) and IIIb (50% versus 26%).

The overall 2-year disease free survival in patients with tumours bigger than 84 cm\(^3\) was significantly longer \( (P = 0.005) \) for the ones undergoing neoadjuvant chemotherapy followed by radiotherapy (87%) than for the ones who underwent exclusive radiotherapy (40%). They have also shown that the 27 women with tumour volume > 84 cm\(^3\) before neoadjuvant chemotherapy who had partial response and underwent radiotherapy have shown a statistically significant difference \( (P = 0.01) \) in 2-year disease free survival when these big tumours regressed to a volume < 34 cm\(^3\) after neoadjuvant chemotherapy, thus giving more evidence for the importance of tumour volume in the treatment modality and outcome of patients with cervical cancer (Sardi et al., 1990b).

Another study involving 42 patients with adenocarcinoma of the cervix (9 FIGO stage Ib/IIa > 4 cm, 19 stage IIb and 14 stage III) who underwent neoadjuvant chemotherapy either with 3 cycles of cisplatin (100 mg/m\(^2\)), bleomycin (15 mg/m\(^2\)) and methotrexate (300 mg/m\(^2\)) plus citrovorum as rescue factor with 21-day interval (18 patients), or 1 or 2 cycles of cisplatin (40 mg/m\(^2\)) and bleomycin (15 mg/m\(^2\)) with 28-day interval (15 patients) or 2 cycles of cisplatin (80 mg/m\(^2\)) and doxorubicin (80 mg/m\(^2\)) with 21-day intervals (Benedetti Panici et al., 1996). They reported an overall response rate on clinical grounds using these combined neoadjuvant chemotherapy protocols of 79% (72% of patients had partial responses and 7% had complete response). Radical hysterectomy
(Piver's type II and IV) was feasible in 29 patients (69%) and histopathologic examination confirmed 3 cases of complete response and 24 cases of partial response [the partial response rate to satisfactory operability being 57% (24/42)], and 2 cases had stable disease or same stage as at inclusion. After a median follow-up of 56 months from enrolment in the study (range 17 to 95), the overall survival rate was 71%, more specifically the survival rate for stages Ib and Iia was 100%, for stages IIb it was 84% and for stages III the survival rate was 36%. After a median follow-up of 54 months from radical surgery (range 15 to 92), the overall disease-free survival rate was 88%, no patient initially with stage Ib or Iia had recurrence, 1 of 15 patients with stage IIb who underwent chemotherapy and radical surgery had recurrence (7%) and 2 of 5 patients with stage III who received chemotherapy followed by radical surgery had recurrence (40%). These results demonstrate a significant tumour response with the use of these combination chemotherapy schemes for the treatment of locally advanced cervical adenocarcinomas.

More scientific evidence that neoadjuvant chemotherapy results in effective response rates in cervical cancer and that tumour volume is a critical factor in prognosis has been published. A study showed that cervical cancer patients (75 enrollees) with stages Ib to III using cisplatin (100 mg/ m²) in day 1, bleomycin 15 mg in days 1 and 8, and methotrexate (300 mg/ m²) in day 8 (with folinic acid rescue) for 3 cycles of 21-day interval had an overall response of 83% which increased to 93% when the initial tumour was ≤ 5 cm, and decreased to 68% when the tumour was bigger than 5 cm. In other words, the size of the tumours significantly affected its responsiveness to chemotherapy (P = 0.02). The extent of disease was another variable significantly influencing chemotherapy response (P = 0.005) since there was only a 14% partial response in patients with stage IIIb tumour bilaterally involving the pelvic bone, compared to 92% partial responses in patients with less extensive disease. Complete response (absence of histologic evidence of cancer in the surgical specimen) was found in 16% of the 62 patients who underwent radical hysterectomy (Piver's type III-IV) plus paraaortic and pelvic lymphadenectomy. Of the 62 patients who had surgery after chemotherapy 81% are disease free after a median follow-up of 27 months. All 12 cases of recurrence occurred within 24 months and the median time of recurrences was 9 months after surgery. There were 31 patients with stage III among which 30% had recurrent disease and 44 were stage Ib-IIb among which 16% had recurrent disease, however it was interesting to observe that there was no statistically significant difference (P = 0.08) in these recurrence rates among patients with stage III or
less extensive disease (Benedetti Panici et al., 1991). When this same study had reached a sample size of 128 patients the overall response rate was 83% and the complete response rate was 15% (Benedetti Panici et al., 1998).

In 79 women with bulky (> 4 mm) stages Ib to IIb squamous cell carcinoma of the cervix, a neoadjuvant chemotherapy protocol using cisplatin (40 mg/m²) weekly for 7 cycles and ifosfamide (3.5 mg/m²) in days 1, 4 and 7 resulted in an overall response of 70% and complete response of 5%; for women with and without lymph node involvement on CT the response rates were respectively 50% and 85% (Bolis, 1992, 1996). Other studies have also described pelvic lymph node involvement after chemotherapy to be associated with reduced survival of patients with cervical cancer (Giaroli et al, 1990; Dottino et al., 1991).

There is evidence that neoadjuvant chemotherapy is useful in the reduction of cervical cancer tumour volume and metastatic lymph node involvement (the major risk factors for poor prognosis), thus increasing the probability of salvage surgery and improving the patient’s outcome and quality of life. There are many questions to be answered though, but it is only with large and well designed randomised phase III clinical trials that they will be sorted out. It is a reality that these types of trials are difficult to achieve in terms of accruing sufficient numbers of cases in developed countries because of the fortunate low incidence of advanced cervical cancers in such countries. On the other hand, where there is a relative excess of advanced cases, there is usually a lack of research funds and qualified personnel. As an international effort to improve the quality of treatment of advanced cervical cancers and to answer scientific questions regarding the role of neoadjuvant chemotherapy as a first line of a multimodality therapeutic approach as soon as possible, willingness and proper communication between key people should suffice.

8. **Chemoprevention**

There is evidence for an important role of cytokines in the control of HPV infection and its progression to HPV-induced neoplasias. Some cytokines such as IL-12 and IFNs present immunostimulatory effects through direct or indirect mechanisms and could be used in combination with other immunomodifiers such as retinoids, vitamin D3 [127], and some non-toxic cytostatics.
8A. Immunomodulators

The drug 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine, coded as S-26308 or R-837, and also called imiquimod, has been shown in animal in vitro assays to induce the synthesis and release of interferon (IFN), interleukin (IL)-6 tumour necrosis factor-α (TNF-α) and probably other cytokines at concentrations of 0.2μg/ml (Reiter et al., 1994).

Imiquimod at 1 to 5μg/ml induces the production of cytokines and subtypes such as IFN-α, TNF-α, IL-1, IL-1RA, IL-6, IL-8, IL-10, IL-12, p40, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and macrophage inflammatory protein 1-α (MIP-1α), MIP-1β, and macrophage chemotactic protein 1 (MCP-1) by human peripheral blood mononuclear cells, particularly monocytes (Weeks & Gibson, 1994; Gibson et al., 1995). These findings suggest immunomodulatory effects on the innate immune response, which may in turn affect viral infections and virus-related tumours. A review article has been published on the immune response modifier effects of imiquimod (Miller et al., 1999).

Studies investigating the use of Imiquimod in clinical practice have shown some immune response and tolerable side effects. A phase III multicentric, randomised, double-blinded, placebo controlled clinical trial involving 180 men and 131 women aged 18 or older who had 2 to 50 external anogenital warts lesion has been undertaken (Edwards et al., 1998). Patients were randomised to topically apply themselves either 1% or 5% imiquimod or placebo cream 3 days a week (separated by no less than 36 hours and no more than 96 hours, and after the 3rd dose a pause of 60 to 120 hours) during 6 to 10 hours of sleep for up to 16 weeks or complete remission of lesions. This study showed that 50% of patients who used 5% imiquimod, 21% who used 1% imiquimod and 11% who used placebo topical cream had complete remission of lesions. The difference between the 5% imiquimod cream and the placebo was significant \( P < 0.0001 \), however it is important to note that 25 to 35% of warty lesions should regress spontaneously (Massing & Epstein, 1963). Women had higher remission rate in the 3 groups (77%, 46% and 28%) compared to men (40%, 10% and 6%) and a median time to clear the lesions of 8 weeks compared to 12 weeks in men. The recurrence rate of lesions that had completely cleared was 13% in the 5% imiquimod group, 0% in the 1% imiquimod group and 10% in the placebo group. Less than 1.2% of participants discontinued treatment due to side effects and the most common adverse effect was local erythema which occurred in 67% of the 5% imiquimod group, in 26% of the 1% imiquimod group and 24% of the placebo group. A
similarly designed phase III clinical trial involving 154 men and 125 women with genital warts was undertaken where patients were randomised to use the topical creams at same concentrations as in the previous study but during 8 hours daily for 16 weeks or complete remission of lesions (Beutner et al., 1998). There was complete remission in 71% of participants using the 5% imiquimod cream, in 16% of participants using 1% imiquimod cream and in 4% of participants who used the placebo cream. The difference in clearance between 5% imiquimod cream and placebo was again significant ($P < 0.0001$), the difference in clearance rates between the 1% imiquimod cream and the placebo was again not significant. A recurrence rate of 19% was observed in the 5% imiquimod group, 17% in the 1% imiquimod group and 0% in the placebo group. The daily therapeutic scheme resulted in increased remission, recurrence and local skin reactions.

The topical 5% imiquimod cream applied 3 times a week for 16 weeks or until complete remission of lesions was also compared to placebo in HIV-positive patients with genital warts in a double-blinded multicentric and multinational study involving 97 men and 3 women (Conant et al., 1998). A remission rate of the genital warts of 11% in the 5% imiquimod group was observed compared to 6% in the placebo group but it is not significantly different. If one analyses the proportion in each group having a wart area reduction over 50% but not complete, then the difference was significant ($P = 0.013$), in the 5% imiquimod group 38% had an over 50% reduction of wart area as opposed to 14% in the placebo group.

A study undertaken in Beijing, China (Chen et al., 1997), including women with mild to severe histologically proven cervical dysplasia, evaluated the use of Retinamide II. This is a synthetic retinoid developed in China (Xu et al., 1981; Cai et al., 1981) which presents marked antineoplastic effect (Chu & Malmgren, 1965; Roberts & Frolik, 1979; Chen et al., 1987). Despite the work's methodological weaknesses, it reports that among 27 participants who used vaginal suppositories of retinamide (10mg) on daily doses for 6 months, after treatment the lesion disappeared completely in 24 women (88.89%) and partially in 26 women (96.29%). Studies at both basic and clinical science levels have shown that a sugar derivative called Glucan induces enhancement of the immunologic response, thus acting as an immune modulator.
8B. HPV Vaccines

Promising but not yet available for clinical use, HPV vaccines are a powerful hope for the control of cervical cancer incidence, morbidity and mortality. After becoming aware that cervical cancer is highly associated with a sexually transmitted viral infection (HPV) and that the immune system produces some response against theses viruses, the idea of creating a vaccine against HPV infection became immediately attractive. In the early 90’s, a vaccine against HPV 16 had been proven successful in the prevention of tumour formation in animal models (Feltkamp et al., 1993). Another study comparing prophylactic vaccines against papillomavirus using natural antigens produced from purified virus and tumour extracts was proven successful in the immunisation of cattle against bovine papillomavirus 2 (BPV 2) (Jarrett et al., 1990).

For a vaccine to be fully effective in the prevention and regression of anogenital tract neoplasias it would have to induce an adequate immune response against the approximately 20 genotypes of HPVs and their variants, particularly HPVs 16, 18, 31 and 45 which account for about 80% of cervical cancers. The use of HPV virus-like particles (VLPs) that induce antigenic response, thus protecting but not exposing the person taking the vaccine from dissemination of oncogenic viruses, since they do not contain viral DNA, are currently being tested. VLPs correspond to empty viral capsids, which contain on their surface epitopes that are recognised by neutralising antibodies of hyperimmune sera and infected patients.

The most promising approach with regard to strategies for creating HPV vaccines is to autoassemble L1 or L1 and L2 viral capsid proteins into empty capsids or VLPs. L1 or L1 and L2 proteins self-assemble into VLPs when expressed from recombinant vaccinia viruses, baculoviruses or yeast expression vectors (Schiller, 1999). In order to prevent HPV replication at its earliest stages one of the best strategies would be to try to activate the immune system against the early HPV proteins E1 and E2. The problem though with this strategy is that E1 and E2 are poorly expressed, particularly after the HPV is integrated into the cell genome, thus making activation of the immune system difficult. The continuous expression of E6 and E7 proteins from latent infection to invasive cervical cancer makes them interesting targets for therapeutic vaccines in humans. It has been reported that E6 and E7 induce cytotoxic T lymphocyte responses in infected
patients and specific antibody responses in a number of patients with cervical cancer (Orth, 1999).

For effective protection it is necessary to have a full-length, nondenaturated protein or intact VLPs (Lin et al., 1993). It is important to realise that the protection obtained with L1 vaccines is type-specific, except for HPV 16, its variants and HPV 33, which produce antibodies that cross-neutralise their genotypes (White et al., 1998; Cheng et al., 1995; Touze et al., 1998). Therefore, it implies that vaccines against HPV infection will have to incorporate type-specific L1 proteins of the most common HPV types associated with cervical neoplasia and genital warts. This fact also emphasises the importance of using PCR methods for detection and typing of HPVs in order to better evaluate the results of HPV vaccine trials. Interesting immunoprophylactic results have been achieved with the use of VLPs in animals. VLP vaccines induce long-term protection (Campo, 1995; Lin et al., 1992, 1993; Donnelly et al., 1996; Sundaram et al., 1997; Breitburd et al., 1995; Christensen et al., 1996; Jansen et al., 1995; Suzich et al., 1995; Kirnbauer et al., 1996).

Vaccines against the L2 protein have shown some protection against papillomavirus affecting rabbits and cattle (BPV 4), although the production of neutralising antibodies with vaccines against L2 was lower than with vaccines against L1. The effect of incorporating L2 into L1 VLPs appeared to provide a better protection against papillomavirus infection in rabbits than either one separately (Breitburd, 1995), although the same enhanced effect related neutralising antibody response was not observed in the bovine model (Kirnbauer, 1996).

In calves with BPV 4 induced papillomas, the use of L2 incorporated into L1 VLPs as therapeutic vaccines, despite showing an apparently quicker tumour regression in the vaccinated group compared to controls, did not result in a significantly improved final outcome (Kirnbauer, 1996). The important cellular infiltrates seen on biopsies from warts undergoing regression characterised a cell-mediated immune response. Therefore, it has been hypothesised that the L2 protein fused to L1 managed to activate a T Lymphocyte response by unmasking epitopes related to intact BPV 4 particles that would have otherwise passed unnoticed (Campo, 1995; Chandrachud et al., 1995). It has been reported that BPV 4 infection induces cell-mediated immune response through E7 specific T cell epitopes (McGarvie et al., 1995) and that BPV 4 E7 shares many
similarities with HPV 16 E7 (Campo, 1995). A study using gene gun-based intracutaneous vaccine with a combination of cottontail rabbit papillomavirus E1, E2, E6 and E7 genes showed protection of rabbits from viral challenge and that the highest cell-mediated immune response in vaccinated rabbits was related to E2 antigenicity (Han et al., 1999). The important antigenicity of E2 has been previously reported also in cases of spontaneous regression of rabbit papillomas (Breitburd et al., 1997; Selvakumar et al., 1995). A vaccine against the major early proteins expressed by papillomaviruses, as described above, might increase the probability of a potent immune response since a larger number of epitopes will be available to trigger the immune system (Han et al., 1999). Another strategy to prevent HPV infection or induce clearance of the viral infection and tumour regression is based on the use of peptide vaccines that ensure sufficient cytotoxic T lymphocyte response.

Synthetic peptides representing tumour-associated T-cell epitopes have been used as antitumour therapy for cervical cancer and have been proven to offer effective response (Ressing et al., 1999). The use of vaccines based on dendritic cells loaded with HPV 16 oncoprotein or HPV 16 as a protein in adjuvant resulted in major histocompatibility complex (MHC) class I-restricted protection against tumours associated with HPV 16 (De Bruijn et al., 1998a).

For therapeutic vaccines against HPV infected tissues, a strategy that has been under investigation is an attempt to improve the cell mediated immune response by the enhancement of viral antigen recognition. Currently there are clinical trials being carried out using vaccines against the oncogenic proteins E6 and E7 of HPVs 16 and 18. Aiming to achieve a broader induction of the immune system by triggering both the production of neutralising antibodies and activation of specific and effective cell mediated responses, chimeric VLP vaccines have been created. These vaccines have chimeric capsid proteins assembled into L1-E7 VLPs and L1/L2-E7 or L1/L2-E2 VLPs (Muller et al., 1997; Greenstone et al., 1998). Studies have shown that these chimeric vaccines are capable of inducing the production of neutralising antibodies and protective cytotoxic T cell responses even to tumours expressing low levels of L1 proteins (Greenstone et al., 1998; Peng et al., 1998; Nieland et al., 1999; Schafer et al., 1999; Rudolf et al., 1999; De Bruijn et al., 1998b; Marais et al., 1999). These VLP vaccines offer potential for both prophylactic and therapeutic immune responses.
It is also possible to assemble VLPs with genes encoding immune response modifiers such as cytokines (i.e. interleukin 12) to produce a vaccine that selectively and powerfully stimulates the Th1 arm of the immune system. A vaccine capable of engineering tumour cells (HPV 16-transformed) to express B7-1 and B7-2, molecules that activate T lymphocytes to secrete interleukin 12, has resulted in potent anticancer effects through E7-directed immunity (Hallez et al., 1999). Another study using a vaccine that induces T lymphocytes to secrete interleukin 12 has shown potentiation of HPV 16 E6 antitumour response (Tan et al., 1999). Laboratories of academic centres and biotechnology companies have been working hard to produce an effective vaccine against HPV. So far VLPs for HPVs 6, 11, 16, 18, 31, 33, 39, 45, 58, and 59 have been produced, which covers more than 90% of HPVs associated with cervical cancer and genital warts. Vaccines have been produced against HPV L1 DNA, recombinant vaccinia (HPV 16 and 18 E6/E7), HPV 16 E7 protein, HPV 16 peptide, HPV 6 L2-E7 protein, and HPV 16 VLPs/E7 (Schiller, 1999; Schiller & Roden, 1995).

The development of efficient HPV specific vaccines may prove to be of a great impact in the epidemiology of cervical cancer.

9. The purpose of this study

In Recife as in many other cities in Brazil and in developing countries in general, cervical cancer is a major public health problem. The hospitals accredited by the Government to treat advanced cervical cancer are often overloaded with this particular cancer affecting women. This creates waiting lists for radiotherapy of sometimes a month or more, which leads to important psychosocial, economic and prognostic implications for the patients. Even hospitals providing general gynaecological care become dysfunctional because of a significant number of hospital beds having to be occupied by patients with advanced cervical cancer and its complications, thus creating waiting lists for benign gynaecological surgery as well that would not exist otherwise.

The result is stress for the management team to sort out ways to solve this serious problem. The logical approach is to implement measures to decrease the incidence of cervical cancer and to improve the quality of treatment provided in order to decrease complication rates. Local screening coverage is far from ideal and even when it reaches...
the patterns of developed countries or what is considered effective coverage (> 80% of eligible population), we would still have to face the problems related to false negative cytology that most authors have been describing ever since cytological screening has been implemented.

The advent of new technologies such as PCR is making clear that chronic diseases like cancer and arthritis, which have been affecting beings on Earth since the era of the dinosaurs, can be much more thoroughly investigated beyond the microscopic eye by studying the interaction of molecules, thus offering a whole new horizon for preventive and therapeutic measures to tackle these so far enigmatic disease processes. A good example of this is the human genome project that is expected to be finished by 2003 or perhaps earlier. Scientists all over the world are mapping the sequences of proteins contained in each of our chromosomes, the genetic messages that give form and function to our cells.

Therefore if there is an effective and safer way to improve the quality cervical cancer screening yet without too much additional cost, it is our impression that this should be the best option for implementing a protocol to decrease cervical cancer incidence morbidity and mortality in developing countries. Bearing this in mind, we decided to evaluate some of the molecular techniques to detect the presence of HPV infection in the cervix and analyse their utility in the identification of women with cervical neoplasia and those at risk of developing it in Recife, a high-risk area for cervical cancer.

II. MATERIALS AND METHODS

All reagents unless otherwise stated were obtained from Merck UK. Restriction enzymes were obtained from Promega UK.

VI. Consensus PCR

Sample preparation
Cervical smears were collected and dispersed into 10ml phosphate buffered saline (PBS) (NaCl 137mM, Na₂HPO₄ 6.5mM, KH₂PO₄ 1.5mM, KCl 3.0mM, pH 7.4) containing antibiotics (penicillin 100 IU/ml, streptomycin 100μg/ml) and fungizone (Amphotericin B 2.5μg/ml).

The clinical specimens were centrifuged at 1600 x g for 5 minutes. The pellets were re-suspended in 1ml PBS and then centrifuged at 10000 x g for 5 minutes. Pellets were re-suspended in 500μl TE (Tris(hydroxymethyl)methylamine 10mM, EDTA 2mM, pH 8.0) containing Proteinase K (200μg/ml) then incubated at 50°C for 1 hour followed by 95°C for 10 minutes. Prepared samples were stored at -20°C until required for PCR. For the consensus PCR amplification of HPV DNA the MY09/11 pair of primers (Manos et al., 1989) was used.

The PCR primers were obtained from Amersham Pharmacia Biotech. They amplify an approximately 450 base from the conserved region of the HPV L1 gene and are described as follows:

Primer. MY09 5'-GCMCAGGGWCATAAYAATGG

MY11 5'-CGTCCMARRGGAWACTGATC

All reagents except primers used for PCR were obtained from Promega UK. Each 25μl reaction contained PCR reaction buffer (KCl 50mM, Tris-HCl 10mM pH 9.0, Triton X-100 0.1%, MgCl₂, 1.5mM), dNTP 0.2mM, MY09/11 primers 0.25μM, Taq DNA polymerase 0.5 units. The reaction mixture was made up as a master mix excluding Taq polymerase and sample (template) DNA and dispensed in 14μl aliquots. Sample DNA, 1μl, was added to the PCR mix just before cycling. Taq polymerase was diluted to 0.5 units per 10μl of PCR reaction buffer. The PCR reaction mixes (excluding Taq polymerase) were cycled thus:

94°C x 1 min
98°C x 3.5 min
72°C HOLD
10μl Taq polymerase mix added to each reaction ("hot start" PCR)

then 35 cycles of

94°C x 30 sec
53°C x 30 sec
72°C x 1 min

and after the 35 cycles, 1 cycle of

72°C x 8 min.

5μl of each PCR product was electrophoresed on a 2% agarose gel in TAE buffer (Tris 40mM, Acetic acid 20mM, EDTA 1mM, pH 8.3).

VII. Restriction fragment length polymorphism (RFLP)

5μl MY09/11 PCR products were restricted with Dde I (2 units Dde I, tris-HCl 6mM, MgCl₂ 6mM, NaCl 150mM, dithiothreitol 1mM pH 7.9, final concentrations) or Rsa I (2 units Rsa I, tris-HCl 10mM, MgCl₂ 10mM, NaCl 50mM, dithiothreitol 1mM pH 7.9, final concentrations). Final volume for each reaction was 10μl. Restriction reactions were carried out at 37°C for 18 hours. Restriction products were electrophoresed on 3% Metaphor gels (Flowgen) in TBE (Tris 44.5mM, Boric acid 44.5mM, EDTA 1mM pH 8.3) (Figure IX).

VIII. Hybrid capture I (Digene corp)

Materials

<table>
<thead>
<tr>
<th>Volume supplied</th>
<th>Item</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30ml</td>
<td>Denaturation reagent</td>
<td>1x</td>
</tr>
<tr>
<td>2x 2.5ml</td>
<td>probe diluent</td>
<td>1x</td>
</tr>
<tr>
<td>55μl</td>
<td>HPV probe mix</td>
<td>50x</td>
</tr>
<tr>
<td>2ml</td>
<td>Negative control</td>
<td>-</td>
</tr>
<tr>
<td>1ml</td>
<td>Positive control</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure IX. RFLP of MY09/11 HPV PCR products. Lanes 1 and 13 size markers (726-717, 553, 506, 427-417, 311, 249, 200, 151, 140, 118, 100, 82, and 66bp). Lane 2 uncut HPV 6, lane 3 HPV 6 restricted with Rsal, lane 4 HPV 6 restricted with DdeI, lane 5 empty, lane 6 uncut HPV 16, lane 7 HPV 16 restricted with Rsal, lane 8 HPV 16 restricted with DdeI, lane 9 empty, lane 10 HPV 18 uncut, lane 11 HPV 18 restricted with Rsal, and lane 12 HPV 18 restricted with DdeI.
15ml Detection reagent 1  1x
15ml Detection reagent 2  1x
60 Capture tubes -
100ml Wash buffer concentrate powder -
350μl Indicator dye -

Before use the probe was diluted 1/50 in probe diluent, wash buffer concentrate was added to 3l of distilled water and 3 drops of indicator dye was added to the denaturing reagent.

The probe mix detects HPV types 16, 18, 31, 33, 35, 45, 51, 52, and 56

Methods
Samples were collected using Digene specimen collection kits. Samples were stored at 4°C until tested.

Denaturing samples
500μl denaturing reagent was added to each sample and the positive calibrator. 1ml denaturing reagent was added to the negative calibrator. Samples and calibrators were vortexed thoroughly until the colour changed throughout the sample (denaturing reagent is purple). Samples were incubated at 65°C for 45 minutes.

Hybridisation
Denatured samples and calibrators were vortexed. 150μl of each sample or calibrator were added to hybridisation microtubes containing 50μl diluted probe mix. The tubes were sealed with a plate sealer and mixed on a rotary shaker at 1100RPM for 3 minutes. All tubes were incubated at 65°C for 1 hour in a water bath.
Hybrid capture
The entire contents of each hybridisation tube were transferred to capture tubes and were shaken at 1100 RPM, at 25°C for 1 hour. The capture tubes were emptied into a sink and blotted dry.

Hybrid detection.
250μl of detection reagent 1 was added to each capture tube. The tubes were covered with parafilm and incubated at 25°C for 30 minutes. The tubes were washed 5 times with wash buffer and blotted dry. 250μl of detection reagent 2 was added to each tube. The tubes were incubated in the dark for 30 minutes. The results were measured using a Digene DCR1 luminometer.

III. RESULTS

Section A. Identification of risk factors associated with cervical neoplasia in Recife.

(1) Experimental design.
This is a prospective nested case-control study designed to identify risk in a high-risk population. Samples were randomly collected from women attending cervical cancer screening programmes at reference services in Recife.

(2) Study population.
Potential volunteers were women coming in for screening either to the Institute of Mother and Child Health in Pernambuco (IMIP) or The Cancer Hospital (HCP), two major screening services in Recife. IMIP is a reference teaching hospital for Gynaecology, Obstetrics and Paediatrics that provides primary to tertiary care to the population in Pernambuco and neighbouring states. The Cancer Hospital is a referral teaching institution for cancer treatment. It offers a cervical cancer screening programme for any woman in the State of Pernambuco.

Women with a Pap smear taken within the last 12 months and women referred from other centres due to suspected cervical neoplasia with or without a Pap smear were recruited.
No age restriction was made for inclusion. The final inclusion criteria for each group was decided on overall clinical diagnosis and histology reports for the case groups, and on overall clinical impression, cytological report and colposcopic examination for the control group. Women in the control group had completely normal cytology and normal colposcopy. Women with cervicitis and no neoplasia were considered separately from the completely normal cervices. Women who were too ill to participate in the study were not included.

(3) Ethics permission and consent.

This study was reviewed and approved by the local Ethics Committee. Details of the study were explained in full and in lay terms to each participant woman. Those willing to participate were invited to sign an informed consent form and attend a personal interview regarding epidemiological information such as monthly family income, number of people dependent on it, level of education, age, age at first intercourse, age at first pregnancy, number of sexual partners, gravidity, number of vaginal deliveries, number of C-sections, number of abortions, number of miscarriages, number of non-obstetrical curettages, history of sexually transmitted diseases, contraceptive use and smoking history.

(4) Procedures.

All participants had cervical scrapes collected on enrolment with an Ayre’s spatula and the cells were dispersed into PBS with penicillin, streptomycin and amphotericin B and stored at -20C. These were later tested for HR HPV using the PCR/RFLP technique (Manos et al., 1989; Bauer et al., 1992; Gravitt et al., 1992; Hildesheim et al., 1994; Bernard et al., 1994). Colposcopic examination was routinely carried out thereafter and a biopsy taken when indicated.

(5) Statistical considerations

For statistical analysis the software packages Epi-info 2000 and SPSS 10 were used. Testing for evidence of association between cervical lesions and presence of HPV DNA by the molecular techniques used required the use of two-tailed Fisher’s exact test for discrete variables and the non-parametric Wilcoxon two-sample test for continuous variables. Chi-squares and *P*-values are Yates corrected and the 95% Confidence Intervals are the exact limits. As an attempt to identify multivariate associations we used
multiple logistic regression. The control group consisted of women with completely normal cervix — normal cytology and normally looking cervical epithelium on colposcopy. Women with cervicitis, even though it is not neoplasia, were considered separately from the completely normal cervices. Statistical significance was considered when $P$-values were $\leq 0.05$.

(6) Results.

A total of 921 women volunteered for the study. Their age range was from 13 to 88 (mean 36.1 ± 13) years. Their age distribution was as follows: 221 (23.9%) were aged 25 years or under, 272 (29.5%) were aged 26 to 35 years, 306 (33.2%) were aged 36 to 50 years, 72 (7.8%) were aged 51 to 60 years and 50 (5.4%) were over 60 years.

In this study population, 430 women (46.7%) had normal cervix, 144 (15.6%) had cervicitis, 7 (0.8%) had condyloma accuminatum, 139 (15.1%) had LSIL, 120 (13%) had HSIL, 81 (8.8%) had invasive cervical cancer. 38 (4.1%) of total enrollees had never had a Pap smear (Table A1).

(6.a) Association with HPV types as detected by PCR/RFLP.

576 (62.5%) women were negative for any HPV. 26 (2.8%) had LR HPV types, 16 HPV6 (1.7%) and 10 HPV11 (1.1%). The 319 (34.6%) HR HPV infections detected were distributed in the total population as follows: 151 HPV16 (16.4%), 13 HPV18 (1.4%), 59 HPV31 (6.4%), 40 HPV33 (4.4%), 13 HPV35 (1.4%), 4 HPV45 (0.4%), 6 HPV52 (0.7%) and 33 HPV58 (3.6%). Table A2 shows the specific risk of cervical lesions in women positive for the HPV types detected in the study population. As expected, any HR HPV type, but HPV16 and HPV18 in particular, posed a high risk for the development of HSIL and cancer.

(6.b) Age.

The majority of lesions occurred in women from 15 to 60 years of age. LSIL was found predominantly in younger women (aged 35 years or younger). HSIL was detected in women at a mean age 39.2 years and cancer at a mean age of 53.9 years (Table A3). At least one girl aged 14 years and another aged 16 years had HSIL, and another girl aged 13
<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Normal</th>
<th>Cervicitis</th>
<th>Condyloma</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 25</td>
<td>106</td>
<td>46</td>
<td>5</td>
<td>54</td>
<td>10</td>
<td>0</td>
<td>221</td>
</tr>
<tr>
<td>26 to 35</td>
<td>130</td>
<td>49</td>
<td>2</td>
<td>51</td>
<td>35</td>
<td>5</td>
<td>272</td>
</tr>
<tr>
<td>36 to 50</td>
<td>139</td>
<td>42</td>
<td>0</td>
<td>31</td>
<td>62</td>
<td>32</td>
<td>306</td>
</tr>
<tr>
<td>51 to 60</td>
<td>44</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>16</td>
<td>72</td>
</tr>
<tr>
<td>≥ 61</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>28</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>430</td>
<td>144</td>
<td>7</td>
<td>139</td>
<td>120</td>
<td>81</td>
<td>921</td>
</tr>
</tbody>
</table>

Table A1. Distribution of final clinical diagnostic categories by age group.
<table>
<thead>
<tr>
<th>HPV status</th>
<th>Cervicitis</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>1 (Referent)</td>
<td>1 (Referent)</td>
<td>1 (Referent)</td>
<td>1 (Referent)</td>
</tr>
<tr>
<td>Any HR HPV</td>
<td>2.05</td>
<td>5.37</td>
<td>17.04</td>
<td>26.12</td>
</tr>
<tr>
<td>HPV 16</td>
<td>1.46</td>
<td>5.41</td>
<td>21.54</td>
<td>44.35</td>
</tr>
<tr>
<td>HPV 18</td>
<td>9.54</td>
<td>12.00</td>
<td>17.37</td>
<td>41.57</td>
</tr>
<tr>
<td>HPV 31</td>
<td>1.16</td>
<td>3.81</td>
<td>1.87</td>
<td>6.89</td>
</tr>
<tr>
<td>HPV 33</td>
<td>5.22</td>
<td>8.11</td>
<td>16.68</td>
<td>14.81</td>
</tr>
<tr>
<td>HPV 35</td>
<td>1.60</td>
<td>9.14</td>
<td>4.39</td>
<td>6.89</td>
</tr>
<tr>
<td>HPV 45</td>
<td>-</td>
<td>Undefined</td>
<td>Undefined</td>
<td>-</td>
</tr>
<tr>
<td>HPV 52</td>
<td>4.82</td>
<td>0.00</td>
<td>0.00</td>
<td>6.89</td>
</tr>
<tr>
<td>HPV 58</td>
<td>1.75</td>
<td>2.16</td>
<td>4.80</td>
<td>2.47</td>
</tr>
</tbody>
</table>

Table A2. Risk of cervical lesions associated with specific HPV types. ORs were calculated relative to total HPV negative women.
<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Normal</th>
<th>Cervicitis</th>
<th>Condyloma</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>35.5</td>
<td>32.4</td>
<td>22.3</td>
<td>29.6</td>
<td>39.2</td>
<td>53.9</td>
</tr>
<tr>
<td>SD</td>
<td>12</td>
<td>10.7</td>
<td>5.3</td>
<td>9.5</td>
<td>11.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Minimum</td>
<td>14</td>
<td>16</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>Maximum</td>
<td>71</td>
<td>73</td>
<td>29</td>
<td>66</td>
<td>79</td>
<td>88</td>
</tr>
</tbody>
</table>

Table A3. Age variables related to clinical conditions of the cervix. (Values in years).
years had LSIL. In addition, 2 women aged 28 years and another 31 years were found to have cervical cancer stages 1B and 2B, respectively.

HR HPV infection was detected over a wide age range (13 to 84 years, Figure A1). HPV16 was detected in both old and young women. Incidence rates peaked at 39.7 years for HPV16, 35.7 years for HPV18, 36.4 years for HPV31, 36.6 years for HPV33 and 36.3 years for HPV58. HPV18 therefore occurred at a slightly earlier age. For comparison with the majority of previously reported studies that basically included only low-risk populations or women with negative cytology, the distribution of latent HR HPV infection in the high-risk population studied is described in Figure A2.

(6.c) Cervical neoplasia in older (\(> 35\) years) or younger (\(\leq 35\) years) women.

Older women were found to be at a higher risk of having HSIL (OR = 2.16) or cancer (OR = 19.69) (Table A4) whereas younger women were not at risk of HSIL or cancer even if they were uninfected or infected with HR HPV (Table A5). This result does not support the use of HR HPV detection in younger women for the identification of HSIL or cancer. In this context, abnormal cytology was not predictive of HSIL or cervical cancer in younger women either (OR for HSIL = 0.48 and OR for cancer = 0.11) (Table A6).

(6.d) Age at first intercourse and at first pregnancy.

The mean ages at first intercourse and first pregnancy among women with normal cervix, LSIL, HSIL and cervical cancer are shown in Table A7. These variables were both associated with any SIL (\(P < 0.005\)) but not with cancer (\(P \geq 0.37\)) suggesting that risk factors other than the acquisition of HR HPV through sexual activities were required for the development of malignancy.

(6.e) Family income.

The mean monthly family income among all participants was 2.8, ranging from 0 (subsistence agriculture) to 115 minimal wages (MW)\(^5\). Table A8 shows the association of cervical neoplasia with the amount of money gathered by the whole family. The

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\(^5\) The minimum wage practised in Brazil when the study was carried out was equivalent to approximately US$120.00 a month.
Figure A1. HR HPV distribution by age in a high-risk population.
Figure A2. HR HPV distribution by age in women with normal cervix from a high-risk population.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>19.69</td>
<td>7.40 – 63.02</td>
<td>70.74</td>
<td>&lt; 0.0000001</td>
</tr>
<tr>
<td>HSIL</td>
<td>2.16</td>
<td>1.42 – 3.31</td>
<td>13.61</td>
<td>0.0002</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.13</td>
<td>0.08 – 0.20</td>
<td>105.61</td>
<td>0.0000001</td>
</tr>
</tbody>
</table>

Table A4. Risk of having cervical neoplasia for older women.
<table>
<thead>
<tr>
<th>HR HPV status</th>
<th>Cervical lesion</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Cancer</td>
<td>0.03</td>
<td>0.01 - 0.09</td>
<td>61.49</td>
<td>&lt; 0.0000001</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>0.33</td>
<td>0.18 - 0.61</td>
<td>13.80</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>LSIL</td>
<td>3.69</td>
<td>1.74 - 8.25</td>
<td>12.59</td>
<td>&lt; 0.0004</td>
</tr>
<tr>
<td>Negative</td>
<td>Cancer</td>
<td>0.14</td>
<td>0.02 - 0.64</td>
<td>7.24</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>0.46</td>
<td>0.19 - 1.06</td>
<td>3.25</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>LSIL</td>
<td>2.12</td>
<td>1.20 - 3.82</td>
<td>6.87</td>
<td>&lt; 0.009</td>
</tr>
</tbody>
</table>

Table A5. Risk of having cervical neoplasia for younger women with or without HR HPV.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>0.11</td>
<td>0.03 - 0.34</td>
<td>19.22</td>
<td>0.00001</td>
</tr>
<tr>
<td>HSIL</td>
<td>0.48</td>
<td>0.28 - 0.82</td>
<td>7.44</td>
<td>0.006</td>
</tr>
<tr>
<td>LSIL</td>
<td>4.34</td>
<td>2.38 - 7.96</td>
<td>26.07</td>
<td>0.0000003</td>
</tr>
</tbody>
</table>

Table A6. Risk of having cervical neoplasia for younger women with ASCUS cytology or above.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Age at first intercourse</th>
<th>Age at first pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>Normal cervix</td>
<td>18.7</td>
<td>Referent</td>
</tr>
<tr>
<td>LSIL</td>
<td>17.2</td>
<td>10.20</td>
</tr>
<tr>
<td>HSIL</td>
<td>16.9</td>
<td>14.64</td>
</tr>
<tr>
<td>Cancer</td>
<td>18.2</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table A7. Association of age at first intercourse and age at first pregnancy with cervical neoplasia.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>≤ 1 MW (Normal)</th>
<th>Mean MW</th>
<th>( \chi^2 )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cervix</td>
<td>24.60%</td>
<td>3.4</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>31.50%</td>
<td>2.8</td>
<td>0.06</td>
<td>0.8</td>
</tr>
<tr>
<td>Condyloma</td>
<td>14.30%</td>
<td>3.3</td>
<td>0.11</td>
<td>0.74</td>
</tr>
<tr>
<td>LSIL</td>
<td>40.30%</td>
<td>2.4</td>
<td>2.59</td>
<td>0.11</td>
</tr>
<tr>
<td>HSIL</td>
<td>43.30%</td>
<td>1.9</td>
<td>15.73</td>
<td>0.00007</td>
</tr>
<tr>
<td>Cancer</td>
<td>51.90%</td>
<td>1.5</td>
<td>32.22</td>
<td>0.00000001</td>
</tr>
</tbody>
</table>

Table A8. The association of monthly family income with the development of cervical neoplasia. MW = minimal wage = US$ 120.00.
monthly family income was significantly lower for women with HSIL or invasive cervical cancer compared to those with normal cervix ($P < 0.00007$). 24.6% of women with a normal cervix had a family income ≤ IMW. This rises to 43.3% and 51.9% in women with HSIL and cancer, respectively (Table A8).

(6.f) Level of education.

Of the population studied, 15% were illiterate, 35.1% had up to the primary school level of education, 27.1% had Junior High and 24.6% had Senior High levels of education, and 2.7% had finished or were at University. The enrollees' level of education ranged from illiterate to university degree. In the cancer group 54.3% were completely illiterate or just knew how to sign their name, and another 38.3% had only early primary school education. In the HSIL group 26.1% were illiterate and 39.5% were educated up to primary school level. No women with a university degree were diagnosed with HSIL or cancer in this study. In the control group 11% were illiterate, 28.5% had up to the primary level of education and 3.7% had university degree. The differences in level of education between women with HSIL or cervical cancer compared to those with normal cervix were significant: $\chi^2 = 49.6$ ($P < 0.0000001$) and $\chi^2 = 74.8$ ($P = 0.0000001$), respectively (Table A.9).

(6.g) Number of partners.

The enrollees referred to having had numbers of lifetime sexual partners ranging from 1 to 50 with a mean of 2.2 partners. In the cancer and HSIL groups the mean number of lifetime sexual partners were respectively 3.3 (range 1 to 50) and 2.8 (range 1 to 15), compared to 1.9 range (1 to 23) in the control group ($\chi^2 = 59.3$ and $P < 0.0000001$) (Table A.9).

71/458 (15.50%) of women who had only 1 lifetime sexual partner had HSIL or cervical cancer. 132/436 (30.27%) of women with more than 1 sexual partner (and up to 23) had a normal cervix and no HR HPV infection (Table A10). These findings emphasise the role of high-risk male partners and possibly the triggering of effective immune response in some women with multiple exposure to HR HPV types over the years. The risk of HSIL or cancer was, therefore, not only related to the number of sexual partners but it was mainly associated with HR HPV infection (Tables A.9 and A.2). The most common HPV
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Education status as (% of women with)</th>
<th>Mean no. of partners</th>
<th>Mean no. of gestation</th>
<th>Mean no. of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Illiterate</td>
<td>Primary education</td>
<td>University education</td>
<td>Unknown of partners</td>
</tr>
<tr>
<td>Normal</td>
<td>11.0</td>
<td>28.5</td>
<td>3.7</td>
<td>56.8</td>
</tr>
<tr>
<td>HSIL</td>
<td>26.1</td>
<td>39.5</td>
<td>0.0</td>
<td>34.4</td>
</tr>
<tr>
<td>Cancer</td>
<td>54.3</td>
<td>38.3</td>
<td>0.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table A9. The association of personal factors with the development of cervical neoplasia.
<table>
<thead>
<tr>
<th>No. of partners</th>
<th>HR HPV status</th>
<th>Normal</th>
<th>Cervicitis</th>
<th>Condyloma</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>28</td>
<td>17</td>
<td>0</td>
<td>18</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>219</td>
<td>67</td>
<td>1</td>
<td>38</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>&gt;1</td>
<td>Positive</td>
<td>27</td>
<td>19</td>
<td>0</td>
<td>48</td>
<td>64</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>132</td>
<td>39</td>
<td>6</td>
<td>35</td>
<td>23</td>
<td>6</td>
</tr>
</tbody>
</table>

Table A10. The association of number of partners with the development of cervical neoplasia.
types associated with having more than one sexual partner in the population studied were HPV 16, 31, 58, 33, 35, 11, 18, and 45 (in order of decreasing frequency).

(6.h) Number of gestations.

The number of gestations for all participants ranged from 0 to 27 with a mean of 3.9. For women with cervical cancer or HSIL the mean numbers of pregnancies were respectively 7.6 (range 0 to 20) and 5.3 (range 0 to 27) compared to 3.4 (range 0 to 22) in the control group (Table A9). This difference was highly significant: $\chi^2 = 129.6$ and $P < 0.0000001$.

(6.i) Parity.

The number of vaginal deliveries varied from 0 to 27 with a mean of 2.9 vaginal births. In the cancer and HSIL groups the means were respectively 6.6 (range 0 to 17) and 4.4 (range 0 to 27), compared to 2.3 (range 0 to 16) in the control group. These differences were also highly significant: $\chi^2 = 149.7$ and $P < 0.0000001$. The number of caesarean sections in the total study population ranged from 0 to 3 with a mean of 0.4. In the cancer and HSIL groups the mean numbers of caesarean sections were respectively 0.1 (range 0 to 2) and 0.3 (range 0 to 3), compared to 0.5 (range 0 to 3) in the control group ($\chi^2 = 27.7$ and $P = 0.00004$) (Table A9).

The ORs in favour of HSIL and cervical cancer when a woman had 4 vaginal deliveries or more were, respectively, 3.88, 95% CI range = 2.53 - 5.96, $\chi^2 = 44.90$, $P < 0.0000001$ and 8.50, 95% CI range = 4.90; 15.15, $\chi^2 = 78.55$, $P < 0.0000001$.

(6.j) Contraception.

65.4% of women used some methods of contraception: 40% had had tubal ligation, 13.4% had used or were using OCP, 6% used condoms, 1.8% had used or were using monthly doses of intramuscular depot medroxyprogesterone acetate or other injectable contraceptive, 1.1% used an intrauterine contraceptive device (IUCD), and 3.1% used other barrier methods. Overall, the use of contraception did not significantly increase the risk of cervical neoplasia in the population studied (Table A11).

The analysis of both crude OR (Table A12) and the OR after adjustment for HR HPV infection (Table A24) failed to show a significant association between the use of the
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>0.23</td>
<td>0.13 - 0.39</td>
<td>37.2</td>
<td>$P &lt; 0.000001$</td>
</tr>
<tr>
<td>HSIL</td>
<td>1.33</td>
<td>0.84 - 2.10</td>
<td>1.39</td>
<td>$P = 0.24$</td>
</tr>
<tr>
<td>LSIL</td>
<td>1.28</td>
<td>0.84 - 1.96</td>
<td>1.22</td>
<td>$P = 0.27$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>0.74</td>
<td>0.55 - 1.00</td>
<td>3.86</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

Table A11. Risk of cervical neoplasia or HR HPV infection associated with contraception as whole.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>0.14</td>
<td>0.02 - 0.59</td>
<td>9.01</td>
<td>$P &lt; 0.003$</td>
</tr>
<tr>
<td>HSIL</td>
<td>0.70</td>
<td>0.36 - 1.34</td>
<td>1.01</td>
<td>$P = 0.31$</td>
</tr>
<tr>
<td>LSIL</td>
<td>1.08</td>
<td>0.62 - 1.87</td>
<td>0.02</td>
<td>$P = 0.88$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>1.10</td>
<td>0.72 - 1.68</td>
<td>0.14</td>
<td>$P = 0.71$</td>
</tr>
</tbody>
</table>

Table A12. Risk of cervical neoplasia or HR HPV infection associated with use of OCP.
contraceptive pill and risk of cervical cancer, SIL or HR HPV infection. When adjusting
for HR HPV infection, the risk of cancer associated with use of OCP decreased from 0.14
to 0.00, the risk of HSIL went from 0.70 to 0.80, and the risk of LSIL increased slightly
from 1.08 to 1.21, but none of these differences were significant (P > 0.88).

The risk of cervical cancer associated with tubal ligation is shown in Table A13. After
adjustment for HR HPV infection the risk of cervical cancer associated with tubal ligation
went from 0.50 to 0.83, the risk of HSIL went from 1.61 to 1.33, and the one of LSIL
from 0.64 to 0.92 (Table A24).

All other methods of contraception (monthly shots of injectable contraceptives, IUCD,
condom, and other barrier methods) when analysed individually as variables possibly
related to risk of cervical neoplasia did not show any statistical significance.

(6.k) Smoking history.

23.3% women in the study were current smokers. 14.9% had smoked but had stopped
some time in the past, and 61.8% had never smoked. 84.7% of smokers preferred
cigarettes, 9.4% used cigars and 5.9% smoked pipes. Of the women with any grade of
SIL, 37.7% were current smokers, 18.9% were ex-smokers, and in the control group
12.2% were smokers and 14.8% were ex-smokers.

The risk associated with current smoking not adjusted (Table A14) or adjusted (Table
A24) for the presence of HR HPV infection showed that it was an independent risk factor.
The adjusted ORs for cervical cancer, HSIL and LSIL when the women were current
smokers were 3.94, 5.79, and 1.85 (Table A24).

A past habit of smoking, even though the patient had since stopped, was a risk factor for
HSIL and cervical cancer if the data was not adjusted for the presence of HR HPV (Table
A15). Adjusted risks (Table A24) were as follows: cervical cancer (1.59), HSIL (1.51)
and LSIL (0.57). Compared to women, who were current smokers, those that quit
smoking decreased the risk of having LSIL, HSIL, HR HPV infection, and cervical
cancer by more than 70%, 60%, 50%, and 30%, respectively. Women who had never
smoked did not have increased risk of cervical neoplasia or HR HPV infection; on the
contrary, never having smoked was protective, particularly against HSIL and cervical
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>( \chi^2 ) or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>0.50</td>
<td>0.28 - 0.88</td>
<td>6.09</td>
<td>( P = 0.01 )</td>
</tr>
<tr>
<td>HSIL</td>
<td>1.61</td>
<td>1.01 - 2.58</td>
<td>4.16</td>
<td>( P = 0.04 )</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.64</td>
<td>0.41 - 0.98</td>
<td>4.25</td>
<td>( P = 0.04 )</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>0.70</td>
<td>0.51 - 0.96</td>
<td>5.00</td>
<td>( P = 0.03 )</td>
</tr>
</tbody>
</table>

Table A13. Risk of cervical neoplasia or HR HPV infection associated with tubal ligation.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>4.17</td>
<td>2.37 - 7.33</td>
<td>29.89</td>
<td>$P &lt; 0.00000005$</td>
</tr>
<tr>
<td>HSIL</td>
<td>5.35</td>
<td>3.34 - 8.58</td>
<td>60.56</td>
<td>$P &lt; 0.00000001$</td>
</tr>
<tr>
<td>LSIL</td>
<td>1.39</td>
<td>0.85 - 2.26</td>
<td>1.68</td>
<td>$P = 0.20$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>2.24</td>
<td>1.58 - 3.16</td>
<td>22.72</td>
<td>$P = 0.000002$</td>
</tr>
</tbody>
</table>

Table A14. Risk of cervical neoplasia or HR HPV infection associated with current smoking habit.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>2.86</td>
<td>1.50 - 5.40</td>
<td>11.58</td>
<td>$P = 0.0007$</td>
</tr>
<tr>
<td>HSIL</td>
<td>2.15</td>
<td>1.22 - 3.75</td>
<td>7.60</td>
<td>$P = 0.006$</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.40</td>
<td>0.18 - 0.86</td>
<td>5.89</td>
<td>$P = 0.02$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>1.11</td>
<td>0.73 - 1.69</td>
<td>0.18</td>
<td>$P = 0.67$</td>
</tr>
</tbody>
</table>

Table A15. Risk of cervical neoplasia or HR HPV infection associated with a past smoking habit.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>0.29</td>
<td>0.17 - 0.48</td>
<td>27.31</td>
<td>$P &lt; 0.0000002$</td>
</tr>
<tr>
<td>HSIL</td>
<td>0.21</td>
<td>0.14 - 0.33</td>
<td>57.17</td>
<td>$P &lt; 0.0000001$</td>
</tr>
<tr>
<td>LSIL</td>
<td>1.15</td>
<td>0.75 - 1.77</td>
<td>0.32</td>
<td>$P = 0.57$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>0.62</td>
<td>0.46 - 0.84</td>
<td>10.28</td>
<td>$P = 0.001$</td>
</tr>
</tbody>
</table>

Table A16. Risk of cervical neoplasia or HR HPV infection associated with never having smoked.
cancer (Table A16). These results clearly showed that smoking is an independent risk factor for SIL (Table A24). The number of smoking years was a significant risk factor for HR HPV infection and the development of HSIL, and cancer but not as much for LSIL (Table A17). Relative to the time ex-smokers with normal cervix had stopped smoking, there was no significant difference between the time since having stopped smoking and the incidence of HR HPV infection, SIL or cancer (Table A18).

The risks for cancer, HSIL, and HR HPV infection varied depending on the type of tobacco smoked (Tables A19, A20, and A21). The risk of cancer was particularly evident in women who smoked a pipe (OR = 13.92) or cigars (OR = 3.56).

The mean age at which women with normal cervix started to smoke was 16.9 years and the mean for women who did not have HR HPV was 16.8 years. The data presented in Table A22 shows that there was a significant association between cervical HR HPV infection, HSIL and cervical cancer with early age at starting to smoke.

The number of cigarettes smoked per day did not have a significant direct effect on the occurrence of cervical cancer and HR HPV infection when compared with the number smoked by women with a normal cervix. However, the mean numbers of cigarettes smoked daily (MDC) by women who were diagnosed with HSIL and LSIL were significantly higher than the mean number smoked by women in the control group (Table A23). The MDC for women in the control group was 12.4 cigarettes.

(7) Conclusion

The association of HR HPV with cervical neoplasia is overwhelming. Other risk factors can only be analysed after adjustment for the presence of HR HPV (Table A24). Overall, HSIL and cancer were found to occur predominantly in women in low the socio-economic groups, smokers, and with little education. It is not known for sure why women with LSIL smoked fewer cigarettes, for less time and starting from an older age than women with HR HPV infection or other cervical neoplasia, although this may be due to the fact that they were in average a decade younger than the ones with HSIL and 24 years younger than those with cervical cancer (Table A3), thus suggesting they are different generations with different habits and behaviour.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Mean time (years)</th>
<th>( \chi^2 ) or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>37.30</td>
<td>48.18</td>
<td>( P &lt; 0.00000001 )</td>
</tr>
<tr>
<td>HSIL</td>
<td>23.60</td>
<td>11.11</td>
<td>( P &lt; 0.0009 )</td>
</tr>
<tr>
<td>LSIL</td>
<td>15.10</td>
<td>3.52</td>
<td>( P = 0.06 )</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>25.60</td>
<td>20.85</td>
<td>( P &lt; 0.0000005 )</td>
</tr>
</tbody>
</table>

Table A17. Association of cervical neoplasia or HR HPV infection with time a woman has smoked any kind of tobacco for.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Mean time (years)</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>11.80</td>
<td>0.12</td>
<td>$P = 0.74$</td>
</tr>
<tr>
<td>HSIL</td>
<td>8.70</td>
<td>0.30</td>
<td>$P = 0.59$</td>
</tr>
<tr>
<td>LSIL</td>
<td>6.10</td>
<td>3.46</td>
<td>$P = 0.06$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>8.90</td>
<td>0.09</td>
<td>$P = 0.76$</td>
</tr>
</tbody>
</table>

Table A18. Association of cervical neoplasia or HR HPV infection with the length of time since stopping smoking. The mean time since having stopped smoking among ex-smokers with normal cervix was 8.1 years, and among women who tested negative for HR HPV the mean was 8.4 years.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>1.15</td>
<td>0.64 - 2.06</td>
<td>0.14</td>
<td>$P = 0.71$</td>
</tr>
<tr>
<td>HSIL</td>
<td>3.01</td>
<td>1.75 - 5.21</td>
<td>17.93</td>
<td>$P = 0.00002$</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.94</td>
<td>0.56 - 1.57</td>
<td>0.02</td>
<td>$P = 0.89$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>1.25</td>
<td>0.88 - 1.76</td>
<td>1.47</td>
<td>$P = 0.22$</td>
</tr>
</tbody>
</table>

Table A19. Risk of cervical neoplasia and HR HPV infection associated with cigarette smoking.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>13.92</td>
<td>4.30 - 47.70</td>
<td>32.36</td>
<td>$P = 0.0000007$</td>
</tr>
<tr>
<td>HSIL</td>
<td>2.33</td>
<td>0.53 - 9.69</td>
<td>0.89</td>
<td>$P = 0.24$</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.50</td>
<td>0.02 - 4.06</td>
<td>0.06</td>
<td>$P = 1.0$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>3.86</td>
<td>1.35 - 11.54</td>
<td>7.22</td>
<td>$P = 0.007$</td>
</tr>
</tbody>
</table>

Table A20. Risk of cervical neoplasia and HR HPV infection associated with pipe smoking.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Crude OR</th>
<th>95% CI</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>3.56</td>
<td>1.47 - 8.58</td>
<td>9.00</td>
<td>$P = 0.003$</td>
</tr>
<tr>
<td>HSIL</td>
<td>2.37</td>
<td>0.99 - 5.62</td>
<td>3.78</td>
<td>$P = 0.05$</td>
</tr>
<tr>
<td>LSIL</td>
<td>0.17</td>
<td>0.01 - 1.20</td>
<td>2.94</td>
<td>$P = 0.06$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>1.63</td>
<td>0.75 - 3.52</td>
<td>1.35</td>
<td>$P = 0.25$</td>
</tr>
</tbody>
</table>

Table A21. Risk of cervical neoplasia and HR HPV infection associated with cigar smoking.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Mean age started smoking (years)</th>
<th>$\chi^2$ or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>13.90</td>
<td>13.54</td>
<td>$P = 0.0002$</td>
</tr>
<tr>
<td>HSIL</td>
<td>14.40</td>
<td>10.51</td>
<td>$P = 0.001$</td>
</tr>
<tr>
<td>LSIL</td>
<td>17.20</td>
<td>3.48</td>
<td>$P = 0.06$</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>14.80</td>
<td>14.80</td>
<td>$P = 0.0001$</td>
</tr>
</tbody>
</table>

Table A22. Association between the presence of cervical neoplasia or HR-HPV infection and mean age participants started smoking.
<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>Mean daily cigarettes (MDC)</th>
<th>MDC in controls</th>
<th>( \chi^2 ) or Wilcoxon two-sample test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>12.80</td>
<td>12.40</td>
<td>0.35</td>
<td>( P = 0.56 )</td>
</tr>
<tr>
<td>HSIL</td>
<td>14.40</td>
<td>12.40</td>
<td>5.57</td>
<td>( P &lt; 0.02 )</td>
</tr>
<tr>
<td>LSIL</td>
<td>8.80</td>
<td>12.40</td>
<td>11.50</td>
<td>( P &lt; 0.0007 )</td>
</tr>
<tr>
<td>HR HPV infection</td>
<td>12.90</td>
<td>12.00</td>
<td>1.48</td>
<td>( P = 0.22 )</td>
</tr>
</tbody>
</table>

Table A23. Mean daily quantity of cigarettes smoked by cases and controls.
<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Odds ratio</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSIL</td>
<td>HSIL</td>
<td>Cancer</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 35 years</td>
<td>1.0 (Referent)</td>
<td>1.0 (Referent)</td>
<td>1.0 (Referent)</td>
</tr>
<tr>
<td>35 to 50 years</td>
<td>0.53</td>
<td>3.37</td>
<td>5.42</td>
</tr>
<tr>
<td>&gt; 50 years</td>
<td>0.16</td>
<td>1.67</td>
<td>50.00</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illiteracy</td>
<td>0.63</td>
<td>3.54</td>
<td>9.33</td>
</tr>
<tr>
<td>High school level</td>
<td>0.55</td>
<td>0.22</td>
<td>0.00</td>
</tr>
<tr>
<td>Obstetric factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of pregnancies ≥ 5</td>
<td>0.84</td>
<td>3.40</td>
<td>11.72</td>
</tr>
<tr>
<td>No. of vaginal deliveries ≥ 3</td>
<td>0.87</td>
<td>3.52</td>
<td>28.17</td>
</tr>
<tr>
<td>Sexual activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of partners ≥ 3</td>
<td>1.61</td>
<td>1.73</td>
<td>0.39</td>
</tr>
<tr>
<td>Contraception</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral contraceptive pills</td>
<td>1.13</td>
<td>0.77</td>
<td>0.00</td>
</tr>
<tr>
<td>Tubal ligation</td>
<td>0.92</td>
<td>1.33</td>
<td>0.83</td>
</tr>
<tr>
<td>Condom</td>
<td>2.15</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>1.0 (Referent)</td>
<td>1.0 (Referent)</td>
<td>1.0 (Referent)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>0.57</td>
<td>1.51</td>
<td>1.59</td>
</tr>
<tr>
<td>Current smoker</td>
<td>1.85</td>
<td>5.79</td>
<td>3.94</td>
</tr>
</tbody>
</table>

Table A24. Risk factors for cervical neoplasias adjusted for presence of HR HPV infection.
Section B. Detection of HR HPV by Hybrid Capture 1 (HC1).

If the detection of HR HPV is to be carried out in cervical cancer screening programmes in Recife, where only limited molecular biological facilities are available, a commercially available, quality controlled test would be more suitable than PCR/RFLP that is not commercially available as a kit yet. For this purpose, HC1 was evaluated for the identification of cancer.

(1) Experimental design.

This is a prospective case-control study designed to evaluate the role of HPV testing by HC1 in the detection of cervical cancer in a high-risk population.

(2) Study population.

Participants were recruited by convenience sampling from the screening services of 3 referral hospitals in Recife: the Institute of Mother and Child Health in Pernambuco (IMIP), the Cancer Hospital (HCP) and Barão de Lucena Hospital.

Since invasive cancer usually occurs in older women, only women aged 35 years or older were included in the control group. Women with SIL were excluded. Pregnant women were not included.

(3) Ethics permission and consent.

The study protocol was reviewed and approved by the local Ethics Committee. After counselling and signing an informed consent form, the participants were interviewed by means of a structured questionnaire addressing epidemiological variables such as age at first pregnancy, number of pregnancies, number of vaginal deliveries and place of residence.

(4) Procedures.

All volunteers had ecto- and endocervical cells collected with a Dacron swab in the Digene collection kit for HC1 followed by colposcopy and biopsy when indicated. Samples were analysed using HC1, which detects collectively HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 56. Inclusion in the case group was based on histopathology. Inclusion in the control group was by a combination of negative cytology and normal colposcopy.
(5) Statistical considerations

Epi-Info 6 and SPSS 6 for Windows were used for statistical analysis. In the analyses of single tables we describe Yates corrected values for Chi-squares and P-values, and exact confidence interval for odds ratio. Statistical significance was considered when P-values were ≤ 0.05.

(6) Results.

A total of 140 women were enrolled in the study, 70 with cervical cancer and 70 with negative cytology and normal colposcopic examination. 90% of the tumours were invasive squamous cell carcinomas, 53% were stage 1B, 22% were stage 2 and 25% were stage 3. 10% were adenocarcinomas. The groups were homogeneous regarding age: the mean age was 43 [range 35 - 60, SD = 6.03] in the control group and 47 [range 16 - 78, SD = 12.77] in the cancer group (P = 0.12).

31 (44%) of the women with cancer had never had a Pap smear and 12 (17%) had false negative cytological results. 15% of women with cervical cancer were 35 years or younger. One case was of particular interest. The patient, aged 16, was diagnosed with stage 1B cervical cancer. She was illiterate, lived in a remote area, had never had a Pap smear and had her first pregnancy at 12 years of age, and was now Gesta 4 Para 3 Abortion 1.

For detection of cervical cancer, HC1 showed sensitivity of 82.9%, a specificity of 41.4%, a positive predictive value of 58.6% and a negative predictive value of 70.7% (Table B1). The odds ratio in favour of cervical cancer when a woman tested positive for HR HPV by HC1 was 3.42 [1.47; 8.20], $\chi^2 = 8.83$ and $P < 0.003$.

Other local risk factors including age at first pregnancy, number of gestations, and number of vaginal deliveries are described in Table B2. A comparison of various risk factors showed that HR HPV was only one of the many factors associated with cancer in this group of women (Table B3).
### Hybrid Capture 1

<table>
<thead>
<tr>
<th>Cervical lesions</th>
<th>Cancer</th>
<th>Normal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>58</td>
<td>41</td>
<td>99</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>29</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>70</td>
<td>140</td>
</tr>
</tbody>
</table>

Table B1. Efficacy of Hybrid Capture I alone for detection of cervical cancer.
<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Population</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first pregnancy (yrs)</td>
<td>Controls</td>
<td>14 to 38</td>
<td>23</td>
<td>5.7</td>
<td>8.40</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Cases</td>
<td>14 to 28</td>
<td>19</td>
<td>3.2</td>
<td>0.05</td>
<td>0.755</td>
</tr>
<tr>
<td>No. of gestations</td>
<td>Controls</td>
<td>0 to 18</td>
<td>4</td>
<td>3.2</td>
<td>12.82</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Cases</td>
<td>1 to 20</td>
<td>7</td>
<td>5.0</td>
<td>0.04</td>
<td>0.835</td>
</tr>
<tr>
<td>No. of vaginal deliveries</td>
<td>Controls</td>
<td>0 to 14</td>
<td>3</td>
<td>3.0</td>
<td>15.06</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Cases</td>
<td>0 to 19</td>
<td>6</td>
<td>4.4</td>
<td>0.05</td>
<td>0.835</td>
</tr>
</tbody>
</table>

Table B2. Relevant local risk factors. SD = Standard deviation.
<table>
<thead>
<tr>
<th>HR HPV by HCl</th>
<th>Local risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age at first pregnancy ≤ 20</td>
</tr>
<tr>
<td>Odds Ratio</td>
<td>3.42</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.43-8.20</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>8.83</td>
</tr>
<tr>
<td>$P$ -value</td>
<td>&lt; 0.003</td>
</tr>
</tbody>
</table>

Table B3. Odds ratio in favor of cervical cancer resulting from local risk factors. CI = Confidence interval.
Section C. Detection of HR HPV by PCR and RFLP

(1) Experimental design.

This is a nested case-control within a cohort study involving 479 women attending cervical cancer screening programmes at the Institute of Mother and Child Health in Pernambuco (IMIP) and the Cancer Hospital in Pernambuco (HCP), both teaching Institutions in Recife (Brazil).

(2) Study population.

Unselected women coming for cervical cancer screening were invited to participate in the study.

(3) Ethics permission and consent.

The local Ethics Committee has reviewed and approved the research protocol.

(4) Procedures.

A Pap smear was collected with an Ayre’s spatula from the ectocervix and with a brush from the endocervical canal. If at the time of smear collection there was clinical suspicion of invasive disease, the woman had immediate colposcopically guided biopsy along with HPV testing. Women whose cytology report was LSIL or above and those with clinical suspicion of cervical cancer, even without accompanying cytology, were initially included as potential cases. Women with negative cytology were initially included as potential controls. Final clinicopathological classification was based on histopathology for cases and on overall clinical decision made upon gynaecological history, examination, and a combination of cytology negative for neoplastic cells and normal colposcopy for controls.

After participants’ informed consent forms were obtained, all volunteers had cervical scrapes collected for HPV testing using an Ayre’s spatula including those with normal cytology. The cervical cells from the spatula were dispersed into phosphate-buffered saline (PBS) solution and were stored at -20°C for HPV analyses by PCR/RFLP. In order to have a better assessment of HPV detection efficacy, all enrollees including those with negative cytology underwent thorough colposcopic examination. Biopsy was done when
clinically indicated. Only after all samples had been analysed for the presence of HPV DNA and typed was comparison with final clinicopathological classification made and the efficacy of the method analysed. The analysis of efficacy of HPV detection was based on HR HPV types only.

(5) Statistical considerations

The analysis of efficacy of HPV detection was based on HR HPV types only. Epi-info 6 and SPSS were used for statistical analysis. All Chi-squares and P-values are Yates corrected. The two-tailed Fisher’s exact test was used when a cell on a 2x2 table had a value less than 5. The 95% Confidence Intervals (CI) are the exact limits. Statistical significance was considered when P-values were ≤ 0.05.

(6) Results.

A total of 479 women aged 13 to 84 (mean 36.5 ± 13) years enrolled in the study. This included 41 (8.6%) aged 20 or younger, 138 (28.8%) aged 21 to 30, 140 (29.2%) aged 31 to 40, 94 (19.6%) 41 to 50 and 66 (13.8%) over 50 years of age. According to final clinicopathological criteria, 215 had normal cervix, 79 had cervicitis, 4 condyloma, 62 LSIL, 60 HSIL and 59 invasive cervical cancer. Among all enrollees, 31 women entered the study without a cytology report but with clinical suspicion of cervical cancer. The cervical cancer stages were as follows: 1 (1.7%) microinvasive, 13 (22%) stage IB, 20 (33.9%) stage II, 22 (37.3%) stage III, and 3 (5.1%) stage IV. Fifty-five cases (93.2%) were squamous cell carcinomas and 4 (6.8%) were adenocarcinomas.

42.2% (202/479) of the population studied were HR HPV positive (Table C1). HPV16 DNA was detected in 101 women representing 21.1% of all specimens tested and 50% of specimens positive for HR HPV. Among these women with HPV 16, 80% had cervical neoplasia, most of which (68%) were HSIL and cancer. HPV 18 infection was detected in only 11 (2%) of all women included in the study. Three were associated with invasive cancers (1 squamous cell carcinoma and 2 adenocarcinomas), 2 with HSIL, 2 with LSIL, 3 with cervicitis and 1 with a normal cervix. Among the 112 women who tested positive for either HPV 16 or 18 thirty-eight (33.9%) had LSIL, cervicitis or normal cervix.

HR HPV detection identified HSIL and cervical cancer with sensitivities of 86.7% and 89.8% respectively, with specificities of 81.5% and 84.7% (Table C1). The negative
<table>
<thead>
<tr>
<th>HPV types</th>
<th>Normal</th>
<th>Cervicitis</th>
<th>Condyloma</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>173</td>
<td>57</td>
<td>1</td>
<td>20</td>
<td>8</td>
<td>6</td>
<td>265</td>
</tr>
<tr>
<td>LR HPV</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Subtotal</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>HR HPV</td>
<td>16</td>
<td>15</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>31</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>2</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td>33</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>35</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>52</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>58</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>Subtotal</td>
<td>33</td>
<td>22</td>
<td>0</td>
<td>42</td>
<td>52</td>
<td>53</td>
<td>202</td>
</tr>
<tr>
<td>Total</td>
<td>215</td>
<td>79</td>
<td>4</td>
<td>62</td>
<td>60</td>
<td>59</td>
<td>479</td>
</tr>
</tbody>
</table>

Table C1. The association of cervical lesions with HPV detectable by PCR/RFLP.
predictive values (NPV) for HSIL and cancer were respectively 87.7% and 96.8%. The efficacy of HR HPV detection alone for identification of cervical neoplasia was clearly observed in the 31 women who entered the study on clinical suspicion of cervical cancer. In these women without cytology, HPV typing identified 2/3 (67%) HSIL and 26/28 (93%) invasive cancers.

Table C2 shows the correlation between cytology and histological grades and Table C3 shows the same data subdivided into HR HPV positive and negative classes. The assessment of cytology efficacy was based on the 448 participants who had a Pap smear test. The sensitivity and specificity of cytology for HSIL and cervical cancer detection are comparable to those achieved by detection of HR HPV (Table C5). However, as shown in Table C3, 9 histologic high-grade lesions (6 invasive cancers and 3 HSIL) were found in the 125 women with smears reported with cellular inflammatory changes but negative for neoplastic cells. 2 HSIL were detected in women with completely negative cytology reports. The false negative rate for cytology reports for women who actually had LSIL, HSIL and cervical cancer were respectively 50%, 8.8% and 19.4%.

The risks of cervical cancer in women with either abnormal cytology (ASCUS or above, LSIL or above), or positive for any HR HPV or for HPV16 or HPV18 in particular are shown in Table C4. There was a strong association between HR HPV positive results and the presence of cervical cancer. Women with HR HPV had about 50-fold increased risk of cervical cancer (OR = 48.72 [18.04; 139.41], \( \chi^2 = 115.8 \) and \( P < 0.00001 \)) and about 36-fold increased risk of HSIL (OR = 35.85 [14.89; 93.73], \( \chi^2 = 108.41 \) and \( P < 0.00001 \)). Of the HR HPV types identified, HPV 16 and 18 were the most oncogenic (i.e. had the strongest association with cancer).

The efficacy of combining HPV typing with cytology as a screening protocol for LSIL, HSIL and cancer is shown in Table C4. Our analysis, which includes cases without cytology but with HPV testing, shows a sensitivity of 96.8% for detection of cervical cancer and 96.6% for detection of HSIL and cervical cancer altogether (Table C5). The negative predictive values of the combined protocol were also high. Based on both the analysis of efficacy stratified by age groups, including the initial 479 participants (Table C6) and all the 921 women included in this thesis (Table C7), a combination of cytology...
<table>
<thead>
<tr>
<th>Cytology</th>
<th>Normal</th>
<th>Cervicitis</th>
<th>Condyloma</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>118</td>
<td>33</td>
<td>2</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>170</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>69</td>
<td>30</td>
<td>1</td>
<td>16</td>
<td>3</td>
<td>6</td>
<td>125</td>
</tr>
<tr>
<td>ASCUS/AGUS</td>
<td>14</td>
<td>10</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>LSIL</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>16</td>
<td>11</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>HSIL</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>34</td>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>Cancer</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>16</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>215</td>
<td>79</td>
<td>4</td>
<td>62</td>
<td>57</td>
<td>31</td>
<td>448</td>
</tr>
</tbody>
</table>

Table C2. Identification of cervical lesions by cytology.
<table>
<thead>
<tr>
<th>HR HPV Cytology</th>
<th>Cervical lesions</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td>Normal</td>
<td>Cervicitis</td>
<td>Condyloma</td>
<td>LSIL</td>
<td>HSIL</td>
<td>Cancer</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCUS/AGUS</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSIL</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>13</td>
<td>10</td>
<td>0</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSIL</td>
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<td>1</td>
<td>0</td>
<td>7</td>
<td>30</td>
<td>6</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>13</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>33</td>
<td>22</td>
<td>0</td>
<td>42</td>
<td>50</td>
<td>27</td>
<td>174</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Negative        | 100             | 25    | 2      | 7           | 1    | 0    | 135 |
| Inflammatory    | 61              | 23    | 1      | 9           | 1    | 1    | 96  |
| ASCUS/AGUS      | 10              | 6     | 1      | 0           | 0    | 0    | 17  |
| LSIL            | 7               | 3     | 0      | 3           | 1    | 0    | 14  |
| HSIL            | 3               | 0     | 0      | 1           | 4    | 0    | 8   |
| Cancer          | 1               | 0     | 0      | 0           | 0    | 3    | 4   |
| Subtotal        | 182             | 57    | 4      | 20          | 7    | 4    | 274 |

Total

215  79  4  62  57  31  448

Table C3. Identification of cervical lesions by HR HPV detection and cytology.
<table>
<thead>
<tr>
<th>Referral tool</th>
<th>Criteria</th>
<th>Detection of cervical cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR</td>
</tr>
<tr>
<td>Cytology</td>
<td>ASCUS or above</td>
<td>27.83</td>
</tr>
<tr>
<td></td>
<td>Mild dyskaryosis or above</td>
<td>35.10</td>
</tr>
<tr>
<td>HPV typing</td>
<td>Any HR HPV</td>
<td>48.72</td>
</tr>
<tr>
<td></td>
<td>HPV 16</td>
<td>67.28</td>
</tr>
<tr>
<td></td>
<td>HPV 18</td>
<td>86.50</td>
</tr>
</tbody>
</table>

Table C4. The utility of cytology or HR HPV as a referral tool for the detection of cervical cancer.
<table>
<thead>
<tr>
<th>Screening tool</th>
<th>Histology</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>SIL+Cancer</td>
<td>71.50%</td>
<td>85.20%</td>
<td>71.10%</td>
<td>85.50%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>87.50%</td>
<td>87.0%</td>
<td>53.80%</td>
<td>94.40%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>80.60%</td>
<td>87.00%</td>
<td>52.80%</td>
<td>96.90%</td>
</tr>
<tr>
<td>HPV typing</td>
<td>SIL+Cancer</td>
<td>81.20%</td>
<td>81.50%</td>
<td>72.80%</td>
<td>87.70%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>88.20%</td>
<td>84.70%</td>
<td>76.10%</td>
<td>92.90%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>89.80%</td>
<td>84.70%</td>
<td>61.60%</td>
<td>96.80%</td>
</tr>
<tr>
<td>Combined</td>
<td>SIL+Cancer</td>
<td>87.30%</td>
<td>71.10%</td>
<td>60.40%</td>
<td>91.80%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>96.60%</td>
<td>74.90%</td>
<td>39.20%</td>
<td>98.20%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>96.80%</td>
<td>74.90%</td>
<td>35.70%</td>
<td>99.40%</td>
</tr>
</tbody>
</table>

Table C5. A comparison of cytology and HR HPV typing as screening tools for cervical neoplasia.
<table>
<thead>
<tr>
<th>Age</th>
<th>Screening tool</th>
<th>For HSIL and Ca</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>PPV</td>
</tr>
<tr>
<td>35 or younger</td>
<td>Cytology</td>
<td>82.10%</td>
<td>93.60%</td>
<td>67.60%</td>
</tr>
<tr>
<td></td>
<td>HR HPV</td>
<td>89.30%</td>
<td>78.90%</td>
<td>40.30%</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>100.00%</td>
<td>74.30%</td>
<td>41.20%</td>
</tr>
<tr>
<td>Older than 35</td>
<td>Cytology</td>
<td>80.00%</td>
<td>92.70%</td>
<td>84.20%</td>
</tr>
<tr>
<td></td>
<td>HR HPV</td>
<td>87.90%</td>
<td>85.40%</td>
<td>81.60%</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>95.00%</td>
<td>79.70%</td>
<td>75.00%</td>
</tr>
</tbody>
</table>

Table C6. Cytology and HR HPV detection as screening tools for HSIL and cervical cancer in younger and older women (total = 448 participants).
<table>
<thead>
<tr>
<th>Age</th>
<th>Screening tool</th>
<th>For HSIL and Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitivity</td>
</tr>
<tr>
<td>35 or younger</td>
<td>Cytology</td>
<td>68.80%</td>
</tr>
<tr>
<td></td>
<td>HR HPV</td>
<td>76.00%</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>95.90%</td>
</tr>
<tr>
<td>Older than 35</td>
<td>Cytology</td>
<td>76.60%</td>
</tr>
<tr>
<td></td>
<td>HR HPV</td>
<td>78.00%</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>96.50%</td>
</tr>
</tbody>
</table>

Table C7. Cytology and HR HPV detection as screening tools for HSIL and cervical cancer in younger and older women included in the whole project (total = 921 participants).
and HR HPV detection represents an effective screening protocol particularly in older women.

Section D. Hormonal influence in the prevalence of cervical HPV infection

(1) Experimental design.

This is a prospective cohort study undertaken at two screening services in Recife (Brazil). The primary end point of this study was to evaluate whether pregnant women from a high-risk population have a higher prevalence of cervical HPV infection compared to non-pregnant non-menopausal (control group) and menopausal women. As a secondary aim, comparisons were made between controls and women in the menopause in order to understand better the clinical significance of the physiologic hormonal changes of menopause in relation to the prevalence of cervical HPV infection. This approach generated data for testing the hypothesis that the decrease in prevalence of HPV infection in older women may be related to the decrease in circulating blood levels of sex steroid hormones that occur in the menopause.

Based on Schneider's report (Schneider et al., 1987) that the prevalence of HPV infection in the lower genital tract of pregnant women was 28.3% compared to 12.5% in non-pregnant women, this study was designed, with a 4:1 ratio of controls to cases, offered a power of over 75% within the 95% confidence interval.

(2) Study population.

Potential enrollees from cervical cancer screening programmes at the Institute of Mother and Child Health in Pernambuco (IMIP) and the Cancer Hospital in Pernambuco (HCP) were invited to participate in the study. Women allegedly known to be HIV negative, without age limit and with or without cytological abnormalities were invited to participate and sign an informed consent form.

Participants were randomly selected and included into one of 3 distinct clinical categories: pregnant women, non-pregnant non-menopausal women, and women in the menopause. All pregnant volunteers had intact amniotic membranes. Women who met the classification criteria of non-pregnant non-menopausal clinical status represented the control group. Pregnant women and menopausal women were analysed individually as
case groups. Women who had surgically induced menopause due to benign disease were also included. Women in the menopause group were those with clinical signs and symptoms of ovarian failure for at least one year. All women who were in the menopause were either on hormone replacement therapy (HRT) or being evaluated for HRT.

(3) Ethics permission and consent.

The procedures followed in this study were in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki and the study protocol was reviewed and approved by the local Ethics Committee.

(4) Procedures.

All volunteers, in addition to a routine Pap smear, had an ectocervical scrape collected with an Ayre’s spatula and an endocervical sample of cells carefully collected with a brush by an experienced colposcopist before having a thorough colposcopic examination. Punch biopsy was performed when colposcopically indicated. Classification of neoplasia was based on histology. Normal cervix was a combination of negative cytology and normal colposcopy. Cells collected were dispersed into phosphate-buffered saline (PBS) solution, stored at -20°C and later tested for HPV infection using PCR/RFLP.

(5) Statistical considerations

The computer package Epi-info 2000 was used for statistical analysis. Significance levels, which intervals were the exact limits, were considered for P-values < 0.05. P-values were Yates corrected or 2-tailed Fisher exact wherever applicable. When analysis of variance showed samples to differ non-parametric results were used.

(6) Results.

A total of 360 women aged 16 to 88 years, mean 36.7 ± 13.7 years, mode and median respectively 20 and 35 years were enrolled in the study. 240 were included in the control group (66.7%), 60 were pregnant (16.7%) and 60 were in the menopause (16.7%). The majority of enrollees (75%) did not have cervical neoplasia. Seventeen (4.7%) had LSIL, 51 (14.2%) had HSIL and 22 (6.1%) had cervical cancer. The majority of HSIL (82.4%) were detected in the control group and the majority of cervical cancers (68.2%) were diagnosed in menopausal women (Table D1).
<table>
<thead>
<tr>
<th>Hormonal status</th>
<th>Cervical lesions</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Condyloma</td>
<td>LSIL</td>
<td>HSIL</td>
<td>Cancer</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>173</td>
<td>2</td>
<td>16</td>
<td>42</td>
<td>7</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>58</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Menopausal</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>15</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>267</td>
<td>3</td>
<td>17</td>
<td>51</td>
<td>22</td>
<td>360</td>
<td></td>
</tr>
</tbody>
</table>

Table D1. Prevalence of clinicopathological conditions in the population studied.
The mean age of women in the pregnant group (24.8 years) was significantly lower than that of those in the control (34.0 years) and menopausal (58.9) groups (P < 0.0000001). The comparison of age distribution among the groups is shown in Table D2. The mean number of gestations ± SD in pregnant, control and menopausal women were respectively 2.2 ± 1.7, 3.7 ± 3.4 and 7.0 ± 5.1 (P < 0.0001). Among pregnant women, the gestational ages ranged from 4.3 to 37.1 weeks, with a mean of 24.7 ± 6.9 weeks, and median and mode were, respectively, 26.3 and 25 weeks. The distribution of pregnant women according to gestational age was as follows: 8.3%, 51.7% and 40% were, respectively, in the first, second and third trimesters.

Figure D1 shows the frequency distribution of HR HPV infection related to participants’ age. The prevalence of HR HPV was frequent in younger women. Table D3 shows that the overall prevalence rates of HPV detected in control, pregnant and menopausal women in the high-risk population studied were 33.3%, 18.3% and 30%, respectively (P = 0.04). When only women with a normal cervix were included in the analysis, the prevalences of cervical HPV infection were respectively, 17.8%, 10% and 21.9% (P = 0.31). In women with a normal cervix, there was no significant difference in the prevalence of HPV infection between pregnant and non-pregnant women either including in (10% x 18.6%) or excluding from (10% x 17.8%) the ones in the menopause as part of the control group (P > 0.22) or between menopausal and non-menopausal non-pregnant women (22.2% vs. 17.8%, P = 0.28). This study revealed decreasing rates of prevalence of cervical HPV infection throughout pregnancy: 16.7%, 12.1% and 9.5%, respectively, during the first, second and third trimesters, although this difference was not significant (P ≥ 0.88).

Four (6.7%) pregnant women had cervical infection with LR HPV types 6 or 11. Only 7 (11.7%) had HR HPV infection and all but one of these (who had LSIL) had no detectable cervical neoplasia (Tables D1 and D3). The risks associated with having cervical HR HPV infection in relation to the different hormonal statuses are summarized in Table D4.

There was no significant difference between the mean gestational age of HR HPV positive pregnant women, which was 24.3 ± 6.7 weeks, compared to 24.8 ± 7.0 weeks in the pregnant women who tested negative for HPV DNA by PCR (P = 0.81). The median and mode for gestational age in the HR HPV positive women were respectively 26 and 12
<table>
<thead>
<tr>
<th>Hormonal status</th>
<th>Age range</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16 to 53</td>
<td>34,0 ± 9,2</td>
<td>34.5</td>
<td>28</td>
</tr>
<tr>
<td>Pregnant</td>
<td>17 to 39</td>
<td>24,8 ± 5,3</td>
<td>23.5</td>
<td>20</td>
</tr>
<tr>
<td>Menopausal</td>
<td>34 to 88</td>
<td>58,9 ± 9,5</td>
<td>57.5</td>
<td>48</td>
</tr>
</tbody>
</table>

Table D2. Age distribution of women in the different phases of hormonal status. All numbers are in years.
Figure D1. Distribution of HR HPV infection by age
<table>
<thead>
<tr>
<th>Hormonal status</th>
<th>None</th>
<th>LR types</th>
<th>HR types</th>
<th>Subtotal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>11</td>
<td>16</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>Control group</td>
<td>160</td>
<td>6</td>
<td>4</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Pregnant</td>
<td>49</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>251</td>
<td>9</td>
<td>5</td>
<td>40</td>
<td>1</td>
</tr>
</tbody>
</table>

Table D3. Prevalence of cervical HPV infection among all women according to different hormonal phases of the life cycle. HPV status is given as the HPV types detected.
<table>
<thead>
<tr>
<th>Hormonal status</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.00 (Referent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>0.32</td>
<td>0.12-0.76</td>
<td>6.81</td>
<td>0.009</td>
</tr>
<tr>
<td>Menopausal</td>
<td>1.04</td>
<td>0.53-2.00</td>
<td>0.00</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table D4. Risk of having HR HPV infection in relation to the different hormonal phases of a woman’s life cycle. CI = Confidence Interval.
weeks compared with 26.3 and 25 weeks in the HR HPV negative women. No pregnant women with a normal cervix and gestational age ≤ 20 weeks had HPV infection. The prevalence of overall HPV infection and HR HPV infection in pregnant women with normal cervix and gestational age > 20 weeks were, respectively, 12.5% (P = 0.57) and 7.5% (P = 1.0).

The contribution of number of lifetime sexual partners as a confounding variable in this study was insignificant (P ≥ 0.08) (Table D5). There was no statistically significant difference in the number of lifetime sexual partners reported by controls and menopausal women (P = 0.29), even after adjustment for the presence of HR HPV (P = 0.44) or SIL (P = 0.39). The same was true for controls and pregnant women (P = 0.06), even after adjustment for the presence of HR HPV (P = 0.27) or SIL (P = 0.35). And for pregnant and menopausal women (P = 0.92), even after adjustment for the presence of HR HPV (P = 0.56) or SIL (P = 0.94).

No clinical complications were observed in either pregnant or non-pregnant women as a result of sample collection.
<table>
<thead>
<tr>
<th></th>
<th>Pregnant</th>
<th>Menopausal</th>
<th>Controls</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>1.7</td>
<td>1.8</td>
<td>2.3</td>
<td>0.08</td>
</tr>
<tr>
<td>HR HPV negative</td>
<td>1.6</td>
<td>1.9</td>
<td>2.1</td>
<td>0.50</td>
</tr>
<tr>
<td>Normal cervix</td>
<td>1.6</td>
<td>1.7</td>
<td>2.0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Table D5. Mean number of lifetime sexual partners in the 3 groups studied.
Section E. Self-sampling versus doctor sampling

(1) Experimental design.
This was a cross-sectional study undertaken at two screening services in Recife (Brazil).

(2) Study population.
Women coming in for screening at the Institute of Mother and Child Health in Pernambuco (IMIP) and the Cancer Hospital (HCP), including pregnant women, without age restriction, were randomly selected to participate.

(3) Ethics permission and consent.
The research protocol was reviewed and approved by the local Ethics Committee. After having the study details explained in lay terms, volunteers were invited to sign an informed consent form and received a sterile cotton-tipped swab to take a self-collected sample from the vagina and, possibly, cervix.

(4) Procedures.
Swabs of different sizes were used. They were classified as small (8 cm), medium (15.2 cm) and large (21.5 cm) as shown in Figure E1. The participants were advised to introduce the swab as far as it would go without leading to discomfort and to rub the swab around the vaginal epithelium in several rotatory movements. After feeling that most of the area had been covered the participant should return the swab to a nurse who would disperse the cells into a universal containing phosphate-buffered saline (PBS) solution and label the tube. A colposcopist doctor also collected samples using an Ayre's spatula for the ectocervix and a cytobrush for the endocervical canal just before performing a thorough colposcopic examination in each participant, including those with a normal cervix, thus avoiding verification bias. All samples were stored \(-20^\circ\)C for HR HPV detection by PCR/RFLP.

(5) Statistical considerations.
The data was entered in and analysed by using the SPSS 10 statistical package. Statistical significance was considered when \(P\)-values were \(\leq 0.05\). Kappa values below 0.40 are considered as poor agreements, between 0.40 and 0.75 it suggests a fair to good
Figure E1. Illustration of the types of swabs used for self-sampling.
agreements, and values over 0.75 are considered excellent agreements beyond what would be expected by chance (Landis & Koch, 1977).

(6) Results.
A total of 253 women volunteered for the study. The number of samples analysed were 506, half were self-collected by the participant women and half were collected by doctors. Among samples collected by participant women, 200 (79.1%) were collected with a small cotton-tipped swab, 10 (4%) were collected with a medium cotton-tipped swab and 43 (17%) were collected with a large cotton-tipped swab (Figure E1). Participants' age ranged from 16 to 88 years with a mean of 38.1 ± 13.7 years, median of 38 years and mode of 28 years. Twenty-seven volunteers (10.7%) were pregnant at enrolment. A description of demographic characteristics of the study population is shown in Table E1. Among the participants, 51.4% had normal cervix, 17.5% had cervicitis, 0.4% condyloma, 4.8% had LSIL, 19.1% had HSIL and 6.8% had cervical cancer.

The overall HPV positivity of specimens from self-sampling and doctor sampling were, respectively, 23% and 29%. There was no significant difference ($P = 0.16$) in the overall results from samples self-collected by the participating women compared to samples collected by doctors (Table E2). However, as shown in Table E3, the prevalence of HR HPV among all self-collected samples was 17% as opposed to 26% in samples collected by doctors ($P < 0.02$). Nevertheless, the concordance rates between self-collected and doctor-collected samples for positive and negative results were 80% and 92%, respectively. There were moderate agreements between the results from samples self-collected and doctor-collected with regards to positivity for HR HPV ($k = 0.60$) and positivity for any HPV type ($k = 0.62$). The results showed fair agreements in patients with HSIL ($k < 0.47$) and extremely poor agreements in patients with invasive cervical cancer ($k < 0.09$) (Table E4).

HPV screening based on self-collected specimens resulted in 50% more missed diagnoses of cervical cancer than HPV screening based on doctor-collected specimens ($P = 0.04$) and for HSIL the increase in missed diagnoses was 2.4% ($P = 0.90$) (Table E5).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Stratification</th>
<th>Number of cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>≤ 34 years</td>
<td>108</td>
<td>42.90%</td>
</tr>
<tr>
<td></td>
<td>35 to 39 years</td>
<td>38</td>
<td>15.00%</td>
</tr>
<tr>
<td></td>
<td>40 to 49 years</td>
<td>63</td>
<td>24.90%</td>
</tr>
<tr>
<td></td>
<td>≥ 50 years</td>
<td>44</td>
<td>17.40%</td>
</tr>
<tr>
<td>Education</td>
<td>None</td>
<td>47</td>
<td>18.60%</td>
</tr>
<tr>
<td></td>
<td>Primary</td>
<td>71</td>
<td>28.40%</td>
</tr>
<tr>
<td></td>
<td>Some high school</td>
<td>65</td>
<td>25.70%</td>
</tr>
<tr>
<td></td>
<td>High school graduate</td>
<td>65</td>
<td>25.70%</td>
</tr>
<tr>
<td></td>
<td>University graduate</td>
<td>4</td>
<td>1.60%</td>
</tr>
<tr>
<td>Age at 1st intercourse</td>
<td>&lt; 16 years</td>
<td>67</td>
<td>26.50%</td>
</tr>
<tr>
<td>No. of sexual partners</td>
<td>One</td>
<td>136</td>
<td>53.70%</td>
</tr>
<tr>
<td></td>
<td>Two</td>
<td>63</td>
<td>24.90%</td>
</tr>
<tr>
<td></td>
<td>Three</td>
<td>27</td>
<td>10.70%</td>
</tr>
<tr>
<td></td>
<td>Four or more</td>
<td>27</td>
<td>10.70%</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>None</td>
<td>20</td>
<td>7.90%</td>
</tr>
<tr>
<td></td>
<td>One to three</td>
<td>128</td>
<td>50.60%</td>
</tr>
<tr>
<td></td>
<td>Four or more</td>
<td>105</td>
<td>41.50%</td>
</tr>
<tr>
<td>Parity</td>
<td>None</td>
<td>60</td>
<td>23.70%</td>
</tr>
<tr>
<td></td>
<td>One to three</td>
<td>110</td>
<td>43.50%</td>
</tr>
<tr>
<td></td>
<td>Four or more</td>
<td>83</td>
<td>32.80%</td>
</tr>
</tbody>
</table>

Table E1. Demographic characteristics of the study population.
<table>
<thead>
<tr>
<th>HPV status</th>
<th>Self-sampling</th>
<th>Doctor-sampling</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>58</td>
<td>73</td>
<td>131</td>
</tr>
<tr>
<td>Negative</td>
<td>195</td>
<td>180</td>
<td>375</td>
</tr>
<tr>
<td>Total</td>
<td>253</td>
<td>253</td>
<td>506</td>
</tr>
</tbody>
</table>

Table E2. HPV prevalence in samples collected by self-sampling versus doctor-sampling.
<table>
<thead>
<tr>
<th>Sampling</th>
<th>None</th>
<th>LR types</th>
<th>HPV status</th>
<th></th>
<th>HR types</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>11</td>
<td>16</td>
<td>18</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Self</td>
<td>195</td>
<td>11</td>
<td>4</td>
<td>21</td>
<td>1</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Doctor</td>
<td>180</td>
<td>5</td>
<td>2</td>
<td>33</td>
<td>2</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>375</td>
<td>16</td>
<td>6</td>
<td>54</td>
<td>3</td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

Table E3. HPV type distribution by self-sampling and doctor-sampling.
### Kappa statistics

<table>
<thead>
<tr>
<th>Cervical lesions</th>
<th>Any HPV type</th>
<th>HR HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.63</td>
<td>0.44</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>0.70</td>
<td>0.81</td>
</tr>
<tr>
<td>LSIL/Condyloma</td>
<td>0.54</td>
<td>0.35</td>
</tr>
<tr>
<td>HSIL</td>
<td>0.47</td>
<td>0.41</td>
</tr>
<tr>
<td>Cancer</td>
<td>0.09</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table E4. Kappa analysis of agreements between self-sampling and doctor-sampling for specific cervical epithelium conditions.
<table>
<thead>
<tr>
<th>Sample</th>
<th>HR HPV status</th>
<th>Normal</th>
<th>Condyloma</th>
<th>Cervicitis</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Ca</th>
<th>Total</th>
</tr>
</thead>
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<td>Patient Positive</td>
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<td>6</td>
<td>5</td>
<td>25</td>
<td>7</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Patient Negative</td>
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<td>39</td>
<td>8</td>
<td>23</td>
<td>10</td>
<td>206</td>
<td></td>
</tr>
<tr>
<td>Doctor Positive</td>
<td>9</td>
<td>0</td>
<td>6</td>
<td>5</td>
<td>33</td>
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<td>67</td>
<td></td>
</tr>
<tr>
<td>Doctor Negative</td>
<td>121</td>
<td>0</td>
<td>39</td>
<td>8</td>
<td>15</td>
<td>3</td>
<td>186</td>
<td></td>
</tr>
</tbody>
</table>

Table E5. HR HPV and cervical lesions associated with samples collected by patients or doctors.
IV. DISCUSSION

Cervical cancer is a major public health problem in Brazil. The incidences (new cases per year per 100,000 women) of cervical cancer in Recife (83.2), Belém (44.4), São Paulo (35.4), Goiânia (26.2), and Porto Alegre (24.6) are amongst the highest in the world (Whelan et al., 1990; Parkin et al., 1997) (Figure V). The incidence of 83.2 per 100,000 women in Recife is the highest reported worldwide although this is already an improvement compared to the incidence of 96.5 per 100,000 women reported in Recife from 1967 to 1979 (Carvalho & Franco, 1986).

Recife is the capital city of the state of Pernambuco. It provides specialised referral services to most women with cervical cancer in the state. The state’s female population is 3,685,000, of whom 29% are illiterate (Brazil - IBGE, 1995). Compared to the findings of our current study (15% of the study population were illiterate and 35.1% had up to the primary level of education), it seems that education is reaching a higher proportion of the female state population or else these findings may still be an underestimation of the extent of the problem. The Local State Health Authority (Pernambuco - FUSAM, 1995) has reported that screening services for cervical cancer are fully implemented in only 5.6% of the state’s counties, partially implemented in 57% and absent in 37.4%. In 1995, 102 (57.63%) of the 177 county towns in the State of Pernambuco had one or more cervical cancer screening centres. In 1996, 34 new screening centres were created but 19 were closed. Since only 7.34% and 9.60% of the county towns have facilities to read cytological specimens and perform colposcopic examination respectively, many cervical smears have to be transported an average of 344 Km before they can be read (Leocádio et al., 1997). The state’s central laboratory reported that 64.5% of local cytology specimens were reported as inflammatory and 5.4% were inadequate (Pernambuco - FUSAM, 1996). Some of these inflammatory cytology reports were known to be false negative for cervical cancer. The mortality rate of cervical cancer in Pernambuco has been increasing since 1986 (Figure VII). It was estimated that 6,815 women died of cervical cancer in Brazil in 1998, approximately 50% of these deaths affected economically productive women aged 40 to 59 years. It is apparent that an alternative surveillance programme other than or in addition to cytology had to be found.

69.1% of the women included in this study, lived in Greater Recife (female population of 1,392,000) and the remainder lived in the surrounding rural areas. In contrast 72.8% of
The women with cervical cancer and 55.5% of the women with HSIL lived in rural areas. This difference reflects the lack of or inadequacy in the current screening programmes for cervical cancer prevention particularly in rural areas.

The inability to deliver cytological screening to all eligible women in Pernambuco is regrettable because cervical cancer is a preventable disease and clinical facilities are in place if only the lesions could be identified for early hospital referral. In Recife there are at least five teaching institutions capable of providing the necessary care for women diagnosed with cervical neoplasia: the Institute of Mother and Child Health (IMIP), the Barao de Lucena Hospital (HBL), the Cancer Hospital (HCP), the Oncology Centre (CEON), the University Hospital (HC-UFPE), the Integrated Health Centre Amaury de Medeiros (CISAM) and the Agamenon Magalhaes Hospital (HAM). All of these institutions offer loop excisions (LLETZ) and all but one can provide radical surgery, radiotherapy and chemotherapy.

In view of the high incidence of cervical cancer, this study was designed to audit current screening practices and to investigate risk factors specific to Recife. Remedial measures were proposed.

It is generally accepted that HSIL and cervical cancer are closely associated with HR HPV and many studies have now shown that the detection of these viruses can facilitate early referral of women with or at an increased risk of developing these lesions in both developed and developing countries. (Cuzick et al., 1995; Manos et al., 1999; Herrero et al., 2000; Schiffman et al., 2000a, 2000b; Solomon et al., 2001). The results described in Section A supported this view.

Two detection methods for HR HPV were evaluated in this study using samples collected from Recife. The first (Section B) was Hybrid Capture 1 (HC1) (Digene Corp., MD, USA), the only kit available commercially at the beginning of this study and this technique was evaluated in women with cervical cancer and normal cervix only. HC1 allows the collective detection of 9 HR HPV types (16, 18, 31, 33, 35, 45, 51, 52 and 56) in one single test. HC1 is a signal amplified solution hybridisation antibody capture test that utilises chemiluminescence detection. Specimens containing the target DNA hybridise with a specific HPV RNA probe cocktail. The resultant RNA:DNA hybrids are
captured onto the surface of a tube coated with antibodies specific for RNA:DNA hybrids. Immobilised hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the RNA:DNA hybrids, and detected with a chemiluminescent substrate. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units (RLUs) in a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen. An RLU measurement equal to or greater than the Cutoff Value (CO = RLU generated by 1 pg of HPV16 DNA) indicates the presence of HPV DNA sequences in the specimen. An RLU measurement less than the CO indicates the absence of the specific HPV DNA sequences tested or HPV DNA levels below the detection limit of the assay. HC1 is simple to perform but each test costs approximately US$ 20.

Table B1 shows that the sensitivity of HC1 for detection of cervical cancer was 82.9%, comparable to that of routine cytology in Recife. Previously sensitivities of 64% (Clavel et al., 1998), 74% (Hatch et al., 1995) and 93% (Cox et al., 1995) for HSIL and 86% for any SIL (Cox et al., 1995) have been reported but figures for cancer have not been reported. The specificity of HC1 for cervical cancer detection in this study was 41.4% (Table B1). Lower specificity is to be expected since HC1 is a test for HR HPV and not for cancer itself. It should be argued that a HC1 test identifies women infected with HR HPV and hence identifies those who are at risk of developing high-grade disease. Koutsky et al. (1992) have published results from a prospective cohort study involving 241 women showing that 28% of HR HPV positive women without cervical lesions at triage developed SIL within 2 years, as opposed to 3% of the HPV negative women.

The second method of HPV detection to be evaluated (Section C) was a low cost non-commercial test involving consensus polymerase chain reaction and restriction fragment length polymorphism (PCR/RFLP). This technique amplifies an approximately 450 nucleotides fragment of DNA from the L1 region of the HPV genome using a pair of consensus primers MY09 and MY11 with subsequent identification of the HPV type based on restriction analysis (Bernard et al., 1994). In contrast to HC1, this technique identifies individual HPV types including those that are HR. Some HR HPV types (HPV16, 18, 31 33) are known to be more oncogenic than others (HPV35, 45, 51, 52, 56) (Lorincz et al., 1992). The results from this study show that HR HPV as detected by
PCR/RFLP identified 94/122 (77%) SIL, 52/60 (87%) HSIL and 53/59 (90%) cancers (Table C1).

1. **Inflammatory cytology**

HPV typing identified 5/6 (83%) of cancers and 2/3 (67%) of HSIL in 125 inflammatory smears with no neoplastic cells (Tables C2 and C3). This showed that unlike cytology, HR HPV typing was not adversely affected by the presence of inflammatory cells (Ayre, 1947; Reagan & Hamonic, 1956; Frisch, 1987; Himmelstein, 1989; Cecchini et al., 1990; Zhou et al., 1998). This is of particular importance in Recife as the Pernambuco state central laboratory reported 64.5% of local cytology as inflammatory and 5.4% as inadequate (Pernambuco - FUSAM, 1996).

2. **ASCUS/AGUS cytology**

38 (8.4%) women had ASCUS/AGUS cytology. 7 (18%) had LSIL, 6 (16%) had HSIL or cancer and 100% of these lesions were positive for HR HPV (Table C3). These results confirm that HSIL and cancers are found in women with mildly abnormal smears and are consistent with data recently reported (Kobelin et al., 1998). 17 (45%) women were tested negative for HR HPV and none (100%) had cervical neoplasia (Table C3). The ASCUS/AGUS cytology false positive rate for cervical neoplasia was 66% (Table C2) and this rate for consensus PCR was 21% ($P = 0.0002$) (Table C3).

3. **Abnormal cytology (ASCUS and above)**

HR HPV was detected in 52/60 (86.67%) HSIL and 53/59 (89.83%) cancers (Table C1). Similar observations have been reported by others (Melbye et al., 1996; Sigurdsson et al., 1997). It is apparent that neither routine cytology nor routine HPV testing alone can detect all HSIL or cancers in this population. However, a combination of HR HPV detection and cytology can detect cervical cancer with a sensitivity of 96.8% and a NPV of 99.4% (Table C3). If this combination for colposcopic referral were to be employed, an initial HPV detection to be followed by cytology in women HR HPV negative would be a logical choice since HPV is the risk factor that initiates the oncogenic process and cytological abnormalities represent cellular reactions to many factors only one of which is infection with HPV. In addition, the detection of HR HPV, the major risk factor for...
cervical neoplasia, is a significantly better marker for assessment of risk compared to cytology \( P = 0.0001 \), thus it improves identification of vulnerable women.

A possible strategy involving HPV detection and cytology (Table C6 and Table C7) was constructed for improved cervical cancer prevention in Recife (Figure C1). In this protocol, all cervical scrapes for HPV detection would be collected at primary screening facilities and sent to a central laboratory for HR HPV detection by PCR/RFLP. Management would proceed as follows:

1. Women negative for HR HPV would go for cytological screening. Women with abnormal cytology (mild or above) would be referred to colposcopy. This would ensure that the 10% of cervical cancer and 13% of HSIL, which are HR HPV negative, would be detected.

Results obtained in this study (Table C3) showed that of the 170 women with completely negative cytology, 135 (79%) had no HR HPV infection and among them there was only one HSIL and no cancer. If the high negative predictive value of a negative HR HPV result with negative cytology (99.4%) could be supported by follow-up studies, women from this group probably would not need annual cytological screening. Considerable savings and convenience could be achieved if the screening intervals for this population were extended to 3 to 5 years. This reassurance should help to alleviate psychological and economic problems associated with cervical cancer screening in Recife.

2. Women with HR HPV would be referred to colposcopy immediately. If colposcopy were normal a woman would have another HR HPV test in 12 months. Incident HPV infection is reported to have a median duration of 8 months, 70% spontaneously resolve within 12 months and 91% by 24 months (Ho et al., 1998). If HR HPV persisted, the woman would again be referred to colposcopy to check for development of SIL. If no lesion was visible on a satisfactory colposcopy, she would be followed yearly with HR HPV detection and colposcopy until either the HPV infection resolved or HSIL was detected. If the HR HPV infection spontaneously resolves, the woman would be advised on taking precautions to try and avoid re-infection, and could be reassured that her screening interval could be safely increased.
Proposed Screening Protocol

All eligible women should be offered primary screening with HPV detection.

- **HR HPV Positive**
  - Colposcopy
  - HSIL and Cancer: Treatment
  - LSIL: Follow-up or treatment
- **HR HPV Negative**
  - Cytology
  - Normal: HPV detection in 12 months
  - Abnormal: Colposcopy
  - Negative: Routine screening*

* Routine screening interval initially could be yearly. Reassurance with a negative HR-HPV test and negative cytology or a normal colposcopy followed by a negative HR-HPV test that initially was positive should allow for a safe routine screening interval of 3 to 5 years.

Figure C1. Proposed screening protocol.
If colposcopy showed minor abnormalities a woman would undergo clinical follow-up with HR HPV detection and colposcopy at 6-month intervals to check for clearance of infection or progression of lesion. Provided the viral infection is cleared, the LSIL should usually regress (Konno et al., 1998). She could then be advised on how to avoid HPV infection and be re-scheduled for routine screening. If the squamo-columnar junction was not satisfactorily seen even after hormone replacement therapy or if the lesion lies within the canal not allowing the margins to be completely seen, an endocervical curettage would help to rule out endocervical lesions.

If HR HPV was persistent and the colposcopic lesion remained mild, a woman would be provided with health education and clinical follow-up, given the increased risk of progression (Hording et al., 1995). If progression of the lesion were detected she would be treated promptly.

In circumstances where the risk of losing a patient with LSIL and HR HPV to follow-up, due to poor education, poverty, taboos, fears, etc., outweighed the probability of spontaneous regression, treatment might be an option, particularly to decrease anxiety. Treating LSIL associated with HR HPV may be regarded as overtreatment from a cervical neoplasia perspective but not from the perspective of a sexually transmitted disease. If no abnormal area was seen on colposcopy of a HR HPV positive cervix, perhaps the use of immune modulators (Weeks & Gibson, 1994; Reiter et al., 1994; Beutner et al., 1998; Edwards et al., 1998; Miller et al., 1999) could be considered to try to avoid transmission of these viruses. Partners also needed to be checked. An effective vaccine against HR HPV types would also be helpful.

Due to insufficient coverage or misdiagnosis, the majority of cervical cancers currently diagnosed in Recife are at advanced stages, 76% of cancers in this study were diagnosed at stage II and above (Figure III). The overall 5-year survival rate for cervical cancer is about 49% and it decreases the more advanced the stage at diagnosis (Meanwell et al., 1988; Thoms et al., 1995). Generally, patients' quality of life decreases significantly in the post-treatment period, particularly after exclusive radiotherapy when vaginal atresia, actinic rectitis, necrosis, bowel obstruction or other complications could occur, and the psychosocial and economic burden on the rest of the family is high.
The Brazilian National Health Service reimburses\textsuperscript{6} US$3.87 for 1 Pap smear test, US$1.41 for 1 colposcopy, and US$1,401.40 for exclusive radiotherapy of advanced cervical cancer restricted to the pelvis. For research purposes, the cost of detection of HR HPV by consensus PCR is US$1.64 and for HPV typing by RFLP was US$3.28 per sample. For each HR HPV negative woman the cost of screening, including PCR and secondary triage with cytology, would be US$5.51 (1.64 + 3.87). For HPV positive women, including PCR/RFLP, the cost would be US$4.92 (1.64 + 3.28). The cost of screening a woman with this combined protocol using HPV detection and cytology would therefore represent only 0.6% of the cost of treating one case of advanced cervical cancer. If all the savings were re-invested in expanding our current screening programme coverage, the incidence, morbidity and mortality of cervical cancer in Recife and many cities alike would be drastically reduced.

To achieve this goal, the detection of HR HPV would have to reach all eligible women in Pernambuco. Two methods to improve coverage have been considered. The first involves sampling women at antenatal visits. This possibility became apparent when the incidence of HR HPV was assessed in pregnant women. The second would involve self-sampling.

The prevalence of cervical HR HPV infection in women in each of 3 physiological hormonal status classifications, namely non-pregnant non-menopausal (control group), pregnant or menopausal was investigated (Section D). The results obtained showed that HR HPV occurs at similar rates in the control and menopausal women (around 30%) (Table D3). The lowest prevalence was observed in pregnant women 18.3% \((P = 0.04)\), more specifically 16.7% in the first trimester, 12.1% in the second trimester and 9.5% in the third trimester. The difference in prevalence of HR HPV infection among trimesters of pregnancy was not significant \((P = 0.88)\). Indeed, the high levels of female steroid hormones during pregnancy apparently had a protective or suppressive effect against HPV infection \((OR = 0.32)\). This result is in contrast to in vitro observations that elements in the long control region (LCR) of the HPV genome are responsive to stimulation by the female sex steroid hormone progesterone (Pater \textit{et al.}, 1988, 1990, 1992, 1993; Mittal \textit{et al.}, 1993a, 1993b, 1994). The results observed here were similar to those obtained in a study investigating the effects of initiating OCP use in women with normal cervix which showed no difference in the long-term occurrence of cervical neoplasia compared to

\textsuperscript{6} The exchange rate at the time the study was carried out was 1 US Dollar = 1.2 Real (Brazilian currency).
women not using OCP. In women with cervical neoplasia, the adjusted effect of the mildly increased circulating levels of oestrogen and progesterone from OCP led to an increased regression rate of the SIL after 2 to 6 months of continuous use, suggesting an initial protective effect. It was only after 6½ years that a marginally significant association (P < 0.05) between the long-term use of OCP and progression of LSIL to HSIL was observed (Stern et al., 1977). The short-term use of hormone replacement therapy in menopausal women also showed no increased risk of cervical HPV infection (Smith et al., 1997).

Routine cervical cancer screening is recommended to all pregnant women (Schmitz et al., 1960). But pregnancy alters some morphophysiologic aspects of cervical cells leading to difficulties in interpretation of cytologic, colposcopic and histologic findings. The physiologic effects of steroid hormones during pregnancy exaggerate the appearance of both normal and abnormal cervical tissues, which could give rise to false positive results (Ostergard & Nieberg, 1979). A recent retrospective study showed that 137/305 (45%) pregnant women presenting with abnormal cytology did not have SIL. 67% were biopsied during pregnancy for colposcopic abnormalities and 34% were found to be histologically normal. This indicates a high false positive rate in cytology during pregnancy. Of 102 pregnant women in whom all biopsies were postponed to the postpartum period, 40% had HSIL or LSIL. Furthermore, in 15% of the biopsies the cytological diagnosis was shown to be an underestimate of disease severity and in 8% it was an overestimate (Palle et al., 2000). The hormonal effects of pregnancy on the cervical epithelium could lead to both cytologic and colposcopic misdiagnosis (Benedet et al., 1987; Economos et al., 1993; Palle et al., 2000).

A comparison of HR HPV detection results obtained in this and other reported studies are summarised in Table D6. HR HPV detection based on PCR showed no significant difference in prevalence between pregnant and non-pregnant women. HR HPV testing during pregnancy would improve coverage in Recife since most pregnant women are seen (at least once) in antenatal clinics. In addition HR HPV testing could also serve as an adjunct to cytology and colposcopy in the identification of high-grade cervical lesions since both of these techniques present difficulties in diagnosis during pregnancy.
<table>
<thead>
<tr>
<th>Sample size</th>
<th>Preg x Non-Preg</th>
<th>Method of collection</th>
<th>Method of HPV detection</th>
<th>Prevalence Preg x Non-preg</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>92 x 96</td>
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<td>Cervical scrapes</td>
<td>Southern blot</td>
<td>28.3% x 12.5% (P = 0.04)</td>
<td>Schneider et al., 1987</td>
</tr>
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<td>101 x 108</td>
<td></td>
<td>Spatula/cotton swab</td>
<td>FISH</td>
<td>34.6% x 20.4% (P = 0.11)</td>
<td>Czegledy et al., 1989</td>
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<tr>
<td>45 x 44</td>
<td></td>
<td>Spatula</td>
<td>FISH/Southern blot</td>
<td>13% x 25% (P = 0.37)</td>
<td>Peng et al, 1990</td>
</tr>
<tr>
<td>107 x 74</td>
<td></td>
<td>Cervicovaginal lavage</td>
<td>FISH</td>
<td>34.6% x 18.9% (P = 0.11)</td>
<td>Morrison et al., 1996</td>
</tr>
<tr>
<td>245 x 494</td>
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<td>Cervicovaginal lavage</td>
<td>ViraType Plus</td>
<td>31% x 18.2% (P = 0.003)</td>
<td>Fife et al., 1996</td>
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<td>748 x 503</td>
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<td>Dacron swab</td>
<td>ViraPap/ViraType</td>
<td>9.6% x 8.9% (P = 0.79)</td>
<td>Soares et al., 1990</td>
</tr>
<tr>
<td>69 x 54</td>
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<td>Cytobrush + cotton swab</td>
<td>ViraPap/ViraType</td>
<td>15.9% x 14.8% (P = 0.92)</td>
<td>Smith et al., 1991</td>
</tr>
<tr>
<td>115 x 160</td>
<td></td>
<td>Dacron swab</td>
<td>ViraPap/ViraType and PCR</td>
<td>42% x 41% (P = 0.95)</td>
<td>Kemp et al., 1992</td>
</tr>
<tr>
<td>73 x 88</td>
<td></td>
<td>Cotton swab (large)</td>
<td>ViraPap</td>
<td>26.7% x 46.6% (P = 0.09)</td>
<td>Chang-Claude et al., 1996</td>
</tr>
<tr>
<td>108 x 192</td>
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<td>Same as above</td>
<td>ViraType</td>
<td>13.9% x 15.1% (P = 0.94)</td>
<td>Chang-Claude et al., 1996</td>
</tr>
<tr>
<td>709 x 3948</td>
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<td>Cervical scrapes</td>
<td>GP 5/6 + Type specific PCR</td>
<td>9.6% x 10.9% (P = 0.37)</td>
<td>de Roda Husman et al., 1995</td>
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<tr>
<td>752 x 1064</td>
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<td>Cervicovaginal lavage</td>
<td>MY09/11 consensus PCR</td>
<td>5.4% x 12.3% (P = 0.00001)</td>
<td>Tenti et al., 1997</td>
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<td>58 x 209</td>
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<td>Spatula + cytobrush</td>
<td>MY09/11 consensus PCR</td>
<td>10% x 18.6% (P = 0.28)</td>
<td>Present et al., 1997</td>
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<td>3222 x 7034</td>
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<td>14.3% x 13.4% (P = 0.29)</td>
<td>Overall</td>
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</table>

Table D6. Studies comparing pregnant and non-pregnant women with regards to prevalence of cervical overall HPV infection in women from low-risk populations or with normal cervix. Preg = pregnant women. FISH = filter in situ hybridization.
The second method to increase coverage involves self-sampling (Section E). The initial studies in cytological surveillance were based on analysis of cervical cells collected by cervicovaginal aspirates (Stockard & Papanicolaou, 1917; Papanicolaou, 1928; Papanicolaou & Traut 1943). More recently cervical vaginal lavage or a cotton or Dacron swab have also been used to collect cervical cells (Morrison et al., 1996; Fife et al., 1996; Kemp et al., 1992; Chang-Claude et al., 1996; Tenti et al., 1997). The rationale is that cells shed from the cervical epithelium travel through the vagina by gravity to reach the external environment. Based on the same principle it is assumed that cells shed from the cervix, including cells from SIL and invasive cervical cancer can be collected by women through self-collection using a cotton-tipped swab. This strategy if proven effective would be more convenient for many women and could increase cervical cancer screening coverage in Recife where taboos related to exposing the genitals to a male doctor still lead to serious anxiety and non-compliance to organised cervical cancer screening programmes. The question is can self-sampling provide enough cells from a representative portion of the cervical ectocervix and canal for HR HPV detection when compared to a cervical scrape collected by the current routine practice with an Ayre’s spatula and a cytobrush?

Some studies have shown a moderate to good correlation between self-sampling and doctor or health professional sampling with Kappa statistics values ranging from 0.45 to 0.70 (Fairley et al., 1992; Harper et al., 1999; Wright et al., 2000). In contrast, this study found a significant difference in HR HPV detection between samples self-collected with a cotton-tipped swab and samples collected by a doctor using a wooden spatula and a cytobrush under direct visualisation of the cervix (Table E4). In particular, there were fair to extremely poor agreements in cases of HSIL and cancer (Table E4). Similar results have also been published showing that doctors' samples collected with a brush were more sensitive than patients' samples collected with a Dacron swab (Peyton et al., 1998). Self-sampling, therefore, is not a method of choice for primary screening.

Although HR HPV is identified as the major risk factor for cervical neoplasia, the role played by other epidemiological variables in Recife were also investigated with a view to promoting public awareness (Section A).
(1) Age

A population-based study undertaken in Holland has shown that the prevalence of cervical HPV infection in sexually active women with normal cytology, the majority using hormonal contraception, was higher in women aged 15 to 34 years than women aged 35 to 55 years (Melkert et al., 1993).

In Recife, younger women were also found to be more likely to have cervical HR HPV infection than older women (Figure A1). The analysis of only women with normal cervix in the high-risk population studied also showed a decrease in the prevalence of latent HR HPV infection in women 40 years or older, even though there were rebound increases in prevalence of latent HR HPV infection in later ages (Figure A2). This finding explains why some women develop LSIL or HSIL in older ages, although the vast majority of these lesions are detected in younger age-groups (Table A1).

HR HPV positivity was related to a higher number of sexual partners (Table A10). However, not all women with more than 1 partner had HR HPV, HSIL or cervical cancer (Table A10). These findings suggest that it is not only the frequency of sexual activities with different partners but also other factors such as the partner’s sexual behaviour, the competence of the woman’s immune system and the specific type of HPV infection may play an important role in the explanation of these findings. The importance of the high-risk male has been previously reported (Prindan & Lillienfeld, 1971; Singer, 1973; Singer et al., 1976; Buckley et al., 1981; Brinton et al., 1989; Zunzunegui et al., 1986, Kessler, 1977). The number of sexual partners reported by males has been strongly correlated with the risk of SIL in their partners (Buckley et al., 1981; Brinton et al., 1989; Zunzunegui et al., 1986, Kessler, 1977). The incidence of cervical cancer is reported to be nearly 3-fold higher in women married to men who previously had a partner diagnosed with cervical cancer (Kessler, 1977).

The low prevalence of cancer and HSIL (Table A1) and higher prevalence of HR HPV (Figure A1) in younger women suggest that the usefulness of HR HPV testing for detection of these lesions in this group of women is questionable. The rates of detection of HSIL and cervical cancer by HR HPV testing was similar to that found by cytology (ASCUS or above), (Tables C1, C2, and C5). However, in combination with cytology, the
detection of HR HPV may provide a role in screening the eligible population for HSIL and cervical cancer, including younger women as well (Tables C3, C6, and C7).

(2) Contraception

No significant association was found between OCP use and cervical cancer, SIL or HR HPV infection (Table A12). The risks of cervical cancer, HSIL and LSIL for women using OCP adjusted for the presence of HR HPV infection were 0.00, 0.77, and 1.13, respectively (Table A24). The association of OCP use with SIL reported in previous studies (Zondervan et al., 1996; Brinton et al., 1990; Brinton, 1991; Franceschi et al., 1986) suggested that young women using OCP may have a more active sexual life and be more exposed to HR HPV infection.

(3) Smoking

The results presented in Table A18 showed that compared to ex-smokers with normal cervix it did not matter how long ago a woman had stopped smoking, if she had ever been a regular smoker. It seems that the DNA damage remains perhaps being passed onto the next generations of cells but other factors are responsible for the difference in developing or not cervical neoplasia. The data from Tables A14, A15, A17, and A22 showed that if she started smoking at an early age or smoked over a long period of years, the risk of HR HPV infection, HSIL or cancer was significant. Some studies (Sasson et al., 1985; Schiffman et al., 1987; McCann et al., 1992; Prokopczyk et al., 1997) have shown the presence of several components of cigarette smoke, such as nicotine and its major metabolite (cotinine) in cervical mucus and the occurrence of smoking-related DNA damage in the cervical epithelium (Phillips et al., 1990, 1994; Cuzick et al., 1990; Simons et al., 1993; Ali et al., 1994). Furthermore, the age at which women start smoking, the number of smoking years, the amount and type of tobacco smoked have been identified as important risk factors for HR HPV infection and SIL (Tables A17 and A19 to A23) and are worth further detailed analysis.
V. CONCLUSION

Cervical cancer is a preventable disease and its pathogenesis is closely associated with infection with HR HPV. This thesis has shown that, although commercial diagnostic kits for HR HPV may have the advantage of ease of use and high sensitivity for the detection of HSIL and cervical cancer, the low specificity limits their clinical use. The PCR/RFLP technique used in this investigation can provide the sensitivity and the specificity required to facilitate early colposcopic referral of affected women to the well equipped hospitals in Recife funded by the state or charities. The reagent cost for this test is low but the procedure is labour intensive. These are both advantages in Recife.

This study has investigated a population of low socio-economic status in Recife where sexual activity begins at an early age and smoking is relatively common. These risk factors can be minimised by public health education.

Overall the control of sexually transmitted HR HPV infections can be achieved by early diagnosis and prevention.
Publications arising from this thesis


Future work

A prospective cohort study to investigate the cost-effectiveness of using HPV detection by PCR and typing by RFLP as a primary screening method for cervical neoplasia combined with cytology is being planned to be undertaken at IMIP Hospital in Recife. It is hoped that funding will be available to initially screen 10,000 women with the possibility of expanding it to 100,000 women.

A prospective randomised double-blind case control study to investigate the clinical use of a sugar derivative called Glucan as a chemoprevention alternative for LSIL management is going to be undertaken in Recife by 5 centres of reference for the management of cervical neoplasia. The pilot study is expected to include 120 women with histologically proven LSIL. Half of the volunteers will be randomly included in the case group which will use Glucan vaginal cream at 4%, with applications 3 times a week for 4 months, and the other half will make the control group which will use a placebo vaginal cream under the same prescription scheme. The follow-up will continue for 5 years.

A prospective randomised and controlled study to compare different management options in cases of LSIL will be carried out in Recife. Three management options will be compared: treatment with trichloroacetic acid (TCA) at 80%, electrocautery and conservative management (observation). Specimens for HPV detection will be collected before treatment and 1, 6 and 12 months after treatment in order to assess whether HPV detection has a role in predicting the clinical outcome and to check the natural history of HPV infection in the different management option within the initial 12 months follow-up. An attempt will be made to follow the participants for a period of 5 years.

A prospective randomised phase III clinical trial to compare exclusive radiotherapy and neoadjuvant chemotherapy with cisplatin, 5-fluorouracil and doxorubicin in cases of cervical cancer stages IIB and above followed by radical surgery after regression to a stage of satisfactory operability is being planned to be undertaken at the Cancer Hospital in Pernambuco. The role of HPV testing plus p53 and Bcl2 expression as prognostic factors will also be considered.
Another study is being considered to be undertaken in England in order to investigate if there is a role for HPV detection as an adjunct tool for the triage of women with atypical glandular cells of undetermined significance (AGUS).
VI. REFERENCES


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SECTION I. Text Amendments to PhD thesis.

INTRODUCTION

1. Location  Page 28
Proposed amendment :-
Label figure clearly.
Amendment :-
Re-label Fig III as follows :-
Figure III. Distribution of clinical stages of cancers detected in study A in this thesis.

2. Location  Page 37. 4A. Age.
Proposed amendment :-
Comment and suggest possible explanations for the decrease in incidence of cervical cancer in women over 60 years of age.
Amendment :-
Append the following :-
However, the incidence and mortality rates of cervical cancer in African-American women continue to rise into the 8th decade (Ries et al., 1994). A fairly high prevalence of cervical HPV infection in women aged over 60 years has been reported (Levert et al., 2000). At the other end of the spectrum, cervical cancer precursors have been detected in girls as young as 11 years old and 30% of 946 Cameroon women diagnosed with CIN had their first intercourse between the ages of 10 and 15 years (Nkегоду et al., 2001). There is also evidence that the age at first intercourse decreases as the woman's current age increases, becoming successively lower in more recent birth cohorts (Wellings & Bradshaw, 1994). The differing sexual practices and other socio-cultural factors in different generations appear to exert an important influence on the association between age and cervical neoplasia rather than an effect of age per se.

Proposed amendment:-

Rigoni-Stern presented data on mortality. He was unable to distinguish cervix uterus from corpus uterus and the analyses were all based on relative mortality.

Amendments:-

a) Replace two occurrences of "cervical cancer" with "uterine cancer".

b) Insert after sentence 1:-

This study presented data on relative mortality by cancer of the uterus without distinguishing cervix uterus from corpus uterus.


Proposed amendment:-

Please cite a good review paper on the topic of oral contraceptives and cervical cancer.

Amendment:-

Insert after para 1, sentence 1:-

In several well-controlled studies of the relationship of oral contraceptives and cervical cancer, residual excess risks of nearly 2-fold persist for users of 5 or more years (reviewed in Brinton, 1991).

5. Location. Page 41. para 1. 4G. Chronic Inflammation.

Proposed amendment:-

Additional references linking chronic inflammation with cancer.

Amendment:-

Append:-

Chronic inflammation contributes to the pathogenesis of several different types of cancer, such as lymphoma, gastric, hepatic and urinary bladder carcinoma among others (Moore, 1991; Correa P, 1992; Kawai et al., 1993; Ames et al., 1995; Baecklund et al., 1998; Christen et al., 1999). Interleukin 1α (IL-1α) and tumour necrosis factor α (TNF-α), which are produced at high levels by macrophages and monocytes, and are expressed by epithelial cells, stimulate proliferation of immortal and malignant cervical epithelial cells by an epidermal growth factor.
(EGF) receptor-dependent pathway requiring autocrine stimulation by amphiregulin (Woodworth et al., 1995).

6. Location Page 43. 4H. Smoking.
Proposed amendment :-


Amendment :-

Append after para 2 :-
In addition to the studies already quoted, a review on the association of smoking and cervical cancer is worth mentioning (Winkelstein, 1990). A study suggesting a strong association between smoking and HSIL described that among 167 women, whose worst ever cervical smear was mild dyskaryosis, 60% of smokers had HSIL compared to only 25% of non-smokers (Luesley et al., 1994). A link between smoking and cervical disease was also supported by the findings of another study in which 82 women with minor-grade lesions on colposcopy (CIN 1 or less) were followed up prospectively. Seventeen participants stopped smoking for at least 6 months and 11 participants reduced their smoking consumption by more than 75% for a similar period. In this group, 82% showed a reduction in lesion size of at least 20% compared to only 28% of the 47 participants who did not reduce their smoking level showing a similar reduction in lesion size (Szarewski et al., 1996).

7. Location Page 47. 4J.b. In cervical neoplasia. Line 3.
Proposed amendment :-

Rephrase the sentence clarifying what the author described in his paper.

Amendment :-

The second sentence should read as follows:-
The study by Franco and collaborators that has been undertaken in Sao Paulo (Brazil) since 1993 in collaboration with the McGill University (Canada) may provide valuable information with regard to the role of HPV variants, viral load
and antibody response in women with persistent HPV infection of the cervix (Franco et al., 1999).

8. Location Page 55. 6A.a). Cytology reporting and colposcopic referral systems.
Proposed amendment :-
Is the study by Raffle et al (from Britain) relevant to the term ASCUS?
Amendment :-
Insert after paral, sentence 3 :-
The study by Raffle et al (1995) was carried out in Bristol (UK) where the terminology used was borderline changes, which is equivalent to ASCUS, and mild dyskaryosis, which is equivalent to LSIL.

9. Location Page 56. Line 2 from the bottom. 6A.a). Cytology reporting and colposcopic referral systems.
Proposed amendment :-
Clarify a statement in the text.
Amendment :-
The "in" just before "54 to 84%" should be changed to "by".
The cumulative colposcopic referral rate in the United Kingdom after about four years of follow-up of mildly abnormal cytology was estimated to range from 14 to 64% and immediate colposcopic referral would reduce the risk of invasion by 54 to 84% (Soutter, 1994).

Proposed amendment :-
There have been several reviews of studies on sampling devices (eg Buntix, Brouwers BMJ 1996).
Amendment :-
Insert after the sentence ending with "... smears."

4
There have been several reviews of studies on sampling devices (Buntinx & Brouwers, 1996; Sasieni, 1996; Martin-Hirsch et al., 1999; 2000).

11. Location Page 63. 6A. c. Liquid-based cytology.
Proposed amendment :-
Comment on the absence of good studies analysing the adequacy of liquid cytology.
Amendment :-
Append :-
There are relatively few studies investigating liquid-base cytology. Many are supported by the manufacturers and use the split-sample method, are not population-based randomised controlled trials of the technologies and use a variety of definitions as to what constitute a positive smear. Only a few have biopsy confirmation of positive results (Australian Health Technology Advisory Committee Report, 1998; American College of Obstetricians and Gynecologists, 1998; Payne et al., 2000).

12. Location Page 83. 7A. Premalignant lesions.
Proposed amendments :-
a) The RELATIVE risk (line 4).
b) Lines 9-12 - sentence does not make sense.
Amendments :-
a) Insert "relative" after the first word in the sentence beginning on line 4.
b) No amendment required since this is a correct statement of the reported data.
To quote the authors :-
"Burchardt and Holzer (1980) state that adequately treated CIS is a totally curable lesion. They report no recurrences in 634 cases treated by conization with complete removal of the lesion. However, the reoccurrence of CIS and the development of invasive carcinoma in adequately treated cases is reported by other authors (Kolstad & Klem, 1976; Creasman & Rutledge, 1972). This latter conclusion is strongly supported by evidence in the present study in which five of the 12 patients who had normal cytology after initial management (group 1) later
developed invasive carcinoma despite complete removal of the original lesion. However, contrary to what would be expected, of the 139 group 1 patients with incomplete excision of the original lesion, only five (3.5%) later developed invasive carcinoma. Thus, whether or not the lesion is completely excised does not appear to influence the possibility of invasion occurring subsequently. (McIndoe et al., 1984).

Proposed amendment :-
"only ONE of these"? (line 6).
Amendment :-
Insert "one" between the words "only" and "of".

14. Location Page 98. 7B.c. Chemotherapy.
Proposed amendment :-
Discuss 2 additional papers published in the NEJM in April 1999 about the use of radiotherapy with concurrent chemotherapy.
Amendment :-
Append :-
The use of concurrent cisplatin- or cisplatin+fluorouracil-based chemotherapy and radiotherapy improved survival rates among women with locally advanced cervical cancer (Morris et al., 1999; Rose et al., 1999). The addition of weekly infusions of cisplatin to pelvic radiotherapy followed by hysterectomy in patients with cervical cancer stage IB (> or = 4cm in diameter) without lymph node involvement significantly reduced the risk of disease recurrence and death (Keys et al., 1999).

Proposed amendment :-
Add comma after "(IL)-6" (line 4).
Amendment :-
Add comma after "(IL)-6" (line 4).

16. Location. Page 105. 9. The purpose of this study. Para 2, sentence 2.
Proposed amendment :-

The human genome project has been completed - please reference.
Amendment :-

Append after sentence 2 :-

A draft sequence has already been assembled and published (Lander et al., 2001).

Reference:

17. Location. Page 105. 9. The purpose of this study.
Proposed amendment :-

Clarify the objectives of each study.
Amendment :-

Insert after para 3 :-

The overall objective of this thesis was to evaluate some of the molecular techniques available to detect the presence of HPV infection in the cervix [Hybrid Capture 1 (HC 1) and PCR] and analyse their utility in the identification of women with cervical neoplasia and those at risk of developing it in Recife, a high-risk area for cervical cancer. The objectives of each study were as follows.

1. The objective of study A was to summarily assess the influence of HR HPV infection on risk factors for cervical neoplasia in Recife.
2. The objective of study B was to evaluate the efficacy of HC 1 to indirectly detect cervical cancer through identification of oncogenic HPV types.
3. The objective of study C was to assess the efficacy of HR HPV detection by consensus PCR and typing by RFLP in identification of cervical neoplasia in a high-risk population.
4. The objective of study D was to evaluate by consensus PCR whether pregnant women have a higher prevalence of cervical HPV infection compared to
non-pregnant non-menopausal and menopausal women in a high-risk population.

5. The objective of study E was to investigate if self-sampling using a cotton-swab for HPV detection and typing can be used to identify HSIL and cervical cancer with similar efficiency as sampling by doctor using an Ayre's spatula and a cytobrush in a high-risk population.

MATERIALS AND METHODS.

18. Location Page 105. MATERIALS AND METHODS

Proposed amendment :-

Insert an overview.

(i) Different methods of Taq polymerase based DNA amplification.

(ii) Different methods of HPV typing.

(iii) Minor modifications used in this investigation for the purpose of "de-skilling".

(iv) Advantage and limitation of the technique used in this investigation.

(not sensitive to detect multiple types, cannot be used for quantitation).

Amendment :-

Insert after main heading :-

I Principle of DNA amplification by PCR

PCR is a simple and relatively quick technique to perform. It is a direct enzymatic amplification process where single-copy genomic sequences can be multiplied by a factor of more than 10 million. There are numerous applications for PCR in both basic sciences and clinical research. The widespread success of PCR comes from the speed, efficiency and reproducibility of the technique. PCR reactions take just minutes to set up and a few hours to run (Saiki et al., 1988; Latchman, 1995).

Any region of a DNA molecule can be chosen to be amplified so long as the contiguous sequences are known. This is necessary, as during the PCR process two short oligonucleotides must hybridise to the DNA molecule, one on each
strand of the sequence. These oligonucleotides act as primers for the subsequent DNA synthesis. The primers must correspond with the sequences flanking the region to be amplified and must be complimentary to their template strand, the 3' ends of the hybridised primers pointing towards one another (Brown, 1995).

Amplification is usually carried out by the DNA polymerase 1 enzyme from Thermus aquaticus (Taq), a bacterium that lives in hot springs and many of its enzymes, including Taq DNA polymerase, are thermostable. Taq polymerase can extend the primers at temperatures up to 72° Celsius. When the synthesis is complete, the whole mixture is heated further (to 95° Celsius) to separate the newly formed DNA duplexes. When the temperature is lowered again, another round of synthesis takes place because excess primer is still present and able to hybridise to the target DNA. The repeated cycles of annealing (cooling), synthesis, and melting (heating) lead to quick amplification of the sequence since the amount of template DNA approximately doubles at each cycle (Saiki et al., 1988; Lodish et al., 2000). The cycle of denaturation-hybridisation-synthesis is repeated many times (30-40 cycles), resulting in the eventual synthesis of several hundred million copies of the desired sequence of DNA (Brown, 1995). Precautions should be taken to guard against contamination of the reaction mixture with trace amounts of DNAs that could serve as templates (Sambrook et al., 1989).

Because HPV cannot be effectively grown in cell culture and serological detection techniques for HPV typing are still not available, HPV genotypes can only be identified by HPV DNA detection techniques and nucleic acid sequence analysis. PCR has allowed the detection and typing of subsets of HPV types. As PCR is a very sensitive method, only small quantities of starting material are required and this is an advantage when dealing with clinical samples, as the virus may be present in minor amounts.

II The basis of consensus primer PCR
A number of different primer combinations amplifying DNA fragments from various regions of the HPV genome have been developed. The primers amplifying DNA fragments in the conserved L1 region, namely MY09/11 (Manos et al, 1989)
and GP5+/GP6+ (de Roda Husman et al, 1995), have become the most widely used in clinical and epidemiological studies. MY09 and MY11 primers are actually mixtures of different primers with sequence variations at certain positions to allow the simultaneous detection of many HPV types which differ only slightly in this conserved region. The MY09/11 and GP5+/GP6+ primer-mediated PCR systems have revealed near equivalent sensitivities in amplifying the major HPV types (i.e. HPV types 16, 18, 31, and 45), and are able to detect as few as 10 to 100 molecules of HPV from a genital sample (Gravitt et al., 1998). As a result of different primer design, they have shown different sensitivities in amplifying other HPV types. Moreover, the GP5+/GP6+ PCR system resulted in the detection of fewer samples infected with multiple HPV types, therefore the MY09/11 PCR system has been proven more robust at this point (Qu et al., 1997).

Consensus PCR using the MY09/11 primers can reach the theoretical detection limit of 1 HPV particle per sample aliquot tested. However, the primers do not show the same sensitivity for each of the 13 HR or intermediate-risk HPV types. The number of mismatches between the MY09 primer and the sequence of HPV 59 (4 mismatches) are more than those in HPV 51 (2 mismatches) and HPV 52 (3 mismatches), while there are no mismatches between HPV 18 sequences and the MY09 primer. As far as the MY11 primer is concerned, there are no mismatches between HPV types 58 and 59 and the primer sequence, while HPV 51 is the HPV type sharing the least number of bases with the primer (5 mismatches). Therefore, consensus PCR amplifies individual HPV types with different specificity and the detection limit of consensus PCR for every HPV type is different. It is generally assumed that there is an inverse association between the number of mismatched base pairs and the ability of the primer to amplify the HPV type. It is estimated that HPV types 18, 33, 58 are amplified with the highest sensitivity and specificity by MY09/11 primers, since not a single mismatch exists between the sequences and primers. On the other hand, it is assumed that HPV 51 is amplified with the lowest sensitivity (7 mismatches in total between the sequences and primers) followed by HPV types 52 and 56 (6 mismatches). Theoretical predictions may not accurately reflect the ability of a given primer to amplify individual HPV types present in clinical samples, since the positions of the mismatches play a crucial role.
III Type-specific PCR

Type-specific PCR relies on the amplification of a defined segment of a particular HPV genome using DNA polymerase. A pair of synthetic primers, each about 20 nucleotides long is designed to specifically bind to regions of the DNA of the HPV type to be tested for. Type-specific PCR has shown a higher detection rate for multiple types of HPV than methods based on amplification with consensus primers (Cuzick et al., 1994).

A problem with type-specific PCR arises if all types of HPV in one specimen are to be identified since many type-specific PCR reactions have to be set up. Therefore, despite its high sensitivity and specificity, type-specific PCR as an alternative for HPV DNA detection is labour intensive and expensive, making it unlikely to become commercially available for routine clinical diagnosis.

IV Gel electrophoresis of PCR products

A portion (5μl) of the amplified DNA sequence is electrophoresed on an agarose gel. A positive result for the presence of HPV DNA is characterised by the visualisation of a band of DNA under UV illumination after ethidium bromide staining and whose distance of migration corresponds to a specific marker, whose molecular weight is known. The mobility of a DNA fragment on gel electrophoresis depends on its molecular weight and charge. The rate of electrophoresis migration varies inversely with the logarithm of the fragment's size and is independent of the DNA sequence or base composition. Therefore, a semi-logarithmic plot of the fragment size versus the distance travelled on the gel will yield a straight line over a specific range of fragment sizes.

Various parameters affect the mobility of fragments on gels: the pore size of the agarose matrix, the salt composition of the buffer, and the electric field applied. The migration rate of nucleic acid fragments on gels is proportional to the electric field, which in turn is dependent on the voltage applied, and the distance between the anode and the cathode of the gel apparatus. The salt concentration of the running buffer determines the resistance of the gel (Latchman, 1995).
Photographs of the gels may be taken using transmitted or incident ultraviolet light. An exposure of few seconds is sufficient to obtain images of bands containing as little as 10ng of DNA (Sambrook et al., 1989).

**V HPV genotyping**

Given that each oncogenic HPV type appears to imply a specific risk for women to have or develop cervical neoplasia, with HPV 16 presenting the highest risk, it is important to identify the specific HPV type present in cervical scrapes. Disease associations and the natural history of each HPV type can be more accurately defined by genotyping. One effective way to do this is by the technique of restriction fragment length polymorphism (RFLP) analysis. This method is capable of identifying specific HPV types, both high- and low-risk HPV types.

For the purpose of de-skilling, minor modifications were used in these investigations. Briefly, an aliquot of a MY09/11 PCR product (5μl) is used in each digestion reaction. The informative restriction enzymes for HPV genotyping include BamHI, HaeIII, HinfI, PstI, RsaI, and Ddel (Bernard et al, 1994). In this thesis, RsaI and Ddel were used. Digested PCR products are electrophoretically separated on a 3% AquaPor gel and a ladder of standard DNA sizes is used as the marker. The restricted band patterns previously stained with ethidium bromide are visualised under ultraviolet light and a photograph is taken. Individual HPV types are then identified by comparison of the restricted bands pattern with standard patterns for the restriction enzymes based on predicted DNA sequences for MY09/11-generated L1 PCR products (Figure IX).

The major limitations of the technique used in these investigations were that it is not sensitive to detect all types in multiple infections and it cannot be used for quantitation.

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19. Location Page 107. VI Consensus PCR.

Proposed amendment :-

Insert scoring system.

(i) Insert a figure (Amendment 1) on page 107 to show reproducibility and scoring criteria.
(ii) Explain why there was no inadequate HPV test.

Amendment :-

Insert before sub-heading VII :-

Markers loaded in the wells just before the first and just after the last test sample on the gel served to identify positive bands of DNA of about 450 base-pairs in size among the samples being tested. The bands were visualised under ultraviolet light on a transilluminator and a photograph was taken to document the results. If there was a band visible at the level of the marker that sample was considered positive. Comparing the intensity of brightness of a band from a test sample to the marker band can give a semi-quantitative estimation of the viral load in that particular sample. However, assessment of viral load was not among the objectives of this thesis.

No category of inadequate HPV test was included since a β-globin test was not carried out. The protocol for HPV detection used included the collection of cells as accurately and carefully as possible, dispersion of the cells into PBS solution, sample digestion with PK and finally, PCR amplification. The HPV data reflect the results that can be obtained in practice with this type of routinely collected sample. It follows that inadequate samples may result in some false negative HPV tests.

New Figure (Figure X) :- Amendment 1.
Lane 1 = Markers (100bp, 200bp, 300bp, 400bp, 500bp identified by giving the strongest signal, 700bp, 900bp, 1kb and 1.5kb).
Lanes 2 to 6 = 5, replicate PCRs with a low positive template (10 copies of HPV16 DNA per reaction).
Lane 7 = Blank
Lanes 8 to 12, replicate PCRs with a high positive template (10,000 copies of HPV16 DNA per reaction).
Lane 13 = Negative control
Lane 14 = Positive control
Lane 15 = Markers as in Lane 1
Figure X. Simple illustration of PCR semi-quatitation.
20. Location Page 110. New section on epidemiological methods.

Proposed amendments:

a) Insert an overview.
   (i) Description of how screening is carried out in the hospitals involved.
   (ii) Eligibility criteria with regards to cytology reports explaining why no inadequate cytology was included in the study.

b) Define terminology used in the thesis.
   (i) Sensitivity. Definition and calculation as used in the thesis.
   (ii) Specificity. Definition and calculation as used in the thesis.
   (iii) Positive predictive value. Definition and calculation as used in the thesis.
   (iv) Negative predictive value. Definition and calculation as used in the thesis.
   (v) Relative risk. Definition and calculation as used in the thesis.

c) Insert an overview of the multivariate analysis with specific reference to the 2 x 2 table.
   (i) Explain the importance of adjustment for confounding variables

d) Insert an overview of odds ratio calculation as used in this thesis.
   (i) Do relative risk rather than odds ratio

Amendments:

Insert immediately before the RESULTS section:

IX. Epidemiological Methods

a) Screening practices at the hospitals involved in this study

At the Cancer Hospital women willing to have a Pap smear test taken have to arrive early in the morning or early afternoon, queue up to receive a number that is distributed from 1 to 400 and wait to be called. They answer a hospital questionnaire to summarily evaluate risk factors for most cancers. Then an experienced nurse assistant introduces a speculum, collects a smear sample and inspects the cervix with the naked eye, after application of 3% acetic acid and iodine solutions. If the nurse assistant believes there is anything abnormal regarding cervical neoplasia the woman is referred to the colposcopy clinic regardless of the cytology result. If clinical cancer is seen, the patient is immediately transferred to the colposcopy clinic where she has a biopsy. Women
with a negative smear without inflammation are referred on to a general gynaecology clinic. Unsatisfactory smear results are repeated and women with smears graded as inflammatory or ASCUS and above are referred to colposcopy.

At IMIP, a general gynaecology service is provided and any women coming in for screening first has an appointment with a gynaecologist. If there are no clinical signs of inflammation or hypoestrogenism the woman will have a Pap smear done and a colposcopy as an attempt to use the combination of tools as primary screening. If signs of inflammation or hypoestrogenism are detected the patient is treated to avoid the likely delay due an unsatisfactory smear, thus saving money and time. If the smear is unsatisfactory it is repeated. So here, all women end up having cytology and colposcopy as a primary screening strategy. The hospital is able to carry out 200 Pap smears and colposcopies a day.

The studies described in this thesis used data collected from women at the colposcopy clinics in the hospitals described above because all enrollees had to have both cytology and colposcopy to reduce verification bias and the objective of the study was to evaluate the efficacy of HPV testing. The eligibility criteria regarding cytology was that women with negative cytology results were considered as potential controls and women with abnormal cytology (ASCUS or above) were considered potential cases. Women with inadequate or unsatisfactory cytology were not included. Only cytology reports from Institutions accredited by the Government were accepted for inclusion in the studies.

b) Definition of Statistical Terms

Sensitivity is the proportion of individuals with the target disease who have a positive test result, the probability that an actual case of disease will be correctly diagnosed by the test. The sensitivity for cervical cancer was calculated by dividing the number of participants whose test was positive in the column of histologically proven cervical cancer by the total number of cases in this column.

Specificity is the probability that an individual without disease will have a correct negative test result. It equates to the true negative rate. The specificity for cervical cancer was calculated by dividing the number of participants whose test was
negative in the column for women with a normal cervix by the total number of 
women with a normal cervix.

Positive predictive value (PPV) is the proportion of individuals with positive test 
results who have the target disease. The PPV for cervical cancer was calculated by 
dividing the number of participants in the column for women with cervical cancer 
whose test was positive by the total number of positive tests.

Negative predictive value (NPV) is the proportion of individuals with negative 
test results who do not have the disease. The NPV for cervical cancer was 
calculated by dividing the number of participants in the column for women with a 
normal cervix whose test was negative by the total number of negative tests.

Analogous calculations were made for the combination of CIN 2/3 and cervical 
cancer, and CIN in general and cervical cancer.

New Figure :-  Amendment 2

<table>
<thead>
<tr>
<th>Detection or screening</th>
<th>Condition status (Gold standard)</th>
<th></th>
<th></th>
</tr>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
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<td>True-negative (TN)</td>
<td>FN + TN</td>
</tr>
<tr>
<td>Total</td>
<td>TP + FN</td>
<td>FP + TN</td>
<td>TP + FP + TN + FN</td>
</tr>
</tbody>
</table>

Sensitivity = TP / (TP + FN)
Specificity = TN / (FP + TN)
PPV = TP / (TP + FP)
NPV = TN / (FN + TN)

In this study,
Positive = cases with histologically confirmed HSIL,
Negative = cases with negative cytology and colposcopy and (therefore no 
histology data) or cases with histology < HSIL found in biopsies taken because of 
abnormal colposcopy (=>cervicitis).

The relative risk quantifies the increase in the probability of disease occurrence 
relative to subjects at the baseline level of risk factors, but does not quantify that 
probability itself. The relative risk, also called hazard ratio, relative rate, rate ratio, 
incidence density ratio or instantaneous relative risk, is a useful measure in
etiologic research that quantifies the strength of the relationship between exposure
and disease.

In the studies reported in this thesis, relative risk can be measured directly and this
was done in study D. However, for the benefit of emphasising the sensitivity and
specificity aspects, the results were analysed as case-control studies in studies A,
B and C where all women presenting for screening were included. In these
studies, the odds ratio was used to give a reasonable estimate of the relative risk.
This mode of analysis has been used in several reported screening studies.

c) Multivariate Analysis

It was not in the scope of this work to carry out a thorough epidemiologic
investigation. The fact that HR HPV infection was observed to be the major risk
factor for cervical neoplasia in Recife (study A), as is the case worldwide, led to
the interest in undertaking a summary assessment of other local risk factors
without the influence of HR HPV by adjustment for its confounding effects.
The use of a logistic regression model allows for estimation of the effects of
multiple covariates on the odds of the outcome variable or response. Several
analyses of 2 x 2 tables based on the conditional regression model can be
undertaken provided specific epidemiologic aims are set.

The calculations of odds ratio described in this thesis were undertaken using
computerised statistical packages. Two by two tables were used to compare the
results from the predictor variables (positive and negative) with the outcome
variables (positive and negative) under investigation. Relative Risk can only be
calculated for cohort studies and this has been done in the case of Study D.


Proposed amendments :-

a) A general description of how women were recruited for the study overall.

b) For each study, define case and control. Exclude from the calculations the
cancers that did not require cytology since they were clinically visible.

Amendments :-
Insert after the new section IX. Epidemiological Methods:

X. Selection of the study population

a) Overall recruitment criteria
Women were recruited from screening services at the Institute of Mother and Child Health in Pernambuco (IMIP) and the Cancer Hospital in Pernambuco (HCP). Volunteers were recruited at the colposcopy clinics in these screening services. It is important to make clear that screening for cervical cancer by these services is performed with both cytology and colposcopy in all women, regardless of the cytology result.

b) Classification of cases and controls
For studies A, C, D, and E the cases were histologically proven CIN 1, CIN 2/3, or cervical cancer. The controls were women with negative cytology and normal colposcopy. Women who had minor alterations on colposcopy and whose histological report on punch biopsies showed only cervicitis were classified as a separate group from the women with a completely normal cervix.

For study B the cases were histologically proven cervical cancer. The controls were women with negative cytology and normal colpocopy (including women with cervicitis). Women with precancerous lesions of the cervix were excluded from study B.

RESULTS

22. Location Page 110 Section A. Risk factors.

Proposed amendments:

a) Insert tables with raw data.

b) Insert a new summary table with relative risk and relative risk adjusted for age.

c) Page 120, Table A5 delete.

d) Page 121, Table A6 delete.

Amendments:
See appropriate new tables in SECTION II (Tables A25 to A30) and delete Tables A5 and A6.

23. Location  Page 145, Section B. Hybrid capture 1. 6 Results.

Proposed amendment :-

Make it clear that cervicitis was included in specificity calculation of this particular study.

Amendment :-

Insert after sentence 1 :-

In this study, women with cervicitis were included in the group of women with negative cytology and normal colposcopy and are included in the specificity calculations.

24. Location  Page 159, Section D. Hormonal influence.

Proposed amendment :-

Match case and control data by age.

Amendments :-

The matching between the different groups of women was inadequate. The effects of hormone level and age could not be clearly distinguished. The results have been over interpreted.

See appropriate table in SECTION II (Table D7).

DISCUSSION.

25. Location  Page 178. para 1.

Proposed amendment :-

Discussion of limitation of cytological screening in general.

Amendment :-

Insert after para 1 :-
As described in the introduction (sections 6A.a and 6A.e) cytological screening for cervical cancer presents some limitations. These include inappropriate sampling of cells, poor fixation, inadequate training of screeners, poor laboratory conditions, lack of quality control, false positive results and no follow up treatment. Indeed, cervical screening has not been successful in controlling cervical cancer in any developing country. In Recife very few reports reviewing cytological screening have been published. They are basically all in Portuguese, reported by the State Central Laboratory or Health Authority, and not by specific Institutions. There is a general lack of confidence in the quality of cytological screening in the State of Pernambuco. This is why some local health Services attempt to screen women for cervical cancer with a combination of cytology and colposcopy in all who attend.


Proposed amendment :-

Discussion of screening with HPV.

Amendment :-

Insert after para 1.:-

According to our findings, the use of HPV testing for primary screening of cervical neoplasia is as effective as cytology. The combination of both may lead to potential improvements in the quality of the screening programme efficacy. Some difficulties in the philosophical approach to screening with HPV testing arise in younger women. Some women will be identified as having cervical HR HPV infection and not having cervical cancer or HSIL. Cervical HR HPV infections frequently occur in young women and usually regress spontaneously. Only persistent HR HPV infections are likely to progress to cervical neoplasia. As a consequence, only a very low proportion of younger women develop cervical cancer compared to older women. However, as HR HPV infections result from sexually transmitted oncogenic viruses (and this is acknowledged by accredited Institutions such as WHO and the American CDC), it may be of use in identifying women who harbour these infections. They not only are at risk of developing cervical neoplasia but also may act as potential vectors in the transmission of these virulent micro-organisms.
Proposed amendments:

a) Add the word "ideal" between "possible" and "strategy" in line 3.
b) Insert "for women aged ≥30 years or pregnant" in line 6.

Amendments:

a) The sentence beginning in line 3 (page 181) should read:
   A possible ideal strategy involving HPV detection and cytology (Table C6 and Table C7) was constructed for improved cervical cancer prevention in Recife (Figure C1).
b) The sentence beginning in line 6 (page 181) should read:
   In this protocol, all cervical scrapes for HPV detection would be collected from women aged ≥30 years or pregnant women at primary screening facilities and sent to a central laboratory for HR HPV detection by PCR/RFLP.

REFERENCES

28. Proposed amendment:

Include the following additional references cited above.


SECTION II. Analysis Amendments to PhD thesis.

Background.

(1) The women studied in this thesis were recruited from self-referred attendees at 2 hospitals in Recife. It is not clear:

(a) What brought them to the hospital.
(b) The nature of their complaints (if any).

(2) At these hospitals, the women were examined by colposcopy as well as by cytology routinely. If colposcopy were abnormal, biopsy (when clinically indicated) with histological examination would be carried out. Since the quality of cytology was considered to be unreliable in Recife, recruitment of most women to this study was based on available histology for cases and all such women had adequate cytology.

(3) Based on (1) and (2), it was considered that the clinical material which could be collected under such conditions would not be appropriate for a population study and no attempt was made to do so. It is unfortunate that this point was not clearly stated in the thesis.

(4) In Recife, the term "cervicitis" as a histological diagnosis was used imprecisely to provide a general guideline to facilitate clinical management. Its relative significance, therefore, depended on the analysis being undertaken. It was viewed as an insignificant grade (i.e. "normal") relative to cancer and was included as normal in Study B and D. It was treated separately from the normal group in studies A, C and E because it could not be perceived clinically as "normal" when considered alongside CIN1. In accordance with the examiners' comments that all individuals in the study population must be included when calculating the predictive values of the screening tests, women with "cervicitis" were included as "normal" in all re-calculations presented in the following amendments for the analyses specifically requested by the examiners.

Specific amendments
(a) Study A (risk factors).

The thesis aimed to clarify the association between exposure to HR HPV and different grades of cervical disease (not the overall disease profile found in the Recife population). If an overwhelming association with high grade disease was found, as one would expect, the detection of HR HPV could form part of a future population study predicting women at risk of HSIL for early hospital referral. Available data for other minor life-style factors were included in the thesis. As these were of less direct clinical utility they were analysed less intensively after adjusting for HR HPV. In accordance with the examiners' comments, these risk factors have now been further analysed within the limitations of the data. A risk factor was considered to be significant only if

\[ p < 0.05 \text{ and lower limit of the 95\% CI } \geq 1.00 \]

Results are presented here only for categories where the numbers are sufficient.

(i) Current smoking as a risk factor for LSIL, HSIL or cancer adjusted for age, number of sexual partners, OCP usage and HR HPV (Table A25).

Result = Not significant.

(ii) Current smoking as a risk factor for LSIL, HSIL or cancer adjusted for HR HPV and illiteracy (Table A25).

Result = Not significant.

(iii) Current OCP usage as a risk factor for LSIL, HSIL or cancer adjusted for age, income and illiteracy (Table A26).

Result = Not significant.

(iv) Vaginal parity (>2) as a risk factor for LSIL, HSIL or cancer adjusted for age, income and illiteracy (Table A27).

Result = Significant in women \( \leq 35 \text{ yrs} \), high income and literate with HSIL.

(v) Number of sexual partners (>2) as a risk factor for LSIL, HSIL or cancer adjusted for age, income and illiteracy (A28).
Result = Significant in women >35 yrs, high income and literate with HSIL.

(vi) HR HPV as a risk factor for LSIL, HSIL or cancer adjusted for age (A29).
Result = Highly significant. In particular HPV16.

(vii) Age >35 years as a risk factor for LSIL, HSIL or cancer adjusted for HR HPV (A30).
Result = Significant only for cancer in HR HPV positive women.

(b) Study B (HC1). Please refer to Background to the Amendments above.

(c) Study C (PCR/RFLP detection of HR HPV types).
The thesis aimed to assess the performance of a simple HPV detection protocol under field conditions. This approach, based on consensus PCR and gel electrophoresis of restricted PCR fragments was first reported by Bernard et al. 1994 and extended by Londesborough et al. 1996 but has not been evaluated as a diagnostic tool. In accordance with the examiners' comments, Tables C4 and C5 have been re-calculated considering women with "cervicitis" only as "normal" and excluding the 31 women who did not have cytology results.

Result = Significant differences were observed between the original and amended analyses. It is evident that HPV typing and cytology, separately or combined, could be used to predict cervical lesions in general but not cervical cancer specifically (Table C9).

(d) Study D (hormone status).
The study aimed to assess the effect of female reproductive hormones on the prevalence and/or detectability of HR HPV with a view to establishing a future study to improve screening coverage by the detection of HR HPV in women attending prenatal clinics. As the hormonal levels were either not measured or unavailable for this study, clinical
criteria were used as substitutes (high level in pregnant women, normal level in nonpregnant nonmenopausal women and low level in menopausal women). The examiners suggested that age adjusted analyses of the data should be included. The number of women in this study was small. The amended analysis has therefore been carried out based on 2 strata (pregnant women 35 years of age or less and menopausal women older than 35 years) for all women or for women with a normal cervix or cervicitis only.

Result = There is a reduced relative risk of having HR HPV detected during pregnancy (Table D7).

(e) Study E (self sampling).

No amendments.
## Table A25

Current smoking as a risk factor for cervical neoplasia (case-control study).

<table>
<thead>
<tr>
<th>Adjustments</th>
<th>Outcome</th>
<th>n</th>
<th>OR</th>
<th>95% CI</th>
<th>( \chi^2 )</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr) Sexual partners OCP HR HPV Illiteracy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 1 none negative -</td>
<td>LSIL</td>
<td>4</td>
<td>11</td>
<td>6.00</td>
<td>0.94 - 36.43</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>1</td>
<td>2</td>
<td>8.25</td>
<td>0.11 - 181.99</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>0</td>
<td>0</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>4</td>
<td>66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 1 none negative -</td>
<td>LSIL</td>
<td>2</td>
<td>10</td>
<td>1.08</td>
<td>0.11 - 5.87</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>1</td>
<td>1</td>
<td>5.40</td>
<td>0.06 - 428.92</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>1</td>
<td>2</td>
<td>2.70</td>
<td>0.04 - 54.26</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>15</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤35 - - negative no</td>
<td>LSIL</td>
<td>10</td>
<td>39</td>
<td>2.19</td>
<td>0.86 - 5.2</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>3</td>
<td>6</td>
<td>4.27</td>
<td>0.64 - 21.39</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>2</td>
<td>0</td>
<td>u</td>
<td>u</td>
<td>8.21</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>24</td>
<td>205</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 - - negative no</td>
<td>LSIL</td>
<td>5</td>
<td>12</td>
<td>1.88</td>
<td>0.48 - 6.29</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>HSIL</td>
<td>5</td>
<td>5</td>
<td>4.52</td>
<td>0.96 - 20.79</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>2</td>
<td>2</td>
<td>4.52</td>
<td>0.31 - 63.88</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>29</td>
<td>131</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n, number of women
- , not adjusted
* , no lesion in this category
u, undefined
### Table A26

**Current OCP usage as a risk factor for cervical neoplasia (case-control study)**

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Income (MW)</th>
<th>Illiteracy</th>
<th>Outcome</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>0.77</td>
<td>0.42 - 1.38</td>
<td>0.60</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>0.78</td>
<td>0.31 - 1.83</td>
<td>0.16</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>0.00</td>
<td>0.00 - 5.81</td>
<td>0.24</td>
<td>0.56</td>
</tr>
<tr>
<td>&gt;35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>0.00</td>
<td>0.00 - 5.10</td>
<td>0.18</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>1.22</td>
<td>0.12 - 6.76</td>
<td>0.04</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>0.00</td>
<td>0.00 - 4.15</td>
<td>0.32</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*MW* = Minimal Wage
Table A27

Vaginal parity >2 as a risk factor for cervical neoplasia (case-control study)

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Income (MW)</th>
<th>Illiteracy</th>
<th>Outcome</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>1.35</td>
<td>0.60 - 2.90</td>
<td>0.40</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>6.83</td>
<td>2.70 - 16.76</td>
<td>22.61</td>
<td>&lt;0.00002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>18.92</td>
<td>0.93 - 1120.53</td>
<td>5.22</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>&gt;35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>1.67</td>
<td>0.67 - 4.32</td>
<td>0.99</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>1.72</td>
<td>0.89 - 3.39</td>
<td>2.44</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>3.82</td>
<td>1.41 - 11.97</td>
<td>7.46</td>
<td>0.01</td>
</tr>
</tbody>
</table>

MW = Minimal Wage
Table A28

No. of sexual partners >2 as a risk factor for cervical neoplasia (case-control study)

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Income (MW)</th>
<th>Illiteracy</th>
<th>Outcome</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>2,33</td>
<td>1.35 - 3.99</td>
<td>9.97</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>1,86</td>
<td>0.78 - 4.19</td>
<td>1.96</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>2,82</td>
<td>0.23 - 25.13</td>
<td>0.36</td>
<td>0.25</td>
</tr>
<tr>
<td>&gt;35</td>
<td>≥1</td>
<td>no</td>
<td>LSIL</td>
<td>3,00</td>
<td>1.01 - 8.14</td>
<td>4.45</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSIL</td>
<td>6,82</td>
<td>3.09 - 14.92</td>
<td>29.26</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
<td>5,03</td>
<td>1.92 - 12.70</td>
<td>13.74</td>
<td>&lt;0.0004</td>
</tr>
</tbody>
</table>

MW = Minimal Wage
### Table A29

**HPV as a risk factor for cervical neoplasia (case-control study, controls include women with cervicitis)**

<table>
<thead>
<tr>
<th>Risk</th>
<th>Outcome</th>
<th>OR</th>
<th>95% CI</th>
<th>( \chi^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any HPV</td>
<td>&lt;35</td>
<td>1.98 - 5.21</td>
<td>25.16</td>
<td>0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>1.29 - 7.29</td>
<td>7.36</td>
<td>&lt;0.0018</td>
<td></td>
</tr>
<tr>
<td>HPV16</td>
<td>&lt;35</td>
<td>2.07 - 9.29</td>
<td>18.26</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>1.88 - 21.32</td>
<td>10.89</td>
<td>&lt;0.0159</td>
<td></td>
</tr>
<tr>
<td>HPV31</td>
<td>&lt;35</td>
<td>2.28 - 11.71</td>
<td>19.72</td>
<td>&lt;0.00009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>3.18 - 25.18</td>
<td>18.82</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td>HPV33</td>
<td>&lt;35</td>
<td>1.09 - 9.13</td>
<td>5.15</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.92 - 11.15</td>
<td>5.15</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>HPV52</td>
<td>&lt;35</td>
<td>1.26 - 31.59</td>
<td>6.35</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>1.22 - 70.13</td>
<td>0.43</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>HPV58</td>
<td>&lt;35</td>
<td>0.00 - 0.759</td>
<td>0.05</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.00 - 0.759</td>
<td>0.58</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.00 - 0.759</td>
<td>0.58</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.00 - 0.759</td>
<td>0.58</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.00 - 0.759</td>
<td>0.58</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>0.00 - 0.759</td>
<td>0.58</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
</tbody>
</table>
Table A30

Age >35 years as a risk factor for cervical neoplasia (case-control study)

<table>
<thead>
<tr>
<th>Adjustments</th>
<th>Outcome</th>
<th>n</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Age &gt;35</td>
<td>Age &lt;=35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR HPV Cytology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Positive**

<table>
<thead>
<tr>
<th>HR HPV Cytology</th>
<th>LSIL</th>
<th>12</th>
<th>54</th>
<th>0.67</th>
<th>0.40 - 1.12</th>
<th>2.16</th>
<th>0.14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSIL</td>
<td>54</td>
<td>35</td>
<td>1.93</td>
<td>1.41 - 2.64</td>
<td>17.02</td>
<td>&lt;0.00004</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>63</td>
<td>3</td>
<td>16.43</td>
<td>5.38 - 50.19</td>
<td>66.82</td>
<td>&lt;0.0000001</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>29</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Negative**

<table>
<thead>
<tr>
<th>HR HPV Cytology</th>
<th>LSIL</th>
<th>13</th>
<th>2</th>
<th>0.57</th>
<th>0.36 - 0.92</th>
<th>5.08</th>
<th>0.02</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSIL</td>
<td>21</td>
<td>10</td>
<td>2.43</td>
<td>1.17 - 5.06</td>
<td>5.19</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>13</td>
<td>2</td>
<td>7.56</td>
<td>1.72 - 33.15</td>
<td>8.57</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>214</td>
<td>262</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**≥ASCUS**

<table>
<thead>
<tr>
<th>HR HPV Cytology</th>
<th>LSIL</th>
<th>13</th>
<th>38</th>
<th>0.46</th>
<th>0.29 - 0.76</th>
<th>10.96</th>
<th>0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSIL</td>
<td>66</td>
<td>38</td>
<td>1.04</td>
<td>0.83 - 1.31</td>
<td>0.03</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>30</td>
<td>4</td>
<td>2.92</td>
<td>1.16 - 7.38</td>
<td>6.32</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>29</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Negative**

<table>
<thead>
<tr>
<th>HR HPV Cytology</th>
<th>LSIL</th>
<th>21</th>
<th>67</th>
<th>0.51</th>
<th>0.32 - 0.80</th>
<th>8.33</th>
<th>&lt;0.004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSIL</td>
<td>6</td>
<td>7</td>
<td>1.24</td>
<td>0.42 - 3.65</td>
<td>0.01</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>12</td>
<td>0</td>
<td>u</td>
<td>u</td>
<td>14.60</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>214</td>
<td>312</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n, number of women
- , not adjusted
u, undefined
### Original Table

Control group ("normal") did not include women with only cervicitis.

<table>
<thead>
<tr>
<th>Referral tool</th>
<th>Criteria</th>
<th>Detection of cervical cancer</th>
<th>OR</th>
<th>95% CI</th>
<th>( \chi^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>ASCUS or above</td>
<td></td>
<td>27.83</td>
<td>9.60 - 88.44</td>
<td>69.35</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>Mild dyskaryosis or above</td>
<td></td>
<td>35.10</td>
<td>12.36 - 103.41</td>
<td>85.02</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>HPV typing</td>
<td>Any HR-HPV</td>
<td></td>
<td>48.72</td>
<td>18.04 - 139.41</td>
<td>115.82</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>HPV 16</td>
<td></td>
<td>67.28</td>
<td>22.15 - 216.37</td>
<td>113.62</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>HPV 18</td>
<td></td>
<td>86.50</td>
<td>6.23 - 2596.55</td>
<td>29.00</td>
<td>&lt;0.000001</td>
</tr>
</tbody>
</table>

Table C4. The utility of cytology or HR HPV as a referral tool for the detection of cervical cancer.

### Amended

Control group ("normal") includes women with only cervicitis.

The utility of cytology and HR HPV as referral tools for the detection of cervical cancer.

<table>
<thead>
<tr>
<th>Referral tool</th>
<th>Criteria</th>
<th>Risk of cervical cancer</th>
<th>OR</th>
<th>95% CI</th>
<th>( \chi^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>ASCUS or above</td>
<td></td>
<td>23.67</td>
<td>8.70 - 73.57</td>
<td>68.46</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>Mild dyskaryosis or above</td>
<td></td>
<td>45.83</td>
<td>15.37 - 150.12</td>
<td>100.32</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>HPV typing</td>
<td>Any HR HPV</td>
<td></td>
<td>38.38</td>
<td>15.26 - 113.26</td>
<td>113.73</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>HPV 16</td>
<td></td>
<td>69.71</td>
<td>24.48 - 220.50</td>
<td>137.39</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>HPV 18</td>
<td></td>
<td>29.88</td>
<td>3.42 - 214.30</td>
<td>21.60</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Original Table - Control group ("normal") did not include women with only cervicitis.

<table>
<thead>
<tr>
<th>Screening tool</th>
<th>Histology</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>SIL+Cancer</td>
<td>71,50%</td>
<td>85,20%</td>
<td>71,10%</td>
<td>85,50%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>87,50%</td>
<td>87,00%</td>
<td>53,80%</td>
<td>94,40%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>80,60%</td>
<td>87,00%</td>
<td>52,80%</td>
<td>96,90%</td>
</tr>
<tr>
<td>HPV typing</td>
<td>SIL+Cancer</td>
<td>81,20%</td>
<td>81,50%</td>
<td>72,80%</td>
<td>87,70%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>88,20%</td>
<td>84,70%</td>
<td>50,30%</td>
<td>84,70%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>89,80%</td>
<td>84,70%</td>
<td>61,60%</td>
<td>96,80%</td>
</tr>
<tr>
<td>Combined</td>
<td>SIL+Cancer</td>
<td>87,30%</td>
<td>71,10%</td>
<td>60,40%</td>
<td>91,80%</td>
</tr>
<tr>
<td></td>
<td>HSIL+Ca</td>
<td>96,60%</td>
<td>74,90%</td>
<td>39,20%</td>
<td>98,20%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>96,80%</td>
<td>74,90%</td>
<td>35,70%</td>
<td>98,40%</td>
</tr>
</tbody>
</table>

Table C5. A comparison of cytology and HR HPV typing as screening tools for cervical neoplasia.

Amended

Table C9 - Control group ("normal") includes women with only cervicitis.

Comparison of cytology and HR HPV typing as screening tools for cervical neoplasia. Cervicitis is scored as normal.

<table>
<thead>
<tr>
<th>Screening tool</th>
<th>Histology</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>SIL + Cancer</td>
<td>72,00%</td>
<td>85,00%</td>
<td>70,60%</td>
<td>84,70%</td>
</tr>
<tr>
<td></td>
<td>HSIL + Ca</td>
<td>87,50%</td>
<td>85,00%</td>
<td>50,30%</td>
<td>84,70%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>80,60%</td>
<td>85,00%</td>
<td>16,30%</td>
<td>84,70%</td>
</tr>
<tr>
<td>HPV typing</td>
<td>SIL + Cancer</td>
<td>79,30%</td>
<td>81,30%</td>
<td>68,40%</td>
<td>87,20%</td>
</tr>
<tr>
<td></td>
<td>HSIL + Ca</td>
<td>87,50%</td>
<td>81,30%</td>
<td>44,30%</td>
<td>87,20%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>87,10%</td>
<td>81,30%</td>
<td>15,50%</td>
<td>87,20%</td>
</tr>
<tr>
<td>Combined</td>
<td>SIL + Cancer</td>
<td>87,30%</td>
<td>71,10%</td>
<td>60,40%</td>
<td>90,50%</td>
</tr>
<tr>
<td></td>
<td>HSIL + Ca</td>
<td>96,60%</td>
<td>71,10%</td>
<td>39,20%</td>
<td>90,50%</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>96,80%</td>
<td>71,10%</td>
<td>13,80%</td>
<td>90,50%</td>
</tr>
<tr>
<td>Hormone exposure</td>
<td>Age (yr)</td>
<td>Disease status (histology)</td>
<td>HPV prevalence (%) (exposed / not exposed)</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------</td>
<td>---------------------------</td>
<td>------------------------------------------</td>
<td>--------</td>
<td>---------------</td>
</tr>
<tr>
<td>Pregnant (high level)</td>
<td>≤35</td>
<td>Normal or cervicitis</td>
<td>10.9 / 27.1</td>
<td>0.33</td>
<td>0.10 - 0.91</td>
</tr>
<tr>
<td>Pregnant (high level)</td>
<td>≤35</td>
<td>Any cervical neoplasia</td>
<td>12.3 / 31.8</td>
<td>0.30</td>
<td>0.11 - 0.75</td>
</tr>
<tr>
<td>Menopausal (low level)</td>
<td>&gt;35</td>
<td>Normal or cervicitis</td>
<td>20.0 / 5.2</td>
<td>4.56</td>
<td>1.05 - 22.60</td>
</tr>
<tr>
<td>Menopausal (low level)</td>
<td>&gt;35</td>
<td>Any cervical neoplasia</td>
<td>30.5 / 26.3</td>
<td>1.23</td>
<td>0.57 - 2.59</td>
</tr>
</tbody>
</table>