THE UTILITY OF HPV DNA TESTING IN TRIAGE OF LOW-GRADE CYTOLOGICAL ABNORMALITIES

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by

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ABSTRACT

This study evaluated the usefulness of human papillomavirus (HPV) DNA testing and repeat cytology in triage of women referred to colposcopy in St. John's, Newfoundland with atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL) cytology. Data were collected on the initial Pap abnormality that prompted referral, HPV test, repeat Pap test, and histology if biopsies were ordered. Of 447 women, 97 with ASCUS and 145 with LSIL had results for all tests. For ASCUS, HPV testing was 100% sensitive for detection of underlying high-grade intraepithelial lesions (HSIL) while reducing referrals to 44.3%. There would have been significant reductions in referrals among women ≥30 years of age (74.3%) compared to younger women (27.4%). Nevertheless, in restricting HPV testing to women aged ≥30 years, 8/16 women with underlying HSIL would not have been referred to colposcopy. Repeat cytology was less sensitive (75%) for triaging all women. For LSIL, any method would have referred approximately 60% or more if a good sensitivity was achieved in any age group. For ASCUS, HPV triage appears to be more useful than repeat cytology. No useful triage strategy was identified for LSIL.

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LIST OF ABBREVIATIONS

ALTS	ASCUS/LSIL Triage Study
ASC	Atypical squamous cells
ASC-H	Atypical squamous cells, cannot exclude HSIL
ASCUS	Atypical squamous cells of undetermined significance
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma *in situ*
DNA	Deoxyribonucleic acid
HC-II	Hybrid Capture II
HPV	Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
HSV-2	Herpes simplex virus-2
IARC	International Agency for Research on Cancer
LBC	Liquid-based cytology
LSIL	Low-grade squamous intraepithelial lesion
NPV	Negative predictive value
PCR	Polymerase chain reaction
Pap	Papanicolaou
PPV	Positive predictive value
RLU	Relative light unit
RNA	Ribonucleic acid
SIL	Squamous intraepithelial lesion

STI	Sexually transmitted infection
STM	Specimen transport medium

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CHAPTER I

INTRODUCTION

1.1 Rationale

Cervical cancer is a slow multi-step process and it is largely preventable with effective screening and adequate treatment (Franco, Duarte-Franco, & Ferenczy, 2001; Schiffman & Kjaer, 2003). The conventional approach to cervical cancer screening has been reliant on women presenting themselves for regular cervical cytology, also known as the Papanicolaou (Pap) test. The main purpose of the Pap test is to detect precancerous cell changes in the cervical epithelium, the cells that line the cervix, which may lead to cancer. If these abnormal cells are detected early, they can be treated before cancer develops.

Research over the past 25 years or so has clearly established that certain oncogenic types of human papillomavirus (HPV) are the underlying necessary cause of cervical cancer (Bosch, Lorincz, Munoz, Meijer, & Shah, 2002; Walboomers et al., 1999). Genital HPV infection is very common (Koutsky, 1997) and it is acquired with sexual activity (Kjaer et al., 2001; Rylander, Ruusuvaara, Almstromer, Evander, & Wadell, 1994). The prevalence of HPV infection is highest among young women, reaching its peak in the early 20s, and declining with advancing age (Herrero et al., 2000; Ho, Bierman, Beardsley, Chang, & Burk, 1998; Ratnam et al., 2000). In most cases, the infection is self-limited and completely asymptomatic without ever being clinically significant

(Evander et al., 1995; Franco et al., 1999; Hildesheim et al., 1994; Ho et al., 1998; Woodman et al., 2001). However, in some women, the infection can become persistent. It is the persistent HPV infection that increases the risk of precancerous abnormalities and its progression to cancer (Bosch & de Sanjose. 2003; Ho et al., 1998; Wallin et al., 1999). Regardless, cervical cancer is an uncommon outcome of HPV infection (Nobbenhuis et al., 1999). Since most HPV infections occur soon after initiation of sexual activity and are temporary, it is therefore those women over the age of 30, who are HPV positive that are most likely to represent persistent carriers (Bosch & de Sanjose, 2003; Ho et al., 1998; Ho et al., 1995). These women are therefore at an increased risk for developing precancerous changes and cervical cancer.

The Pap test has been proven to reduce the incidence and mortality of cervical cancer in developed countries (Anderson et al, 1988; Canadian Cancer Statistics 2007; Gustafsson, Pontén, Zack, & Adami, 1997a). However, this test has limitations, the most serious being false negative results (Cuzick et al., 2006). Further to this, there is remarkable variation in its performance indicators (Cuzick et al., 2006; Fahey, Irwig, & Macaskill, 1995; Nanda et al., 2000). A more precise and efficient method for screening is called for. Ideally, the Pap test should detect precancerous changes. Unfortunately, the majority of changes detected are not related to cervical cancer risk (Ho et al., 1998; Kinney, Manos, Hurley, & Ransley, 1998; Solomon, Schiffman, & Tarone, 2001). In fact, the majority of all abnormal Pap reports, specifically atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesions

(LSIL), are minor cellular changes that are not associated with cervical cancer risk (Ho et al., 1998; Solomon et al., 2001). In most cases, these changes are not predictive of cervical cancer risk and will regress spontaneously (Ostor, 1993). However, because a small number of cases are associated with high-grade disease (ASCUS/LSIL Triage Study (ALTS) Group, 2003a; Ho et al., 1998; Sherman et al., 2003), combined with the fact that Pap cytology is a screening test and not a diagnostic one, family physicians routinely refer women with these low-grade cytological abnormalities to gynecologists for further assessment or follow them up with repeat Pap testing at 4-6 month intervals over 2-3 years (Stuart et al., 2004). Referrals and repeat Pap testing involve delays in testing for those with true underlying disease, resulting in loss to follow-up and much anxiety on the part of these women, most of whom are not at risk (Bell et al., 1995; Flannelly et al., 1994; Peters, Somerset, Baxter, & Wilkinson, 1999).

Gynecologists routinely conduct additional tests, including an invasive and expensive procedure called a colposcopy on all those women referred for follow-up in order to identify the few of them who have high-grade disease. This testing may also involve invasive procedures such as biopsies or excisional procedures, so as to provide a histological diagnosis. At the end of this lengthy and costly process, most of these women with low-grade cytological abnormalities are not at risk for developing cervical cancer (Ho et al., 1998; Solomon et al., 2001). Since the majority of women who are found to have low-grade cytological abnormalities do not have an increased risk for developing cervical cancer, these repeat visits and procedures are unnecessary for most of

them. This represents poor resource utilization at a considerably unnecessary expense in health care costs, as well as unnecessary overtreatment with potential for negative health outcomes. In this context, recent studies indicate that HPV deoxyribonucleic acid (DNA) testing could be useful in identifying the small proportion of women who are at greatest risk of developing cervical cancer, while returning the majority to routine screening (Arbyn et al., 2004; Arbyn et al., 2005; Manos et al., 1999; Solomon et al., 2001).

The HPV DNA test is a sophisticated, molecular test that looks specifically for HPV DNA in cervical cells. To date, the Hybrid Capture II (HC-II) assay (Digene Corporation, Gaithersburg, Maryland, USA) is the most extensively used test. The HC-II assay is an *in vitro*, signal-amplified test for detecting DNA, in which ribonucleic acid (RNA) probes for HPV DNA are hybridized in solution with the sample DNA. It tests for 13 of the 15 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68. It is highly reproducible, easy to perform, and provides an objective result (Castle et al., 2002; Castle, Wheeler, Solomon, Schiffman, & Peyton, 2004). Published studies have consistently reported a superior sensitivity of the HPV DNA test to cytology (Bigras & de Marval, 2005; Clavel et al., 2001; Cuzick et al., 2006; Cuzick et al., 2003; Mayrand et al., 2007; Ratnam, Franco, & Ferenczy, 2000; Schiffman et al., 2000).

In 1998, the Newfoundland Public Health Laboratory began offering the HPV DNA test on a routine basis through gynecologists across the Province as an adjunct test for further stratification of clarifying low-grade cytological abnormalities. The Canadian and

American guidelines now recommend the HPV DNA test as an adjunct test in triage of most low-grade Pap abnormalities, namely ASCUS (Provencher & Murphy, 2007; Stuart et al., 2004; Wright et al., 2007). However, there is a difference in the recommendations. The American recommendation is for all women to be tested for HPV to clarify these abnormalities (Wright et al., 2007), while the Canadian recommendation is to test only women 30 years of age and older (Provencher & Murphy, 2007; Stuart et al., 2004). The rationale for this age-restricted testing recommendation is based on the fact that women 30 years of age and older who are HPV positive most likely represent persistent carriers (Bosch & de Sanjose, 2003; Ho et al., 1998), thereby reflecting an increased risk for developing cervical cancer. As well, because of the high prevalence of HPV in younger women, the specificity of the HPV test in these women is low (Sherman, Schiffman & Cox, 2002; Shlay, Dunn, Byers, Baron, & Douglas, 2000). In Canada and the United States, HPV testing is not recommended for women with LSIL Pap results, as it most likely represents a self-limited HPV infection; rather colposcopy is the recommended management option (Provencher & Murphy, 2007; Wright et al., 2007). Regardless, the usefulness of this test in the triage of low-grade Pap abnormalities in Newfoundland needs to be determined, and Canadian data is lacking. It has been recommended that more Canadian data be generated examining the usefulness of HPV DNA testing in triaging women with low-grade cytological abnormalities (Duarte-Franco & Franco, 2004).

In this retrospective cohort study, we evaluated the usefulness of HPV DNA testing compared to repeat Pap cytology in the triage of low-grade cytological abnormalities in women referred to a colposcopy clinic in St. John's, Newfoundland.

1.2 Objectives

The objectives of the present study are as follows:

1. To describe the association of HPV, repeat Pap cytology and histology results in women with low-grade cytological abnormalities.
2. To assess the performance of the HPV DNA test in triage of women with low-grade cytological abnormalities.
3. To assess the performance of the repeat Pap test in triage of women with low-grade cytological abnormalities.
4. To compare the performance of both triage tests.
5. To determine if age affects triage performance.

CHAPTER II

LITERATURE REVIEW

2.1 Burden of Cervical Cancer

Globally, cervical cancer is the second most common cancer among women and the third most frequent cause of cancer-related deaths (Parkin, Bray, Ferlay, & Piscani, 2005). In 2002, it was estimated that 493,000 new cases of cervical cancer were diagnosed worldwide, and that 274,000 women died from cervical cancer that same year. The disease incidence shows clear geographical variation. Eighty-three per cent of these cases were diagnosed in developing countries, where cervical cancer accounts for approximately 15% of cancers in women. It is the most common cancer in women in many regions, and is in fact the leading cause of cancer-related deaths among women in developing countries. In developed countries, however, cervical cancer accounts for only 3.6% of cancers in women.

Incidence and mortality rates in Canada are relatively low. Table 1 shows Canada's incidence and mortality rates for cervical cancer and estimated numbers of new cases and deaths for 2007 (Canadian cancer statistics, 2007). Approximately 1350 new cases of cervical cancer were estimated to have been diagnosed in Canadian women in 2007, and an estimated 390 women died from the disease in the same year. The provinces with the highest incidence rates are Nova Scotia, Prince Edward Island and Alberta, with rates of 10 per 100,000 women or greater; Newfoundland and Labrador has the highest mortality

Table 1: Estimated age-standardized incidence and mortality rates* for cervical cancer and estimated new cases and deaths in 2007 in Canada

Province	Incidence rate per 100 000	Mortality rate per 100 000	Estimated no. of new cases	Estimated no. of deaths
Newfoundland	8†	4	25†	10
Prince Edward Island	10	3	10	5
Nova Scotia	11	3	55	20
New Brunswick	8	3	35	15
Quebec	6	1	280	75
Ontario	7	2	500	140
Manitoba	7	2	45	15
Saskatchewan	9	2	45	15
Alberta	10	2	160	40
British Columbia	7	2	170	50
Canada‡	7	2	1350	390

* Rates are age-standardized according to the 1991 Canadian population.
† Likely an underestimate of the number of cases for the years used to generate estimates.
‡ Canada totals include provincial and territorial estimates. Territories are not listed separately due to small numbers.
Source: Canadian Cancer Statistics 2007

rate in the country with 4 per 100,000 women, twice that of the Canadian rate; Quebec was the only province with an incidence rate below 7 per 100,000 women and a mortality rate below 2 per 100,000 women. Figures 1 and 2 show the time trends in age-standardized incidence and mortality of cervical cancer for Canada since 1978. Generally, incidence and mortality rates of cervical cancer have declined in Canada during the last 50 years.

The lower risk for cervical cancer in developed countries is a relatively recent phenomenon. This trend is attributed to effective cervical cytology screening programmes. Incidence rates of invasive cervical cancer have dropped by 70% since the introduction of cytological screening in some populations (Gustafsson et al., 1997a). Before the introduction of screening programmes in the 1950s and 1960s, the incidence rates in most developed countries were similar to those found in developing countries today (Anderson et al, 1988; Gustafsson, Pontén, Bergström, & Adami, 1997b). In fact, screening for cervical cancer has been regarded as having a greater influence in reducing incidence and mortality than screening for any other cancer.

2.2 HPV and Cervical Cancer

2.2.1 HPV as Etiological Agent

For more than a century, a link between cervical cancer and sexual activity has been suspected. In 1842, Rigoni-Stern reported that there was a relatively low incidence of cervical cancer in virgins and nuns and a high frequency of cervical cancer in prostitutes

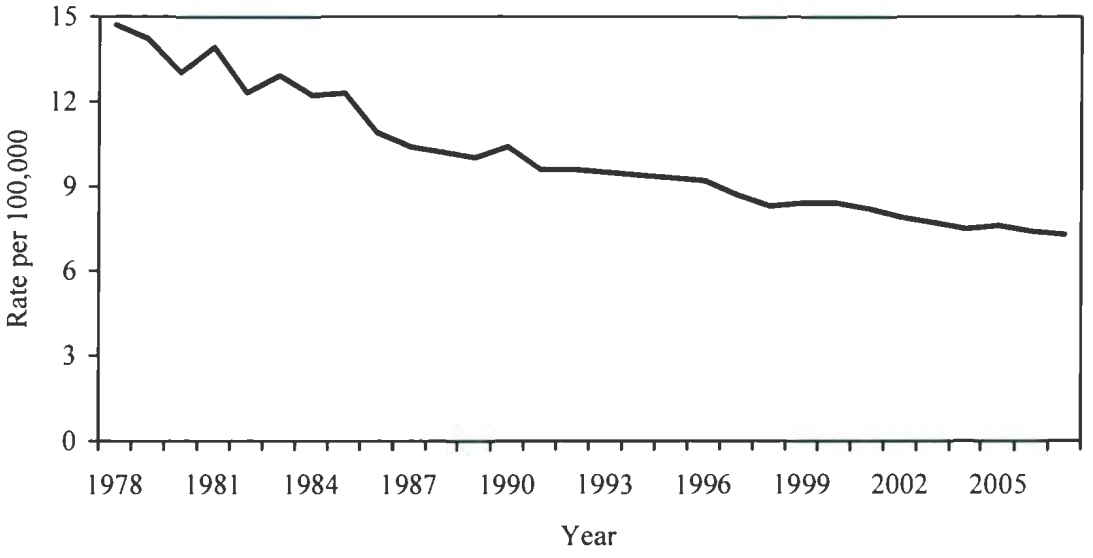

Figure 1: Age-standardized incidence rate for cervical cancer, Canada, 1978-2007

Note: Rates are age-standardized according to the 1991 Canadian population.
Source: Canadian Cancer Statistics 2007.

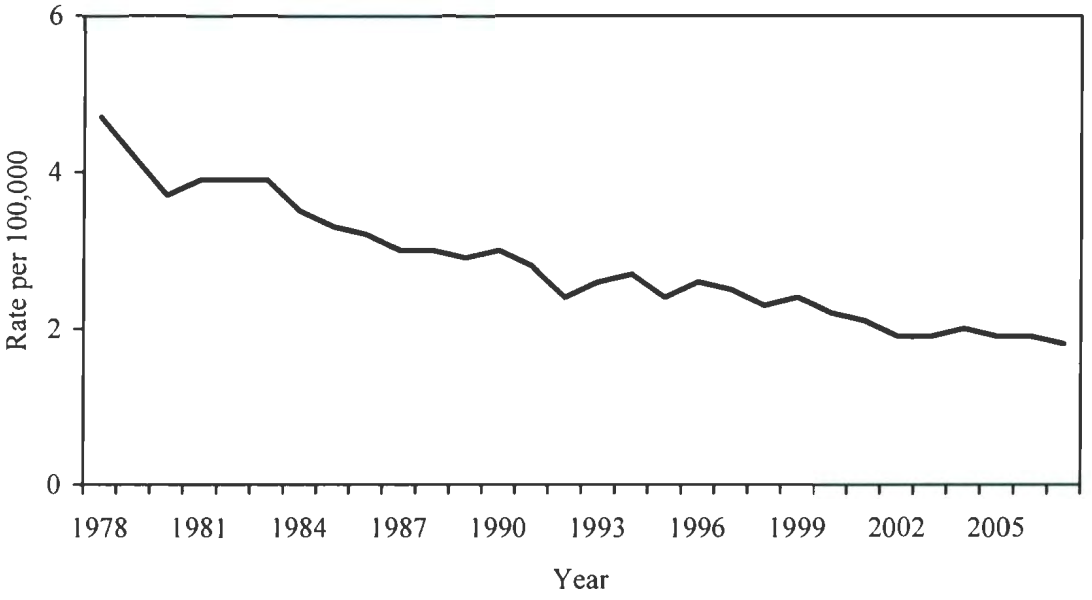

Figure 2: Age-standardized mortality rate for cervical cancer, Canada, 1978-2007

Note: Rates are age-standardized according to the 1991 Canadian population.
Source: Canadian Cancer Statistics 2007.

(Griffiths, 1991). Since that time, several epidemiological studies of women with cervical cancer showing strong associations with promiscuity and early age of first sexual intercourse further supported the notion that a sexually transmitted infection (STI) was involved in the development of cervical cancer (Brinton et al., 1987; Buckley, Harris, Doll, Vessey, & Williams, 1981; Harris et al., 1980; Kessler, 1977). Over the years, several infectious agents were put forth including syphilis, gonorrhea, and herpes simplex virus-2 (HSV-2) (zur Hausen, 1991). An association between the human papillomavirus (HPV) and cervical cancer was first suggested in the 1970s (Meisels, Fortin, & Roy, 1977; zur Hausen, 1976). In recent years, research has now firmly shown that a subset of oncogenic types of HPV are the necessary, though not sufficient cause, of nearly all of cervical cancers (Bosch et al., 2002; Walboomers et al., 1999) and its precursors (Cuzick et al., 2003; Elfgren et al., 2005; Kjaer et al., 1996; Kjaer et al., 2002; Koutsky et al., 1992; Nobbenhuis et al., 1999; Schiffman et al., 1993; Schlecht et al., 2001). It is the first ever identified necessary cause of a human cancer (Walboomers et al., 1999). The International Agency for Research on Cancer (IARC) has coordinated 22 studies that have shown unequivocally that HPV can be detected in 99.7% of adequate cervical cancer specimens (Walboomers et al. 1999).

2.2.2 Basic Virology

HPV is a small, non-enveloped, double-stranded circular deoxyribonucleic acid (DNA) tumour virus, which is part of a family of viruses classified in the *Papillomaviridae* family (de Villiers, Fauquet, Broker, Bernard, & zur Hausen, 2004). All HPVs have the

same basic genomic organization and have been divided into three major portions based on their function. The first is a long control region, which is a non-coding region that regulates DNA replication. The second is an early transcription region that encodes transcripts (E1, E2, E4, E5, E6 and E7) for viral proteins involved in viral DNA replication, initiation of RNA transcription and disruption of the cell cycle. The third is a late transcription region that encodes transcripts (L1 and L2) for two viral proteins that make up the viral capsid (Munger et al., 2004).

2.2.3 Classification

More than 120 different HPV types have been fully sequenced and characterized, with about 40 types infecting the epithelium of the human anogenital tract (de Villiers et al. 2004). Based on IARC pooled data from 11 case-control studies of the association between cervical cancer and HPV infection from multiple countries (Munoz et al., 2003), 15 HPV oncogenic types have been classified as high-risk for development of cervical cancer, 3 have been classified as probable high-risk, 12 have been classified as low risk, and 3 are considered to have undetermined risk (Table 2). The two most prevalent HPV types are 16 and 18, accounting for more than 60% of cervical cancer cases worldwide. HPV 16 accounts for approximately 50% of cases of cervical cancer, while HPV 18 accounts for the other 10% to 12% (Munoz et al., 2003). In Canada, there are indicators that type 31 is the second most frequent genotype associated with precancer or cancer (Antonishyn, Horsman, Kelln, Saggar, & Severini, 2008). Types 6 and 11 are the most

Table 2: Classification of HPV types by the association with cervical cancer

Risk classification	HPV types
High-risk	16, 18, 31, 33, 35, 39, 45, 51
	52, 56, 58, 59, 68, 73, 82
Probable high-risk	26, 53, 66
Low-risk	6, 11, 40, 42, 43, 44, 54
	61, 70, 72, 81, CP6108
Undetermined risk	34, 57, 83

HPV = Human papillomavirus

Source: Munoz et al., 2003.

frequently detected low-risk types and can often be detected in cases of genital warts (Brown, Schroeder, Bryan, Stoler, & Fife, 1999; Greer et al., 1995).

2.2.4 Natural History of HPV

HPV infections are among the most common STIs worldwide (Koutsky, 1997; Schiffman & Kjaer, 2003). In fact, the lifetime risk of acquiring HPV is approximately 70% (Bosch & de Sanjose, 2003). HPV prevalence peaks in women during the early 20s (Ho et al., 1998; Ratnam et al., 2000) and drops to less than 10% in women over 30 years reaching about 5% or less with advancing age (Herrero et al., 2000; Ratnam et al., 2000). In the vast majority of cases, infections are self-limited and asymptomatic, and resolve without treatment, cleared by the woman's immunity within one to two years (Evander et al., 1995; Franco et al., 1999; Hildesheim et al., 1994; Ho et al., 1998; Woodman et al., 2001). However, in a small proportion of women the infection can become persistent, and it is this persistent infection that predisposes to precancerous changes and cancer (Bosch & de Sanjose. 2003; Ho et al., 1998; Wallin et al., 1999). Since most HPV infections occur soon after initiation of sexual activity and are self-limited, women over the age of 30 include those who are more likely to be persistent carriers (Bosch & de Sanjose. 2003; Ho et al., 1998; Ho et al., 1995).

2.2.5 Other Co-Factors

Since only a minority of HPV infections will eventually lead to cervical cancer, HPV infection alone is not sufficient for cervical cancer development (Walboomers et al.,

1999). Therefore, other factors must act as co-factors by influencing HPV persistence and cancer progression. Factors that have been established as co-factors include long-term use of oral contraceptives (Moreno et al., 2002), smoking (International Collaboration of Epidemiological Studies of Cervical Cancer, 2006), high parity (Munoz et al., 2002), and HIV co-infection (Palefsky & Holly, 2003). Probable co-factors include nutritional deficiencies (Garcia-Closas, Castellsague, Bosch, & Gonzalez, 2005) and other STIs, namely HSV-2 (Smith et al., 2002) and *Chlamydia trachomatis* (Smith et al., 2004).

2.2.6 Pathogenesis of Cervical Cancer

Cervical cancer is a proliferation of abnormal cells of the cervix, the lower part of the uterus. The cancer develops gradually over time, and in most cases, the cells go through a series of precancerous changes over a period of years before they become cancer (zur Hausen, 2002). The process of abnormal cell changes is initially limited to the cervical squamous epithelium, the cells that line the outer cervix. This precancerous overgrowth of cells is referred to as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL), and is classified into a three- or two-tier system. The CIN system has three categories according to the proportion of the epithelial layer showing CIN (Richart, 1973). CIN1 refers to abnormal cells that occupy the lower one third of the cervical epithelium, and is most often indicative of a self-limited HPV infection. CIN2 indicates that two thirds of the cervical epithelium is occupied by abnormal cells, and CIN3 indicates that the entire epithelial layer is occupied. The Bethesda system classifies

SIL as either low-grade SIL (LSIL) or high-grade SIL (HSIL), depending on the proportion of the epithelial layer showing SIL (Solomon et al., 2002). LSIL corresponds to CIN1 and HPV infection, and HSIL corresponds to CIN2, CIN3 and carcinoma *in situ* (CIS).

The development of cervical cancer is a multi-step process. The major steps include HPV infection with a high-risk oncogenic type, persistence of the infection, progression from mild precancerous lesions (LSIL) to the more severe precancerous lesions (HSIL), and eventually cancer (Figure 3). Provided that the latter step has not yet occurred, prior to the development of cancer, this process is reversible, including clearance of HPV infection and regression of precancerous lesions (Ostor, 1993). It is generally accepted that HSIL has a higher likelihood of progression to cancer, so if diagnosed, HSIL are treated. The peak incidence of HSIL in women is between 25 to 29 years of age, a full 10 years earlier than the peak incidence of cervical cancer (Kitchener, Castle, & Cox, 2006; Schiffman & Kjaer, 2003). Based on this, progression to cervical cancer takes 10 to 15 years from the development of HSIL.

2.2.7 Role of HPV Oncoproteins

In the process of HPV-induced carcinogenesis, the HPV genome integrates itself into the host chromosomes, leading to the continued expression of high-risk HPV oncoproteins, E6 and E7 (Munger et al., 2004). These oncoproteins interact with host cellular proteins that play central roles in the regulation of cell growth (zur Hausen, 2002). Consequently,

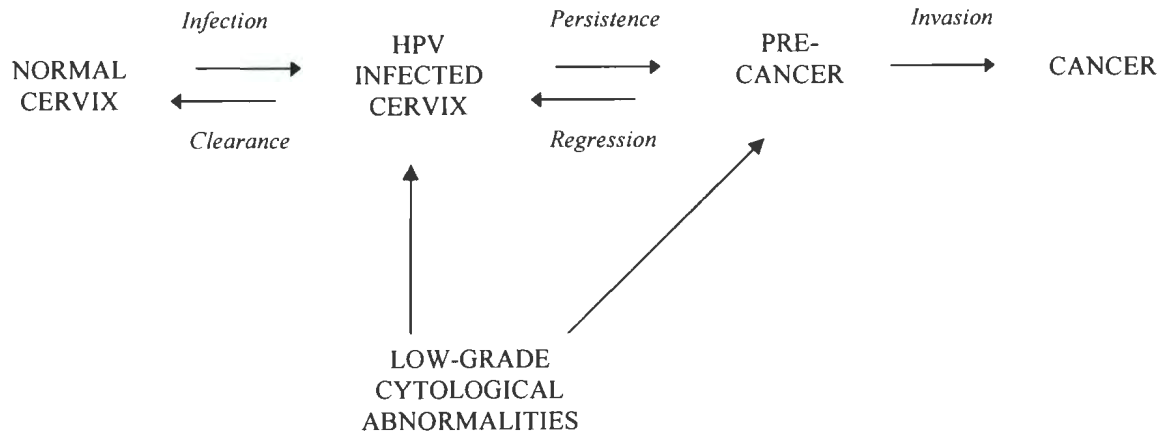

Figure 3: Natural history of cervical cancer

Source: Schiffman & Kjaer, 2003.

the normal cell cycle is disrupted, resulting in genome instability, hyperproliferation, and ultimately immortal cells (Munger & Howley, 2002).

High-risk E6 HPV proteins bind and direct the degradation of the cellular tumor suppressor protein p53 (Scheffner, Werness, Huibregtse, Levine, & Howley, 1990; Werness, Levine, & Howley, 1990). The p53 protein is expressed when the cell experiences stressful conditions, such as DNA damage, low levels of oxygen and nucleotide depletion or depletion of products or processes dependent on nucleotides (Graeber et al., 1994; Linke, Clarkin, Di Leonardo, Tsou, & Wahl, 1996). The major events induced by p53 are cell growth arrest in order to allow DNA repair and survival or, elimination of cells with abnormal growth properties through programmed cell death (Munger & Howley, 2002). As a result of the disruption in the function of p53 in the cell by the E6 protein, cell cycle arrest and programmed cell death are not possible. This leads to impairment of DNA repair mechanisms, accumulation of DNA mutations, leading to cellular transformation and ultimately malignancy (Gu, Pim, Labrecque, Banks, & Matlashewski, 1994; Havre, Yuan, Hedrick, Cho, & Glazer, 1995).

E7 proteins of high-risk HPVs bind to, and inactivate, cellular tumor suppressor protein pRb (Dyson, Howley, Munger, & Harlow, 1989). The pRb protein acts as a regulator in cells about to progress into DNA replication by binding to E2Fs, a family of transcription factors that stimulate genes required for DNA replication and entry into the phase of replication (Weinberg, 1995). E7 binds to pRb, resulting in the release of E2Fs, causing

unscheduled progression through the cell cycle and leading to cell proliferation (Munger et al., 2001; zur Hausen, 2000).

2.3 Screening

Screening is defined as population-based testing of apparently healthy individuals in order to classify them as likely or unlikely to have a certain disease (Last, 2001). People identified to be at risk for the disease are further investigated through diagnostic tests. Those who are found to have disease are then treated. The goals of reducing morbidity and mortality among the screened are achieved by early diagnosis and treatment. To be suitable for screening, a disease has to go through a phase during which it would be detectable but unnoticed if not investigated. Further, the treatment should provide benefits as a result of detecting cases at an early stage. As was stated earlier, this is the case with cervical cancer screening.

Sensitivity is the ability of the test to detect women who have a significant abnormality in the cervix while specificity is the ability of the test to correctly identify normal women. Both are important in screening programmes; the higher the quality of the test, the higher its sensitivity and specificity. Unfortunately, measures taken to increase sensitivity (reduce false negatives) often result in decreasing specificity (increasing false positives). Therefore, attempts to reduce the number of false negatives may lead to more normal women being recalled for repeat further testing.

2.3.1 Cytology

2.3.1.1 Conventional Cytology

The conventional Pap test has been regarded as the most successful screening tool for cancer in the history of medicine. Developed by Dr. George Papanicolaou in the 1920s (Vilos, 1999), the conventional Pap test involves taking a sample of cells from the lining of the cervix with a wooden spatula or a plastic brush. The cells are then transferred to a slide, stained and examined under a microscope to establish the presence or absence of abnormal cells. As this test is a screening tool, and not a diagnostic test, subsequent confirmation of these abnormalities is done by diagnostic histological examination of tissue biopsy via a colposcopic examination. These changes are often caused by HPV.

The Pap test reporting classification has changed over time. Currently used in North America, the Bethesda System for classification and reporting of abnormal squamous and glandular cervical cytology was developed in 1988 (National Cancer Institute, 1989) and revised in 2001 (Table 3) (Soloman et al. 2002). It groups squamous cell abnormalities into four categories: atypical squamous cells (ASC), LSIL, HSIL, and cancer (Table 3). The ASC category is subdivided into 2 categories: ASC of unknown significance (ASCUS) and ASC in which high-grade lesions cannot be excluded (ASC-H). ASCUS may represent reactive changes that mimic, but are unrelated to, cellular changes caused by HPV, and HPV-associated changes, but are not distinguishable as a LSIL. ASC-H includes cellular changes that are suggestive of HSIL, but are lacking in the criteria to categorize it as such. As stated earlier, LSIL refers to abnormal cells that occupy the

Table 3: The 2001 Bethesda classification system for cervical squamous cell abnormalities

Result	Interpretation
ASCUS (Atypical squamous cells of undetermined significance)	Squamous cells are abnormal, but may or may not be precancerous
ASC-H (atypical squamous cells, cannot exclude HSIL)	Squamous cells are abnormal, but may or may not be HSIL
LSIL (low-grade squamous intraepithelial lesions)	Mildly abnormal squamous cells; changes are almost always due to temporary HPV infection
HSIL (high-grade squamous intraepithelial lesions)	Moderately to severely abnormal squamous cells
Cancer	Possibility of cancer cells present in the cervix is very high

Source: Solomon et al., 2002.

lower one third of the cervical epithelium, and is most often indicative of a self-limited HPV infection (Solomon et al., 2002). HSIL refers to abnormal cells that occupy the lower two thirds or the entire cervical epithelium.

2.3.1.2 Limitations of Conventional Cytology

Although the conventional Pap test is important in its place as the most widely used cancer screening test in the world, and its impact on the incidence of cervical cancer, cervical screening, by conventional cytology, has considerable limitations. The sample must be representative of the cells that line the cervix. Often, an inadequate cell sample is taken, and/or the cells are not properly transferred or preserved on the slide. Also, the ability to fully evaluate the slide due to obscuring material, such as blood, mucus, overlapping cells, or inflammation, can also be a source of error. As well, the interpretation of changes in the cells is very subjective and poorly reproducible, even among expert cytologists (Stoler & Schiffman, 2001). Consequently, a wide range of false-negative and false-positive results has been reported (Cuzick et al., 2006; Fahey et al., 1995; Nanda et al., 2000), which indicates that Pap screening can fail by under- or over-diagnosis. A meta-analysis of 62 studies conducted between 1984-92, reported the mean sensitivity was 58% (range = 11-99%) and mean specificity 68% (range = 14-97%) (Fahey et al., 1995). In a more recent meta-analysis, Nanda et al. (2000) found the sensitivity of conventional cytology to be 47% and to range from 30-87%, while specificity was 95% ranging from 86-100%.

2.3.1.3 Liquid-Based Cytology

In order to increase the sensitivity and specificity of cervical screening, alternative approaches have been proposed. Liquid-based cytology (LBC) is an improved method of preparing cervical samples for cytological examination. Two technologies are available: SurePath (BD TriPath Imaging Inc, Burlington, North Carolina, USA) and ThinPrep (Halogic Cytyc Corporation, Boxborough, Massachusetts, USA). It is a modification of the conventional Pap test, in which slides are not prepared at the time of collection. However, specimens are collected like a conventional Pap test. The ability to interpret the slide is improved because this approach ensures better specimen yield, the cells are more representative and randomized, and there is less obscuring material such as blood, mucus and inflammation. This leads to a better quality smear, therefore reducing the number of unsatisfactory reports (Karnon et al., 2004). Several smears can be made and tested from one sample and the residual fluid is also suitable for ancillary testing, such as those for HPV DNA and other STIs. However, despite these advancements in cytological screening for cervical cancer, LBC is still limited by moderate sensitivity, low reproducibility, and the subjective nature of the interpretation of results.

2.3.2 HPV DNA Testing

HPV DNA testing relies on molecular techniques to detect HPV in cervical specimens. There are currently two techniques available to test for the majority of high-risk HPV types responsible for the development of cervical cancer.

The first category is the signal-amplified nucleic assay, and to date, the Hybrid Capture II (HC-II) assay (Digene Corporation, Gaithersburg, Maryland, USA) is the most extensively used. It tests for 13 of the 15 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68. The HPV DNA test is a molecular test to detect HPV DNA in infected cells, in which RNA probes for target HPV DNA are hybridized in solution with the sample DNA.

The second category is the target-amplified assay, such as the polymerase chain reaction (PCR) technique. This technique produces highly concentrated samples of specific HPV DNA sequences which are then probed to identify the HPV types present. These types of assays require very small amounts of cervical specimen to detect HPV DNA and in fact can identify as few as 10-100 copies of HPV genome.

Unlike the Pap test, HPV DNA testing is objective and highly reproducible (Castle et al., 2002). Since HPV is the necessary cause of cervical cancer, screening for HPV specifically is significantly more sensitive than the Pap test to detect pre-cancerous lesions and cervical cancer (Bigras & de Marval, 2005; Clavel et al., 2001; Cuzick et al., 2006; Cuzick et al., 2003; Mayrand et al., 2007; Ratnam et al., 2000; Schiffman et al., 2000). A recent meta-analysis assessed the value of HPV DNA testing compared to the Pap test in countries in North America and Europe with well-established cytology-based screening programmes (Cuzick et al., 2006), using histology as the gold standard. Their conclusion was that the sensitivity of cytology for detecting underlying HSIL or cancer

was substantially less than for HPV DNA testing with considerable variation between studies. The overall sensitivity of cytology was 53% (95% CI = 48.6-57.4) with a wide range from 18.6% to 76.7%, reflective of the interpretative nature of the test. HPV DNA testing was consistently very sensitive in all studies, with an overall sensitivity for HSIL or worse of 96.1% (95% CI = 64.2-97.4). However, the overall specificity of HPV DNA testing was lower than that of cytology (90.7%; 95% CI = 90.4-91.1 and 96.3%; 95% CI = 96.1-96.5 respectively). A recently published Canadian study of 10,154 women also suggests that HPV DNA testing has greater sensitivity for detecting HSIL, as compared to Pap testing (Mayrand et al., 2007). Again, using histology as the gold standard, the authors found that the sensitivity of HPV DNA testing was 94.6% (95% CI = 84.2-100), while the sensitivity of Pap testing was 55.4% (95% CI = 33.6-77.2). The specificity of HPV DNA testing was 94.1% (95% CI = 93.4-94.8), which was lower than that of Pap testing (96.8%; 95% CI = 96.3-97.3).

2.3.2.1 Clinical Applications of HPV DNA Testing

Considering the limitations of Pap cytology and the fact that HPV is present in virtually all cervical cancers and pre-cancerous lesions, it has been suggested that detection of high-risk HPV could be useful in three clinical applications: a) as a primary screening test used alone or in combination with a Pap test to detect pre-cancerous lesions; b) as an adjunct test in triage of low-grade cytological abnormalities to identify women who need referral for diagnosis and treatment; and c) as a follow-up test for women who have been treated to predict cure or failure of treatment (Cuschieri & Cubie, 2005). The most

recommended clinical application of HPV testing at this time is in triage of specific low-grade cytological abnormalities, namely ASCUS.

2.3.3 Low-Grade Cytological Abnormalities

Low-grade cytological abnormalities, as interpreted by the Pap test, are the most common Pap abnormalities (Solomon et al., 2002). They include ASCUS and LSIL. As described previously, ASCUS is an equivocal result that may encompass both reactive changes that mimic, but are unrelated to, HPV and HPV-associated cell abnormalities but fall below the diagnostic threshold for a definitive diagnosis of LSIL. It is difficult to reliably distinguish between the two conditions (Pitman, Cibas, Powers, Renshaw, & Frable, 2002; Stoler & Schiffman, 2001; Sherman et al., 1994). It is the most common cytological abnormality, accounting for up to two-thirds of all reported Pap abnormalities (Solomon et al., 2002). LSIL results represent low-grade cytological abnormalities that are due to HPV infections that most often resolve spontaneously. However, because the Pap test is a screening tool, and not a diagnostic test, a small proportion of women with these low-grade cytological abnormalities results will have underlying HSIL or cancer by histology (Davey, Woodhouse, Styer, Stastny, & Mody, 2000). It has been reported that approximately 7% of women with ASCUS Pap results and 15% of women with LSIL Pap results have underlying HSIL or cancer (Davey et al., 2000; Kinney et al., 1998; Shlay et al., 2000). Because the prevalence of low-grade cytological abnormalities is the highest of all abnormal Pap categories, they are the source of the majority of histologically confirmed HSIL and cancer. A substantial proportion of HSIL and cancer occurs among

women presenting with these low-grade Pap results (Kinney et al., 1998). This creates a dilemma for clinical management; follow-up or further testing is necessary to identify those at greatest risk; however, it should not result in over referring and over diagnosing, leading to increasing costs and patient anxiety.

2.3.3.1 Management Options

The management of women with ASCUS and LSIL is problematic because only a small proportion will have or progress to HSIL and cancer. Until recently, management options of women with low-grade cytological abnormalities were limited to immediate referral to colposcopy or repeat Pap testing at four to six month intervals until two consecutive normal results were obtained, with immediate colposcopy if ASCUS or more significant cytologic abnormalities were reported on any subsequent tests (Stuart et al., 2004). Both options require repeated clinic visits, patient adherence, and represent poor resource utilization and potential for unnecessary treatment, not to mention considerable anxiety. HPV DNA testing for the triage of these women has been a subject of great interest in the last decade (Arbyn et al., 2004; Arbyn et al., 2005; ALTS Group, 2000; Manos et al., 1999; Schiffman & Adrianza, 2000; Solomon et al., 2001).

2.3.3.2 Triage

Since the evolution of HPV DNA testing, many studies have evaluated its role in the triage of women with low-grade cytological abnormalities. However, most notably, two

landmark studies have contributed substantially to our knowledge of the value of HPV DNA testing in the follow-up of low-grade cytological abnormalities.

In the first study, Manos et al. (1999) carried out an observational study in Northern California comparing HPV DNA testing to repeat Pap testing in a sample of 995 women with ASCUS. All women had specimens taken for a repeat Pap test and HPV DNA testing, followed by a colposcopically-directed biopsy to confirm the diagnoses. The gold standard was a histological diagnosis of HSIL or cancer, as the current clinical practice is to treat histologically confirmed HSIL, in addition to cancer. The sensitivity of the HPV DNA test was 89.2% for detection of underlying HSIL or cancer. This was higher than repeat Pap testing at an ASCUS threshold of referral, which had a sensitivity of 76.2%. However, the specificity of HPV DNA testing was similar to that of repeat cytology (64.1% and 63.8%, respectively). It was estimated that triage based on HPV DNA testing or on repeat Pap testing with referral to colposcopy set at a repeat Pap result interpreted as ASCUS or more severe, would have resulted in approximately the same number of referrals for colposcopy (40%). The authors concluded that for women with ASCUS Pap results, a single HPV DNA test can help identify the majority of women with underlying HSIL or cancer, thereby replacing the practice of repeated cytology following and ASCUS diagnosis.

In the second study, The National Cancer Institute of America initiated the ASCUS LSIL Triage Study (ALTS) to evaluate the management of women with ASCUS or LSIL Pap

results (Schiffman & Adrianza, 2000). It is the largest study, to date, examining HPV DNA testing as a way of triaging women with low-grade cytological abnormalities. ALTS compared three management strategies, namely referral for immediate colposcopy (considered to be the reference standard), HPV DNA triage with referral if positive, or triage based on repeat Pap testing with referral set at a repeat Pap result of ASCUS or greater. They enrolled 3,488 eligible women with ASCUS and 1,572 women with LSIL, and randomized them to one of the three management strategies. The gold standard was histologically confirmed HSIL or cancer. In women with ASCUS cytology, the sensitivities of immediate colposcopy, HPV DNA triage, and repeat Pap were 100, 95.9, and 85.0%, respectively (Solomon et al., 2001). Compared to all women being sent to immediate colposcopy, just over half (56.1%) of the women having HPV DNA testing would have been referred to colposcopy, and 58.6% of the women having a single repeat Pap test would have been referred. The HPV DNA test showed a greater sensitivity for detection of histologically confirmed HSIL or cancer than a single repeat Pap at a threshold for referral of ASCUS or worse (95.9% and 85.0%, respectively), and a comparable specificity (48.4% and 44.7%, respectively). The authors concluded that HPV DNA testing is an option for managing women with ASCUS to determine if colposcopy is warranted. Nevertheless, the referral rate of women by HPV DNA testing is still high compared to the low percentage of true HSIL diagnosed in women with ASCUS, but is better than that of repeat cytology with the need for multiple repeat visits and multiple costs associated with further testing.

In women with LSIL Pap results, HPV DNA was detected in 82.9% (ALTS Group, 2000). Despite the sensitivity of this approach, the high prevalence of HPV DNA in this group would not reduce referrals, clearly limiting its practicality and cost-effectiveness as a triage test. The authors concluded that HPV DNA testing would be ineffective in triage. The ALTS Group (2003b) also came to the same conclusion that repeat cytology was not justified, as over 80% of women at the ASCUS threshold would have been referred. Direct referral to colposcopy was suggested as the best management option for this group of women (ALTS Group, 2000; ALTS Group, 2003b).

A recent systematic review of 10 published studies also came to the conclusion that due to the low specificity (28.8%; 95% CI = 22.0-36.0) of the HPV DNA test in women with LSIL cytology, as a result of the high HPV positivity (77.2%), the test would not be effective (Arbyn et al., 2005). However, in a study conducted in Israel of 503 women, Fait et al. (2000) reported that a positive HPV DNA test result had a sensitivity of 88.2% and a specificity of 94.7% for detecting histologically confirmed HSIL in women with two consecutive LSIL Pap test results. They concluded that HPV DNA testing could have a place in the triage of these women, but suggested that the test be used after one cytology result of LSIL rather than two.

A recent meta-analysis of 8 published studies also examined HPV DNA testing compared with repeat Pap testing for triage of ASCUS Pap results (Arbyn et al., 2004). It was concluded that HPV DNA testing has a significantly higher sensitivity than repeat Pap

cytology at a referral threshold of ASCUS (94.8%; 95% CI = 92.7-96.9 and 81.8%; 95% CI = 73.5-84.3 respectively) for detection of HSIL and cancer, while maintaining similar specificity (67.3%; 95% CI = 58.2-76.4 and 57.6%; 95% CI = 49.5-65.7 respectively).

In one of few Canadian studies conducted in this area, Lytwyn et al. (2000) examined HPV DNA testing and repeat Pap testing in 212 women from Ontario with ASCUS and LSIL. They found the HPV DNA test to be more sensitive (87.5%) than repeat Pap testing (55.6%) at an ASCUS threshold for referral in these women. However, they reported the performance of the tests without distinguishing between women with ASCUS or LSIL.

Kim, Wright, & Goldie (2002) reported a modeling analysis of immediate colposcopy, HPV DNA testing, and repeat Pap cytology for managing ASCUS cytology. They came to the conclusion that HPV triage is more cost-effective than repeat Pap cytology or colposcopy, while maintaining the same health benefits as immediate colposcopy. However, even in the context of ASCUS cytology and HPV DNA testing, the specificity is somewhat low. Only about one quarter of women with ASCUS cytology and who are HPV positive will have underlying HSIL.

Because the specificity of the HPV DNA test is relatively low, it is a priority to identify strategies that could be used to improve it. Since HPV prevalence varies with age, the specificity of the HPV DNA test in triage depends on age (Arbyn et al., 2006).

Therefore, using age-restricted HPV DNA testing could possibly improve specificity, as well as significantly reduce the number of referrals to colposcopy, while maintaining test sensitivity. A few studies have examined the performance of HPV DNA testing, stratified by age. The ALTS data were further analysed by age group to compare test performance (Sherman et al., 2002). In women with ASCUS, HPV prevalence was much lower in women older than 28 years of age (31.2%) compared with younger women (more than 65%), which could represent a significant cost saving in referrals. The sensitivity of the HPV test varied minimally between age groups (range, 93.9% to 97.8%). In women with LSIL, more than 74% would have been referred regardless of the age group under which they fell. In another study, Shlay et al. (2000) compared the performance of HPV DNA testing in women with ASCUS in two age categories. In women 30 years of age and older, only 20.2% would have been referred to colposcopy, compared to 48.7% of women younger than 30 years of age. The sensitivity was somewhat lower in older women (85.7% in women ≥ 30 years and 100% in women < 30 years, respectively). The specificity of HPV DNA testing was significantly higher in older women (83.9%) versus younger women (57.4%) ($P < 0.01$). However, if HPV DNA testing had been restricted to women 30 years and older, 8 women younger than 30 years of age with underlying HSIL would have been missed.

2.3.3.3 HPV Triage Recommendations for Low-Grade Cytological Abnormalities

The Pan-Canadian Forum on Cervical Cancer Prevention and Control in 2004 recommended that HPV testing should be used to triage women 30 years and older with

ASCUS cytology (Stuart et al., 2004). No recommendation was given for women with LSIL cytology with respect to HPV DNA testing, but it was recommended that a national consensus management algorithm be developed. Canadian Consensus Guidelines on HPV published in 2007 also state that HPV DNA testing is recommended for women aged 30 years or more with ASCUS cytology (Provencher & Murphy, 2007). However, it is recommended that HPV testing should not be done on LSIL cytology.

Largely based on the ALTS-trial findings, the American Society for Colposcopy and Cervical Pathology (ASCCP) Concensus Conference 2006 recommended that a programme of repeat Pap tests, colposcopy, or HPV DNA testing are all acceptable methods of management for ASCUS cytology (Wright et al., 2007). However, when reflex HPV DNA testing is available, it is the preferred approach, as it makes a second clinic visit unnecessary. For women with LSIL cytology, colposcopy is the recommended management option.

CHAPTER III

MATERIALS AND METHODS

3.1 Study Population and Data Collection

The Health Sciences Centre in St. John's, Newfoundland, currently operates a referral colposcopic clinic. The gynecologists regularly see women who are referred with abnormal Papanicolaou (Pap) reports. These gynecologists routinely use the human papillomavirus (HPV) deoxyribonucleic acid (DNA) test as part of their standard patient care, in addition to repeat Pap tests, colposcopies, and biopsies.

In the present study, data were collected on 447 women with low-grade Pap abnormalities, namely atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSIL), referred to the above clinic during the period of November 2003 to March 2006. Data were systematically collected on the initial Pap abnormality that prompted referral, the results of the HPV DNA test and repeat Pap test, along with histology, if biopsies were taken. Cervical specimens for HPV DNA testing and Pap testing were either co-collected using separate cytobrushes, or collected in liquid-based cytology (LBC) with reflex testing. All data were retrieved from the Meditech laboratory information system (Boston). Data analysis was limited to those women who had HPV DNA testing and repeat Pap testing at the colposcopy clinic, and for whom histology results were available within one year of follow-up.

3.2 Ethics

Ethics approval for the present study was obtained from the Memorial University of Newfoundland Faculty of Medicine, Human Investigation Committee (Appendix A). All files are kept in a locked storage cabinet in a locked room. All computer files are password protected.

3.3 Cytology

All women had a conventional Pap test done within one year prior to referral, performed by community physicians. These baseline smears were interpreted in four different cytology laboratories in Newfoundland, and were used as the prompting referral Pap smear diagnoses.

The repeat Pap specimens at referral were collected using either conventional Pap test methods or LBC. In the conventional Pap test method, a cervical sample was taken with a wooden spatula and/or cytobrush. The cells were then directly transferred to a glass slide and immediately sprayed with an alcohol fixative. The collection device was discarded and the sample was sent to the regional cytology laboratory, Eastern Health, in St. John's, where it was processed and interpreted by qualified cytotechnologists and pathologists.

For the LBC portion of the study, SurePath System (BD TriPath Imaging Inc, Burlington, North Carolina, USA) was used. Cervical samples were collected using a plastic broom

and placed into a vial of SurePath preservative fluid. The collection devices were detached from the handle and were left in the vial. The vial was then sent to the regional cytology laboratory in St. John's for processing. In the laboratory, the preserved sample was mixed by vortexing. The cell suspension was then layered onto a density reagent in a centrifuge tube; centrifugation of the suspension removed debris, mucus, and excess inflammatory cells from the sample, producing a concentrated pellet of cells. After centrifugation, the pelleted cells were resuspended, mixed and transferred to a settling chamber mounted on a glass slide. The cells were sedimented by gravity, stained and examined under a microscope. The residual sample was forwarded to the Public Health Laboratory for HPV DNA testing. Technologists at the regional cytology laboratory performed all cytology. Cervical cytology results were classified according to the 2001 Bethesda System (Solomon et al., 2002) by qualified cytotechnologists and pathologists.

3.4 HPV DNA Testing

HPV DNA testing was done using the Hybrid Capture II (HC-II) test (Digene Corporation, Gaithersburg, Maryland, USA). For this, cervical specimens collected in either the Digene specimen transport medium (STM) or the leftover SurePath media were used. Specimens collected in the SurePath media were validated for use for HPV DNA testing. STM specimens were processed according to the manufacturer's instructions for HPV testing. SurePath specimens were centrifuged in STM and processed as in the STM protocol.

The HC-II assay detects the presence of DNA by ribonucleic acid (RNA)-DNA hybridization technology using an RNA probe cocktail. The RNA probe cocktail recognizes a wide range of high- and low-risk HPV types (high-risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68; low-risk: 6, 11, 42, 43, 44). The study used the high-risk probe cocktail to detect 13 high-risk types. The test procedure is as follows: The DNA was extracted and denatured. The RNA probes then hybridized in solution with the denatured target HPV DNA from the specimen. The DNA-RNA hybrids were subsequently bound to the surface of a micro plate well, which is coated with antibodies specific for DNA-RNA hybrids. HPV positive wells were detected with alkaline phosphatase-conjugated antibodies specific for DNA-RNA hybrids in combination with a chemiluminescent substrate. The light emitted is measured in relative light units (RLU) using a plate luminometer. The intensity of light measured is proportional to the amount of target DNA in the specimen. A positive test was defined as an RLU measurement of 1 pg/mL HPV DNA. That level corresponds to approximately 5000 genomic copies of HPV DNA in the test. Samples with less than 1 pg/mL oncogenic HPV DNA indicate the specimen is negative for the 13 HPV DNA types included in the test or that the HPV DNA levels for these 13 types is below the detection threshold of this assay. A positive result indicates the presence of at least one of the types of HPV in the panel, but does not specify which of the types are present. Technologists at the Public Health Laboratory, St. John's, tested all specimens.

3.5 Histology

Histology was performed at the discretion of the managing clinicians. Standard processing included three levels for each biopsy and further testing as deemed appropriate by the pathologist. Interpretation of hematoxylin and eosin slides was given by qualified pathologists. In assessing the performance of the Pap and HPV tests, histological diagnoses were used as the gold standard. We used squamous intraepithelial lesion (SIL) nomenclature to describe histological outcomes (Solomon et al., 2002). We considered only the clinical outcome of high-grade squamous intraepithelial lesions (HSIL) or worse, as it is standard practice to treat a histological diagnosis of HSIL or more severe (Wright et al., 2007).

3.6 Data Analysis

This analysis examines the performance of repeat Pap cytology and HPV DNA testing using a histological diagnosis of HSIL or worse as the primary study endpoint. Data for the women with ASCUS and LSIL referral Pap test results were analysed separately.

3.6.1 Definition of Variables

Cervical Cytology – A categorical variable reported in this format.

Negative	Negative for malignancy
ASCUS	Atypical Squamous Cells of Undetermined Significance
ASC-H	Atypical Squamous, Cannot Exclude HSIL

LSIL	Low-grade Squamous Intra-epithelial Lesion
HSIL	High-grade Squamous Intra-epithelial Lesion

The variable was dichotomized for analysis of performance indicators in two different ways. The performance of repeat Pap cytology was evaluated at two thresholds for colposcopy referral: 1) ASCUS or more severe interpretation and 2) LSIL or more severe interpretation.

HPV DNA – A categorical variable reported as a semi-quantitative measure in RLU by the laboratory.

Negative	Negative or below detection threshold for all of 13 high-risk types listed earlier.
Positive	≥1.0 RLU.

HPV DNA testing was assessed at one threshold for a positive result: ≥1.0 pg/mL.

Histology - A categorical variable reported in this format.

Negative	Negative for malignancy
LSIL	Low-grade Squamous Intra-epithelial Lesion
HSIL	High-grade Squamous Intra-epithelial Lesion

The variable was dichotomized as follows for analysis:

Negative	≤ LSIL
Positive	≥ HSIL

However, because there were no cases of cancer, we refer to the primary end-point as HSIL as there is a general consensus that this SIL has a strong risk for progression to cancer and therefore requires treatment (Wright et al., 2007).

Age – Provided by the laboratory and categorized according to the format that is commonly reported in the literature and the recommended age threshold for HPV triage in women with ASCUS cytology by the Pan-Canadian Forum on Cervical Cancer Prevention and Control (Stuart et al., 2004) and the Canadian Consensus Guidelines on Human Papillomavirus (Provencher & Murphy, 2007).

1	Age < 30
2	Age ≥ 30

3.6.2 Statistical Methods

Statistical analyses were performed using SPSS® and SAS® software packages (SPSS, version 13.0 and SAS, version 9.1). Conventional 2 x 2 contingency tables were compiled and analysed to assess the association between categorical variables. For these analyses, the Pearson chi-square test was used, and in cases where the expected cell count for at least one cell was less than five, Fisher's exact test was used. Performance indicators of sensitivity, specificity, positive predictive value (PPV) and negative

predictive value (NPV) for detecting histologically confirmed HSIL based on repeat cytology and HPV DNA testing were calculated using the conventional 2 x 2 contingency tables, with a 95% confidence interval (CI) around the estimates. Because the study sample size was small, 95% exact CIs based on binomial probabilities were calculated (Deeks & Altman, 1999). If CIs did not overlap, differences between proportions were considered statistically significant. All statistical tests were two-sided and the values $P \leq 0.05$ were regarded as statistically significant.

CHAPTER IV

RESULTS

4.1 Introduction

In this retrospective cohort study, we collected data on women who were referred to a colposcopy clinic in St. John's with a prompting Papanicolaou (Pap) test result of atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSIL). During the study period, data were collected from a total of 447 women, representing 186 referred to the clinic with ASCUS cytology and 261 with LSIL cytology. Since not all women had a human papillomavirus (HPV) deoxyribonucleic acid (DNA) test, for further analyses we considered only those that had a valid HPV DNA test. Study inclusion criteria also entered only those with a repeat Pap test at enrollment and histology within a year of enrollment. This reduced the total numbers evaluated to 242, 97 women with ASCUS and 145 with LSIL, and this constituted the study population. The study analyzed the data to explore the association of HPV, repeat cytology and histology results in these women. The study also analyzed the data to assess the performance of HPV DNA testing in triage of women with low-grade cytological abnormalities, comparing it with that of repeat Pap testing, while determining if age affects test performance.

In the 97 women referred with an ASCUS result, the mean age was 36.3 years (ranging from 18 to 64), 36.1% were under 30 years of age, and 63.9% were 30 years of age and

older. In the 145 women referred with an LSIL result, the mean age was 27.8 years old (range 16 to 58), 70.3% were under 30 years of age, and 29.7% were 30 years of age and older.

4.2 HPV Prevalence

The overall HPV prevalence in women referred with ASCUS was 44.3% (43/97). When stratified by age, in women referred with an ASCUS Pap, HPV prevalence was significantly higher among women less than 30 years of age (74.3%) as compared with those beyond that age (27.4%) ($P < 0.001$). The prevalence of HPV among women referred to the colposcopy clinic with a baseline Pap of ASCUS or LSIL, stratified by age, is shown in Table 4. In women referred with LSIL cytology, 79.3% (115/145) tested positive for HPV. There was no significant difference in the prevalence of HPV among younger and older women referred with LSIL when stratified by age ($P = 0.065$). However, the prevalence of HPV was significantly higher in women with a baseline Pap result of LSIL (79.3%) than in women with a baseline Pap result of ASCUS (44.3%) ($P < 0.001$).

4.3 Association of HPV with Repeat Pap Cytology

The association between HPV and the repeat Pap test result for the 97 women referred with ASCUS cytology is presented in Table 5. Although all of these women had an ASCUS Pap in the community-based routine Pap screening, the repeat Pap test results at referral were heterogeneous. The repeat Pap test was negative in 59 (60.8%) women,

Table 4: Association between baseline Pap and HPV result

Baseline Pap	HPV result		Total
	Positive	Negative	
Age <30 years			
ASCUS	26 (74.3%)	9 (25.7%)	35
LSIL	85 (83.3%)	17 (16.7%)	102
Age ≥30 years			
ASCUS	17 (27.4%)	45 (72.6%)	62
LSIL	30 (69.8%)	13 (30.2%)	43
All ages			
ASCUS	43 (44.3%)	54 (55.7%)	97
LSIL	115 (79.3%)	30 (20.7%)	145

ASCUS = Atypical squamous cells of undetermined significance; HPV = Human papillomavirus; LSIL = Low-grade squamous intraepithelial lesions; Pap = Papanicolaou

$P < 0.001$ from Pearson chi-square test for prevalence of HPV in women ≤29 years versus ≥30 years who were referred with ASCUS.
$P = 0.065$ from Pearson chi-square test for prevalence of HPV in women ≤29 years versus ≥30 years who were referred with LSIL.
$P < 0.001$ from Pearson chi-square test for prevalence of HPV in women with LSIL versus ASCUS.

Table 5: Association between repeat Pap and HPV DNA result in women referred with ASCUS Pap results

Repeat Pap	HPV result		
	Positive (row %)	Negative (row %)	Total (column %)
Negative	11 (18.6)	48 (81.4)	59 (60.8)
ASCUS	11 (78.6)	3 (21.4)	14 (14.4)
LSIL	15 (83.3)	3 (16.7)	18 (18.6)
HSIL	6 (100.0)	0 (0.0)	6 (6.2)
Total	43 (44.3)	54 (55.7)	97 (100.0)

ASCUS = Atypical squamous cells of undetermined significance; DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou

ASCUS in 14 (14.4%), LSIL in 18 (18.6%) and high-grade squamous intraepithelial lesions (HSIL) in 6 (6.2%). The prevalence of HPV increased with the increasing severity of the repeat Pap test. Specifically, 100.0% (6/6) of women with a repeat Pap showing HSIL were HPV positive compared with 83.3% (15/18) of women with a repeat Pap showing LSIL, 78.6% (11/14) with a repeat Pap of ASCUS and 19.0% (11/58) with a negative Pap test. Because the expected cell count for at least one cell was less than five, this trend could not be tested for significance.

Table 6 shows the repeat Pap test results compared with HPV DNA testing results in the 145 women who were referred to colposcopy with LSIL Pap test results. As with the women referred with an ASCUS Pap, the repeat Pap test results were heterogeneous for women who had been referred with an LSIL Pap in the community-based routine Pap screening. Only 37.2% (54/145) were again interpreted as having LSIL. However, the prevalence of HPV increased in parallel with the increasing severity of the repeat Pap test from 60.7% (37/61) in cases with negative Pap test results up to 100% (14/14) among those with a HSIL Pap test. Nevertheless, as with the ASCUS group, this trend could not be tested for significance because the expected cell count for at least one cell was less than five.

4.4 Histological Diagnoses

Histologically confirmed HSIL was present in 16.5% (16/97) and 20% (29/145) of women with ASCUS and LSIL baseline Pap results, respectively (Tables 7 and 8). No

Table 6: Association between repeat Pap and HPV DNA result in women referred with LSIL Pap results

Repeat Pap	HPV result		
	Positive (row %)	Negative (row %)	Total (column %)
Negative	37 (60.7)	24 (39.3)	61 (42.1)
ASCUS	12 (80.0%)	3 (20.0%)	15 (10.3)
LSIL	51 (94.4%)	3 (5.6%)	54 (37.2)
ASC-H	1 (100.0%)	0 (0.0%)	1 (0.7)
HSIL	14 (100.0%)	0 (0.0%)	14 (9.7)
Total	115 (79.3)	30 (20.7)	145 (100.0)

ASC-H = Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASCUS = Atypical squamous cells of undetermined significance; DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou

Table 7: Association between histology and HPV DNA result in women referred with ASCUS Pap results, stratified by age

HPV Result	Histology		Total	Significance*
	No. of women with HSIL	No. of women with ≤LSIL		
All ages				
HPV (+)	16 (37.2%)	27 (62.8%)	43	$P < 0.001$
HPV (-)	0(0.0%)	54 (100.0%)	54	
Age ≥30 yrs				
HPV (+)	8 (47.1%)	9 (52.9%)	17	$P < 0.001$
HPV (-)	0 (0.0%)	45 (100.0%)	45	
Age <30 yrs				
HPV (+)	8 (30.8%)	18 (69.2%)	26	$P = 0.058$
HPV (-)	0 (0.0%)	9(100.0%)	9	

ASCUS = Atypical squamous cells of undetermined significance; DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion

*Fisher's exact test for association of HSIL confirmation with HPV positivity

Table 8: Association between histology and HPV DNA result in women referred with LSIL Pap results, stratified by age

HPV Result	Histology		Total	Significance
	No. of women with HSIL	No. of women with ≤LSIL		
All ages				
HPV (+)	28 (24.3%)	87 (75.7%)	115	$P < 0.05$†
HPV (-)	1 (3.3%)	29 (96.7%)	30	
Age ≥30 yrs				
HPV (+)	9(30.0%)	21(70.0%)	30	$P < 0.05$‡
HPV (-)	0 (0.0%)	13 (100.0%)	13	
Age <30 yrs				
HPV (+)	19 (22.4%)	66 (77.6%)	85	$P = 0.182$‡
HPV (-)	1 (5.9%)	16 (94.1%)	17	

DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion

†Pearson chi-square test for association of HSIL confirmation with HPV positivity
‡Fisher's exact test for association of HSIL confirmation with HPV positivity

cervical cancer was detected in any of the women. After stratification for age in the ASCUS group, histologically confirmed HSIL was found in 12.9% (8/62) of women 30 years of age and older and in 22.9% (8/35) of those younger than 30 years (Table 7). In women with LSIL baseline cytology, 20.9% (9/43) of women 30 years and older and 19.6% (20/102) of younger women were found to have underlying HSIL by histology (Table 8).

4.5 Association of HPV and Histological Diagnoses

Overall, there was a highly significant correlation ($P < 0.001$) between a positive test for HPV and a histological diagnosis of HSIL in women referred to colposcopy with ASCUS (Table 7). In this group, 16.5% (16/97) of the women were found to have underlying HSIL by histology, and HPV was detected in all of these women. Among women aged 30 years and older, a strong association was also observed between HPV positivity and underlying HSIL by histology ($P < 0.001$). However, this association was not statistically significant in women younger than 30 years of age ($P = 0.058$), even though all 8 women with underlying HSIL by histology were positive for HPV.

The association between histological diagnoses and HPV results among women with an LSIL baseline interpretation is shown in Table 8. Twenty-nine (20%) of the women had histologically confirmed HSIL. Among these women, all except one of the 29 cases of HSIL detected by histology occurred in women with an HPV positive result. In total, HPV positivity was associated with a seven-fold increase (24.3% vs. 3.3%) in the

histological diagnosis of HSIL ($P < 0.05$). When stratified by age, a strong association between histologically confirmed HSIL and HPV positivity was also seen in women 30 years of age and older ($P < 0.05$). Among women younger than 30 years of age, the association between a positive HPV DNA test and underlying HSIL by histology was not statistically significant ($P = 0.182$), even though a higher percentage of women who were HPV positive were diagnosed with HSIL histology than those who were HPV negative (22.4% vs. 5.9%).

4.6 Association of Repeat Pap Cytology and Histological Diagnoses

The relationship between repeat Pap testing and histology in women referred with ASCUS is shown in Table 9. With the exception of a repeat Pap test result of ASCUS, the percentage of women with underlying HSIL by histology increased with the increasing severity of the repeat Pap category, from 6.8% in a negative result to 66.7% in HSIL. Because the expected cell count for at least one cell was less than five, this trend could not be tested for significance.

The association between repeat Pap cytology results and histology in women referred with LSIL cytology are presented in Table 10. The percentage of women with underlying HSIL by histology increased with the increasing severity of the repeat Pap interpretation, from a range of 6.7 - 20.4% in negative or low-grade cytology categories to 78.6 – 100% in categories regarded as high-grade cytology. This trend could not be

Table 9: Association between repeat Pap and HSIL confirmation in women referred with ASCUS Pap results

Repeat Pap	Histology		Total
	No. of women with HSIL	No. of women with ≤LSIL	
Negative	4 (6.8%)	55 (93.2%)	59
ASCUS[1]	5 (35.7%)	9 (64.3%)	14
LSIL[1]	3 (16.7%)	15 (83.3%)	18
HSIL[2]	4 (66.7%)	2 (33.3%)	6

ASCUS = Atypical squamous cells of undetermined significance; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou

[1] Regarded as low-grade cytology
[2] Regarded as high-grade cytology

Table 10: Association between repeat Pap and HSIL confirmation in women referred with LSIL Pap results

Repeat Pap	Histology		Total
	No. of women with HSIL	No. of women with ≤LSIL	
Negative	5 (8.2%)	56 (91.8%)	61
ASCUS[1]	1 (6.7%)	14 (93.3%)	15
LSIL[1]	11 (20.4%)	43 (77.8%)	54
ASC-H[2]	1 (100%)	0 (0.0%)	1
HSIL[2]	11 (78.6%)	3 (21.4%)	14

ASC-H = Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; ASCUS = Atypical squamous cells of undetermined significance; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou

[1] Regarded as low-grade cytology
[2] Regarded as high-grade cytology

tested for significance because the expected cell count for at least one cell was less than five.

4.7 Performance of Different Triage Protocols

On the basis of our data, we calculated the performance of three triage protocols in women referred to colposcopy with low-grade cytological abnormalities.

Table 11 summarizes the performance indicators for detecting histologically confirmed HSIL and the percentage of women referred for colposcopy based on HPV DNA testing and repeat Pap cytology according to age for women referred with ASCUS. For all women, the most sensitive triage strategy would have been to refer those positive for HPV. HPV DNA testing detected 100% (95% CI = 79.4-100) of the cases of histologically confirmed HSIL. Repeat Pap testing showing ASCUS or more severe interpretation detected 75% of these cases (95% CI = 47.6-92.7), but this difference did not reach statistical significance. Repeat Pap testing at a higher threshold of LSIL would have resulted in a significantly lower sensitivity (43.8%; 95% CI = 19.8-70.1). The specificity, positive predictive value (PPV) and negative predictive value (NPV) of repeat Pap testing at the two thresholds of referral did not differ significantly from those of HPV DNA testing. All three triage strategies would have referred less than half of the women with ASCUS to colposcopy.

Table 11: Performance of HPV DNA testing and repeat cytology in detecting histologically confirmed HSIL in women with ASCUS Pap results, stratified by age

	HPV DNA testing % (95% CI)	Repeat Pap:≥ASCUS % (95% CI)	Repeat Pap:≥LSIL % (95% CI)
All ages (16 HSILs)*			
Sensitivity	100.0 (79.4-100.0)	75.0 (47.6-92.7)	43.8 (19.8-70.1)
Specificity	66.7 (55.3-76.8)	67.9 (56.6-77.9)	79.0 (68.5-87.3)
PPV	37.2 (23.0-53.3)	31.6 (17.5-48.7)	29.2 (12.6-51.1)
NPV	100.0 (93.4-100.0)	93.2 (83.5-98.1)	87.7 (77.9-94.2)
Referral	44.3 (34.4-54.2)	39.2 (29.5-48.9)	24.7 (16.2-33.3)
Age ≥30 yrs (8 HSILs)			
Sensitivity	100.0 (63.1-100.0)	75.0 (34.9-96.8)	50.0 (15.7-84.3)
Specificity	83.3 (70.7-92.1)	81.5 (68.6-90.8)	87.0 (75.1-94.6)
PPV	47.1 (23.0-72.2)	37.5 (15.2-64.6)	36.4 (10.9-69.2)
NPV	100.0 (92.1-100.0)	95.7 (85.2-99.5)	92.2 (81.1-97.8)
Referral	27.4 (16.3-38.5)	25.8 (14.9-36.7)	17.4 (8.2-27.3)
Age <30 yrs (8 HSILs)			
Sensitivity	100.0 (63.1-100.0)	75.0 (34.9-96.8)	37.5 (8.5-75.5)
Specificity	33.3 (16.5-54.0)	40.7 (22.4-61.2)	63.0 (42.4-80.6)
PPV	30.8 (14.3-51.8)	27.3 (10.7-50.2)	23.1 (5.0-53.8)
NPV	100.0 (66.4-100.0)	84.6 (54.6-98.1)	77.3 (54.6-92.2)
Referral	74.3 (59.8-88.8)	62.9 (46.8-78.9)	37.1 (21.1-53.2)

ASCUS = Atypical squamous cells of undetermined significance; CI = Confidence interval; DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou; PPV = Positive predictive value; NPV = Negative predictive value

*Number of women with histologically confirmed HSIL

When stratified by age, the specificity of a positive HPV DNA test differed considerably between the two age categories of women, whereas the sensitivity and NPV remained the same at 100% (Table 11). In women younger than 30 years of age, the HPV DNA test revealed a significantly lower specificity than in older women (33.3%; 95% CI = 16.5-54.0 and 83.3%; 95% CI = 70.7-92.1 respectively). Among these older women, only 27.4% (95% CI = 16.3-38.5) would have been referred for colposcopy, a difference of more than 45% compared with women younger than 30 years (74.3%; 95% CI = 59.8-88.8). For repeat cytology at both referral thresholds, sensitivity, NPV and PPV did not differ significantly between the two age categories. However, specificity of repeat cytology of ASCUS or worse was significantly higher in older women compared with those younger than 30 years (81.5%; 95% CI = 68.6-90.8 and 40.7%; 95% CI = 22.4-61.2 respectively).

Table 12 summarizes the performance indicators of HPV DNA testing and repeat Pap testing for women referred with LSIL and stratified by age. The most sensitive triage strategy for all women was HPV DNA testing, correctly identifying 96.6% (95% CI = 82.2-99.9) of women with underlying HSIL by histology. However, the difference in sensitivities between the three triage protocols did not reach statistical significance. The specificity of HPV DNA testing was significantly lower than repeat Pap cytology at both thresholds for referral. The PPV and NPV varied minimally among the triage protocols, while HPV DNA testing would have required the referral of significantly more women than repeat Pap cytology.

Table 12: Performance of HPV DNA testing and repeat cytology in detecting histologically confirmed HSIL in women with LSIL Pap results, stratified by age

	HPV DNA testing % (95% CI)	Repeat Pap:≥ASCUS % (95% CI)	Repeat Pap:≥LSIL % (95% CI)
All ages (29 HSILs)*			
Sensitivity	96.6 (82.2-99.9)	82.8 (64.2-94.2)	79.3 (60.3-92.0)
Specificity	25.0 (17.4-33.9)	48.3 (38.9-57.7)	60.3 (50.8-69.3)
PPV	24.3 (16.8-33.2)	28.6 (19.2-39.5)	33.3 (22.4-45.7)
NPV	96.7 (82.8-99.9)	91.8 (81.9-97.3)	92.1 (83.6-97.1)
Referral	79.3 (72.7-85.9)	57.9 (49.9-66.0)	47.6 (39.5-55.7)
Age ≥30 yrs (9 HSILs)			
Sensitivity	100.0 (66.4-100.0)	100.0 (66.4-100.0)	100.0 (66.4-100.0)
Specificity	38.2 (22.2-56.4)	44.1 (27.2-62.1)	52.9 (35.1-70.2)
PPV	30.0 (14.7-49.4)	32.1 (15.9-52.4)	36.0 (18.0-57.5)
NPV	100.0 (75.3-100.0)	100.0 (78.2-100.0)	100.0 (81.5-100.0)
Referral	69.8 (56.0-83.5)	65.1 (50.9-79.4)	58.1 (43.4-72.9)
Age <30 yrs (20 HSILs)			
Sensitivity	95.0 (75.1-99.9)	75.0 (50.9-91.3)	70.0 (45.7-88.1)
Specificity	19.5 (11.6-29.7)	50.0 (38.8-61.3)	63.4 (52.1-73.8)
PPV	22.4 (14.0-32.7)	26.8 (15.8-40.3)	31.8 (18.6-47.6)
NPV	94.1 (71.3-99.9)	89.1 (76.4-96.4)	89.7 (78.8-96.1)
Referral	83.3 (76.1-90.6)	54.9 (45.2-64.6)	43.1 (33.5-52.7)

ASCUS = Atypical squamous cells of undetermined significance; CI = Confidence interval; DNA = Deoxyribonucleic acid; HPV = Human papillomavirus; HSIL = High-grade squamous intraepithelial lesion; LSIL = Low-grade squamous intraepithelial lesion; Pap = Papanicolaou; PPV = Positive predictive value; NPV = Negative predictive value

* Number of women with histologically confirmed HSIL

When stratified by age, the sensitivity and NPV of all three triage protocols was 100% among women 30 years of age and older, with statistically non-significant declines in younger women (Table 12). The specificity of the HPV DNA test was higher in older women, but it was not significantly different from younger women. Based on HPV DNA testing, colposcopy referrals would have declined only slightly among women 30 years of age and older, from 83.3% (95% CI = 76.1-90.6) in younger women to 69.8% (95% CI = 56.0-83.5). For repeat cytology at both referral thresholds, specificity and the referral rate did not differ significantly between the two age categories.

CHAPTER V

DISCUSSION

5.1 Introduction

This retrospective cohort study was carried out to: (1) describe the association of human papillomavirus (HPV), repeat Papanicolaou (Pap) cytology and histology results in women with low-grade cytological abnormalities; (2) assess the performance of the HPV deoxyribonucleic acid (DNA) test in triage of women with low-grade cytological abnormalities, namely atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesions (LSIL); (3) assess the performance of the repeat Pap test in triage of women with low-grade cytological abnormalities, namely ASCUS and LSIL; (4) compare the performance of both triage tests; and (5) determine if age affects triage performance.

5.2 HPV Prevalence

The prevalence of HPV in the present study population is similar to that from other published research using the Hybrid Capture II (HC-II) assay (Digene Corporation, Gaithersburg, Maryland, USA) (ALTS Group, 2000; Manos et al., 1999; Solomon et al., 2001). In the current study, 44.3% of women with ASCUS Pap test results were positive for HPV, thereby potentially reducing the number of women with ASCUS cytology who are referred to colposcopy by more than one half. Manos et al. (1999) reported a HPV prevalence of 39.5% and the ALTS trial demonstrated a slightly higher HPV prevalence

of 56.1% (Solomon et al., 2001). In contrast, the prevalence of HPV in our study sample was significantly higher in women with a referral Pap test result of LSIL (79.3%) ($P < 0.001$) than in women with an ASCUS cytology interpretation (44.3%). A recent review article suggests that HPV prevalence rates in LSIL cytology were consistently higher than in ASCUS cytology (Arbyn et al., 2006). Other studies have also reported similar levels of HPV prevalence in LSIL cytology (Clavel et al., 1999; ALTS Group, 2000). Clavel et al. (1999) found that 76.8% of women with LSIL in their study were HPV positive. In the ALTS trial, HPV was detected in 82.9% of women with LSIL, and consequently, they closed the enrollment of women to the HPV arm of the study early (ALTS Group, 2000). Because of the high prevalence of HPV in women with LSIL cytology, they concluded that an LSIL cytology result is likely indicative of HPV infection, and it appears that there is limited potential for the use of HPV DNA testing in triage for the evaluation of LSIL.

HPV prevalence was clearly age-dependent in women with an ASCUS Pap test result, being significantly higher among women younger than 30 years of age (74.3%) than older women (27.4%) ($P < 0.001$). On the other hand, this age distinction seems to be not as great in women with an LSIL interpretation, where HPV prevalence is high in both younger (83.3%) and older women (69.8%) ($P = 0.065$). Nevertheless, the decreasing prevalence of HPV with age is consistent with the natural history of HPV infection and is similar to previous studies (Ho et al., 1998; Sherman et al., 2002; Shlay et al., 2000).

5.3 Association between HPV and Repeat Cytology

Among women referred with ASCUS cytology, repeat Pap results were heterogeneous. Only 14.4% of repeat Pap tests were read as ASCUS. Repeat Pap results were also heterogeneous among women referred with LSIL cytology. However, a higher percentage of LSIL results were again LSIL when repeated (37.2%). These findings reflect those of other research elsewhere that ASCUS results are less reproducible than LSIL results (Stoler & Schiffman, 2001).

In the present study, the prevalence of HPV increased with the increasing severity of the repeat Pap test result in women with baseline cytology of ASCUS and LSIL. We could not test this trend because the expected cell count for at least one cell was less than five. Other studies, however, were able to test this trend. Solomon et al. (2001), reported that among women with ASCUS, the trend towards increasing HPV positivity with increasing severity of repeat cytology diagnoses was significant ($P < 0.001$). The ALTS Group (2003b) also found a significant trend ($P < 0.001$) in a group of women with LSIL cytology.

5.4 Association between HPV, Repeat Cytology and Histology

Women referred for an ASCUS Pap abnormality generally have a lower prevalence of histologically diagnosed high-grade squamous intraepithelial lesions (HSIL) than do women referred for a LSIL Pap abnormality (ALTS Group, 2003a; ALTS Group, 2003b;

Lytwyn et al., 2000; Sherman et al., 2002). In the present study, the overall prevalence of histologically diagnosed HSIL in women with ASCUS was 16.5%. Solomon et al. (2001) reported a similar percentage (15.4%). In women with LSIL Pap test results, we found 20% to have underlying HSIL. The overall detection of HSIL as diagnosed by histology in the ALTS trial was slightly higher at 25% (ALTS Group, 2003b). However, the rate reported by the ALTS study was a cumulative 2-year percentage, whereas our study followed up for only one year. These findings indicate that women with low-grade cytological abnormalities require some form of additional evaluation and follow-up.

A significant association ($P < 0.001$) was found between a positive HPV result and underlying HSIL in women with ASCUS cytology. All women with a histological diagnosis of HSIL were HPV positive. When stratified by age, however, the association between HPV and confirmed HSIL was no longer significant among women younger than 30 years of age ($P = 0.058$). This was likely due to the small sample size.

In women with a referral diagnosis of LSIL, there was also a significant association between HPV positivity and HSIL confirmation ($P < 0.05$). Among these women, all except one of the 29 cases of HSIL detected by histology occurred in women with an HPV positive result. This single exception may represent a woman with a regressing lesion who already had cleared the virus. It is also possible that the HPV negative case may have been due to false negative results as a result of low viral copy number or poor sample collection. When stratified by age, there was no longer a statistically significant

association between HPV and histological HSIL among women younger than 30 years of age (P = 0.182). Again, the failure to reach significance is likely due to the small study sample.

In women with a baseline Pap result of ASCUS or LSIL, the percentage with HSIL by histology increased with the increasing severity of the repeat Pap category. However, because the expected cell count for at least one cell was less than five, this trend could not be tested. Previous research has shown that the more severe the Pap category, the greater the risk of having underlying HSIL (Kinney et al., 1998).

5.5 Performance of Different Triage Protocols

5.5.1 Triage of ASCUS Cytology

In assessing the performance indicators in women with baseline ASCUS cytology, we found that a single HPV DNA test would have appropriately triaged 100% of the women who had a histological diagnosis of HSIL, while referring only 44.3% of the total ASCUS population with a negative predictive value (NPV) of 100%. The relatively low prevalence of HPV in women with ASCUS, combined with the high sensitivity and NPV of the HPV DNA test, is compatible with the Canadian and American recommendations to use HPV DNA testing as an immediate adjunct screening test for triage of ASCUS Pap cytology results (Provencher & Murphy, 2007; Stuart et al., 2004; Wright et al., 2007). The strategy of repeat cytology was less sensitive (75%) than the HPV DNA test in detecting underlying HSIL at an ASCUS threshold of referral, referring 39.2% of women.

Although repeat cytology at a LSIL threshold of referral would have referred the least number of women to colposcopy (24.7%), it was the most insensitive triage strategy, identifying only 43.8% of underlying HSIL. These results are similar to those of previous research (Arbyn et al., 2004; Manos et al., 1999; Solomon et al., 2001).

Manos et al. (1999) compared HPV DNA testing to repeat Pap testing in a sample of 995 women with ASCUS. All women had specimens taken for a repeat Pap test and HPV DNA testing, followed by a colposcopically-directed biopsy to confirm the diagnoses. As in the present study, the gold standard was a histological diagnosis of HSIL or cancer since the current clinical practice is to treat histologically confirmed HSIL, in addition to cancer. The sensitivity of the HPV DNA test was 89.2% for detection of underlying HSIL or cancer. This was higher than repeat Pap testing at an ASCUS threshold of referral which had a sensitivity of 76.2%. However, the specificity of HPV DNA testing was similar to that of repeat cytology (64.1% and 63.8%, respectively). It was estimated that triage based on HPV DNA testing or on repeat Pap testing with referral to colposcopy set at a repeat Pap result interpreted as ASCUS or more severe, would have resulted in approximately the same number of referrals for colposcopy (40%). The authors concluded that for women with ASCUS Pap results, the HPV DNA test can help identify the majority of women with underlying HSIL or cancer.

The ALTS Group compared three management strategies, immediate colposcopy, HPV DNA triage with referral if positive, or triage based on repeat Pap testing in 3,488 women

with ASCUS (Solomon et al., 2001). The sensitivities of immediate colposcopy, HPV DNA triage, and repeat Pap at an ASCUS threshold of referral were 100, 95.9, and 85.0%, respectively. Just over half (56.1%) of the women having HPV DNA testing would have been referred to colposcopy, and 58.6% of the women having a single repeat Pap test would have been referred. Given that the HPV DNA test showed a greater sensitivity for detection of histologically confirmed HSIL or cancer than a single repeat Pap at a threshold of ASCUS or worse (95.9% and 85.0%, respectively), and a comparable specificity (48.4% and 44.7%, respectively), the authors concluded that HPV DNA testing is an option for managing women with ASCUS to determine if colposcopy is needed. Nevertheless, the referral rate of women by HPV DNA testing is still high compared to the low percentage of true HSIL diagnosed in women with ASCUS.

Arbyn et al. (2004) recently conducted a meta-analysis that compared HPV DNA testing with repeat Pap testing for triaging ASCUS. They reported that restriction of colposcopy examination to women with a positive HPV DNA test had a sensitivity of 94.8% for underlying HSIL, compared with 81.8% for repeat cytology at an ASCUS threshold.

The fact that differences in age distribution may affect the performance of the HPV DNA test became apparent in this study. We found that in women younger than 30 years of age, the HPV DNA test showed a significantly lower specificity than in older women (33.3% and 83.3%, respectively). The poor specificity in younger women is reflective of the high prevalence of HPV in this age group. On the other hand, the sensitivity and

NPV were largely unaffected by age. HPV DNA testing would have shown a significant reduction in referrals to colposcopy among women 30 years of age and older compared to younger women (27.4% and 74.3%, respectively). However, had we restricted HPV testing to women 30 years of age and older, 8 women younger than 30 years of age with HSIL by histology would not have been referred to colposcopy.

The association between age and HPV test performance in our study is consistent with that observed in other studies (Rebello, Hallam, Smart, Farquharson, McCafferty, 2001; Sherman et al., 2002; Shlay et al., 2000). In a study by Rebello et al. (2001), the sensitivity was 94% in women under 30 years of age versus 91% among women at least 30 years old, while the specificity was 33% and 72% in younger versus older women, respectively. Shlay et al. (2000) reported that the specificity was 57.4% in women under 30 years of age versus 83.9% among women at least 30 years old. However, the sensitivity was somewhat lower in older women (85.7% in women ≥30 years and 100% women in <30 years, respectively). Sherman et al. (2002) reported that sensitivity varied minimally with age (range, 93.9% to 97.8%) and specificity increased with age. In age groups 18-22, 23-28 and 29 and older the specificity was 34%, 41% and 52%, respectively. Consistent with the present study, HPV testing in these studies all demonstrated a significant reduction in referrals between older and younger women.

The results for repeat Pap cytology in the current study demonstrated similar differences in performance indicators when stratified by age, as was seen for HPV DNA testing. The

sensitivity of repeat cytology at an ASCUS threshold of referral remained the same in both age groups, and like HPV testing, the test was significantly more specific in women 30 years of age and older (81.5% in women ≥ 30 years and 40.7% in women < 30 years, respectively). Nevertheless, HPV DNA testing remained the most sensitive test among all age groups.

5.5.2 Triage of LSIL Cytology

Unlike women with baseline ASCUS cytology, we did not find an appropriate triage method for women with LSIL of any age. A single HPV DNA test would have appropriately triaged 96.6% of the women who were found to have HSIL as diagnosed by histology, but the test would have referred the greater majority (79.3%) of the total LSIL population, limiting its usefulness for triage of these women. A program of triage is generally not considered acceptable if 75% or more of women tested would be referred to colposcopy because of a positive result (Wright et al., 2007). As discussed previously, the ALTS Group (2000) closed the enrollment of women to the HPV arm of their study due to the high prevalence of HPV in the LSIL population (82.9%). Consistent with previous results of women with LSIL cytology (Sherman et al., 2002), the percentage of women with positive HPV results did not decline substantially with age. Approximately 70% of women at least 30 years of age would still have been referred by this triage method.

In examining the performance of repeat cytology in women with LSIL, repeat cytology at a threshold of ASCUS would have referred 57.9%; however, the sensitivity of this approach was low at 82.8%. In fact, in any age group and for both thresholds of repeat cytology, either method that had a good sensitivity required referral of the majority of women.

When the ALTS Group (2003b) estimated the performance of repeat cytology in women with LSIL, a single repeat Pap result at the ASCUS threshold referred more than 80%. They concluded that this percentage was too high to justify the use of repeat cytology for the triage of these women.

5.6 Strengths and Limitations of Study

There are strengths and limitations with the current study. The main strength of this study was the opportunity to evaluate the utility of the HPV DNA test in triage of women with low-grade cytological abnormalities in Newfoundland, Canada. This is one of few studies in Canada that have examined the HPV test in triage of these women. The opportunity to assess HPV DNA testing in secondary screening presents new options for the cervical cancer screening programme. Lytwyn et al. (2000) conducted a study comparing HPV DNA testing and repeat Pap testing in 212 women from Ontario with ASCUS and LSIL cytology. They found the HPV DNA test to be more sensitive (87.5%) than repeat cytology (55.6%) at an ASCUS threshold for referral in these women.

However, they reported the performance of the tests without distinguishing between women with ASCUS or LSIL.

This study does have limitations. A reliable gold standard was not available for all women in the study. Ideally, all women should have undergone colposcopy and biopsy to determine disease status. However, because this was a retrospective cohort study and not a randomized control trial, histology was taken at the clinician's discretion. It is likely that a number of women did not undergo biopsies because of a negative colposcopy, and this is an indication of no disease. However, some research suggests that the accuracy of colposcopy is imperfect (Barker, Garcia, Warner, Lozerski, & Hatch, 2002; Massad & Collins, 2003).

The small sample size was also a limitation. Some statistical tests could not be carried out because of the small numbers. This may help explain why certain significant associations or differences were not observed. However, while the small sample size could also limit the generalizability of the conclusions, the findings of this study are similar to those of large randomized trials.

A further limitation is that the study does not take into account regression, persistence, or progression of HPV infection, or histological outcomes.

Finally, we did not distinguish between women with a history of abnormal cytology and those without one. This may have influenced the performance of the HPV DNA test. However, other published studies have found there to be no difference in HPV DNA test performance in women independent of history of cytological abnormalities. (Manos et al., 1999; Shlay et al., 2000).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

This study confirms that human papillomavirus (HPV) deoxyribonucleic acid (DNA) testing can be used to triage women with atypical squamous cells of undetermined significance (ASCUS). The HPV DNA test reached 100% sensitivity for detection of underlying high-grade intraepithelial lesions (HSIL) and would have reduced referrals to 44.3%. The specificity of this approach was at 66.7%, but when stratified by age, specificity increased significantly in older women (83.3% in women ≥ 30 years and 33.3% in women < 30 years, respectively). As well, referrals to colposcopy would have been significantly reduced among older women compared to younger women (27.4% and 74.3%, respectively). However, restricting HPV testing to women 30 years of age and older would have resulted in eight out of the sixteen women with HSIL by histology not being referred to coloposcopy. Further study should be untaken to investigate the usefulness of age-restricted HPV testing, examining various age thresholds of referral. Repeat cytology for women with ASCUS was estimated to be a less sensitive (75%) approach for triaging women in all age groups.

In contrast to ASCUS, we did not find a suitable triage strategy for women with low-grade squamous intraepithelial lesion (LSIL) cytology. Approximately 80% of these women were positive for HPV, and this percentage did not decline substantially with age. In women 30 years of age and older, the HPV prevalence was 69.8% and in women

younger than 30 years of age, the prevalence of HPV was 83.3%. Because of this high HPV prevalence, triage using HPV DNA testing may not be useful for the LSIL population. Repeat cytology using either an ASCUS or LSIL threshold of referral was also determined to be an ineffective method of triage in any age group. Any method that had a good sensitivity (approximately 90%) required referral of the majority (approximately 60%) of women. It is therefore recommended, as is the recommendation of others (Wright et al., 2007), that women with LSIL Pap results be referred to colposcopy immediately.

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APPENDICES

Appendix A

Memorial
University of Newfoundland

Office of Research and Graduate Studies (Medicine)
Faculty of Medicine
The Health Sciences Centre

November 1, 2002

Reference #02.205

Dr. Sam Ratnam
Newfoundland Public Health Laboratory
Leonard A. Miller Centre
PO Box 8800
St. John's, NF A1B 3T2

Dear Dr. Ratnam:

Your research application entitled **"Predictive value of HPV DNA test in low grade cytologic abnormalities"** was distributed for expedited review to a Sub-Committee of the Human Investigation Committee and ***full approval*** was granted. This will be formally reported to the full Human Investigation Committee at the meeting scheduled for **November 14, 2002.**

Please be advised that the Human Investigation Committee currently operates according to the Good Clinical Practice Guidelines, the Tri-Council Policy Statement and applicable laws and regulations.

We wish you success with your study.

Sincerely,

Sharon K. Buehler, PhD
Co-Chair
Human Investigation Committee

Richard Norgan, PhD
Co-Chair
Human Investigation Committee

SKB:RN/mc

C Dr. C. Loomis, Vice-President (Research)
Dr. R. Williams, Vice-President, Medical Affairs, HCC