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HPV mRNA and $p16^{INK4A}$/Ki-67 detection for improved diagnosis and management of cervical neoplasia in smokers

By

Christine White, B.Sc. (Hons.)

Department of Histopathology and Morbid Anatomy,

Trinity College, Dublin.

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University of Dublin,

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Under the supervision of

Doctor Cara Martin and Professor John O'Leary

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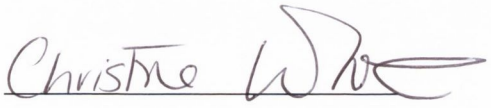

Christine White

Dedicated in loving memory to my Dad, Tommy

Forever in my heart

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Summary

Persistent infection with high risk human papillomavirus (HR HPV) is the main etiological agent in the development of cervical cancer. The use of HPV DNA testing in cervical screening programs is becoming increasingly common worldwide. However, due to the high prevalence of transient HPV infections in low grade lesions, HPV DNA testing is limited. Early evidence suggests that detection of HPV E6/E7 mRNA transcripts and $p16^{INK4A}$/Ki-67 over expression is more indicative of a clinically relevant infection than HPV DNA testing.

Co-factors are believed to be involved in the transition from transient to transforming infections. Local immunosuppression in the cervix, induced by smoking, could facilitate in persistent HPV infection, promoting viral integration.

The utility of testing for HPV E6/E7 transforming gene expression and $p16^{INK4A}$/Ki-67 both as standalone tests and in combination to HPV DNA testing, for the detection of high grade cervical pre-cancer was investigated. Findings indicated HPV E6/E7 and $p16^{INK4A}$/Ki-67 expression offered a high specificity for detection of CIN 2+ (69.2% and 83.2%) compared to HPV DNA testing (46.7%), while HPV DNA testing yielded a higher sensitivity (91.5%). By combining the strengths of each test it was establish that merging HPV DNA testing with $p16^{INK4A}$/Ki-67 offered the most efficient approach for stratifying women presenting with minor cytological abnormalities at true risk of high grade pre-cancer. This approach had potential to reduce colposcopy referrals by up to one third.

Additionally, exposure to tobacco smoke, measured through urinary cotinine concentration was found to increase risk of HPV DNA and HPV mRNA. It was further identified that only higher concentrations of urinary cotinine induced risk of CIN 2+. Suggesting heavy smoking is a risk factor for high grade pre-cancer in women with minor cytological abnormalities

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Abbreviations

^{o}C	Degrees Celsius
µ	Micro
AB 9700	Applied Biosystems Gold-plated 96-well GeneAmp PCR System
ADAT1	Adenosine deaminase 1
AGC	Atypical Glandular Cells
AIS	Adenocarcinoma in Situ
AL	Lysis Buffer
ALTS	ASCUS-LSIL HPV triage study
AMV-RT	Avian Myeloblastosis Virus Reverse Transcriptase
AP	Alkaline phosphatase
ASC-H	Atypical Squamous Cells cannot exclude High Grade
ASCUS	Atypical Squamous Cells of Undetermined Significance
ATL	Tissue Lysis Buffer
AW2	Wash Buffer 2
AWE	Acetowhitening
BSCCP	British Society for Colposcopy and Cervical Pathology
CADM	Cell Adhesion Molecule
CAR	Carrier RNA
CC	Cervical Cancer
CDK	Cyclin Dependant Kinase
cDNA	copy Deoxyribonucleic Acid
CERVIVA	The Irish cervical screening research consortium
CFS	Common Fragile Sites

CIN	Cervical Intraepithelial Neoplasia
CIT	Citrate Concentrate
CLM	Column
CO	Cut-off
CpG	Cytosine Phosphate Guanine
DAPK1	Death-associated Protein Kinase 1
dATP	Deoxy Adenosine Triphosphate
dCTP	Deoxy Cytosine Triphosphate
dGTP	Deoxy Guanine Triphosphate
diH_2O	Deionised water
DNA	Deoxyribonucleic Acid
DNR	Denaturation Reagent Mixture
DNR	Denaturation Reagent Mixture
dTTP	Deoxy Thiamine Triphosphate
dUTP	Deoxy Uracil Triphosphate
E	Early Region
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
ETL	Elution Tube
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
GP	General Practitioner
HART	HPV in Addition to Routine Testing
hc2	Hybrid Capture 2

HPV	Human Papillomavirus
HR	High Risk
HR HPV	High Risk HPV
HRC	High-Risk Calibrator
HRCx	High-Risk HPV Calibrator Mean
HR-HPV	High-risk HPV
HRP	Horseradish Peroxidise
HSE	Health Service Executive
HSIL	High-grade Squamous Intraepithelial Lesion
IARC	International Association for Research on Cancer
ICE	Interdisciplinary Capacity Enhancement
IUCD	Intrauterine Contraceptive Device
IUS	Intrauterine System
L	Late Region
l	Litre
LBC	Liquid Based Cytology
LC	Langerhan Cells
LLETZ	Large Loop Excision of the Transformation Zone
LR HPV	Low-risk Human Papillomavirus
LSIL	Low-grade Squamous Intraepithelial Lesion
MALT	T lymphocyte Maturation Association Protein
MCM	Minichromosome Maintenance
Mg^{2+}	LINEAR ARRAY HPV Magnesium Solution
MgCl	Magnesium Chloride

Mins	Minutes
ml	Millilitre
mM	Millimolar
MMX	LINEAR ARRAY HPV Master Mix
mRNA	Messenger RNA
MSP	Methylation specific PCR
MW	Elution Buffer
n	Cohort number
NASBA	Nucleic Acid Sequence Based Amplification
NC	Negative Calibrator
NCRI	National Cancer Registry Ireland
NCx	Negative Calibrator Mean
Ng	Nanograms
NHS	National Health Service
NHSCSP	National Health Service Cervical Screening Program
NNK	Nitrosanime 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone
NPV	Negative Predictive Value
NTCC	New Technologies for Cervical Cancer
OCP	Oral Contraceptive Pill
oligo	Oligonucleotide
OR	Odds Ratio
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
POBOSCAM	POpulation Based SCreening study AMsterdam

PPD	Proofer Plate Design Software
PPV	Positive Predictive Value
pRB	Retinoblastoma Protein
pRB	Retina Blastoma Protein
QC1-LR	Quality Control 1 Low-Risk
QC2-HR	Quality Control 2-High-Risk
RBS35	RBS35 Tray Cleaning Solution
RCF	Relative Centrifugal Force
RCT	Randomised Controlled Trials
RFU	Relative Flourescence Units
RLT	Tissue Lysis Buffer
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RPE	RPE Wash Buffer
Rpm	Revolutions Per Minute
RT	Room Temperature
SA-HRP	Streptavidin-Horseradish Peroxidase Conjugate
SCC	Squamous Cell Carcinomas
SCJ	Squamocolumnar Junction
SDS	20% Sodium lauryl sulphate and 1% ProClin® 150
SIL	Squamous Intraepithelial Lesion
SSPE	Sodium Phosphate Solution
STD	Sexually Transmitted Disease
STM	Specimen Transport Medium

Strip	Linear Array HPV Genotyping Strips
TBS	The Bethesda System
TMA	Transcription Mediated Amplification
TMB	Tetramethylbenzidine
TNA	Total Nucleic Acid
TOMBOLA	The Trial of Management of Borderline and other Low Grade Abnormal smears
TOT2A	Topoisomerase 2 Alpha
U1A	Human U1 small nuclear ribonucleoprotein specific protein A
VAIN 1	Vaginal Intraepithelial Neoplasia 1

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-Chapter 1-

General Introduction

1 General Introduction

1.1 Overview

Cervical cancer is the third most frequent malignancy in women worldwide with an estimated 530,000 cases and 275 deaths each year (Jemal et al., 2011). In Ireland, the incidence in 2007 was 286 cases, resulting in 83 deaths (CervicalCheck, 2009-2010). The rate of cervical cancer is decreasing in developed countries, this is mainly due to organised cervical screening programs. Thus cervical cancer is more often fatal in developing countries; more than 85% of cervical cancers occur in low income nations. This disproportionate burden of cervical cancer is largely due to the lack of adequate recourses in cervical cancer screening and vaccination programs. Population based cervical screening can reduce cancer incidence by an estimated 80% (IARC, 2005) by allowing for early detection and treatment of cervical disease.

Prior to the introduction of the Irish cervical screening program, CervicalCheck, cervical screening in Ireland was one of opportunistic screening for women attending general practitioners, post natal examinations, family planning and STD clinics and self-referral to community clinics. There was a lack of standardisation with screening intervals varying amongst smear takers, additionally, the lack of a registry made it difficult to target vulnerable groups within the population. Phase one of the National Cervical Screening Programme began in the Mid-Western Area in October 2000. Under the programme, women in the 25 to 60 age group were screened at five-year intervals free of charge. In 2008, the program went nationwide offering free pap smears to women between the ages of 25-60 over three/five-year intervals depending on age. Since its launch the program has proved very successful, with an average of 1,000 women availing of a free smear per day, during the first two years of operation (Cervical Check, 2009-2010). A vaccination program was introduced in 2011, and is offered to all girls who are starting 1st and 2nd year in secondary schools.

Cervical screening has undergone transformation in recent years and will continue to do so. HPV testing has been implemented into a number of screening programs leading to changes in screening algorithms. Vaccination is

likely to change the landscape of cervical screening. Cytological abnormalities will become less frequent, and methods such as HPV testing and biomarkers will be more appropriate.

1.2 History of Cervical Screening

Cervical screening has aided in earlier detection and prevention of cervical cancers. Screening by the pap smear is one of the most successful cancer screening techniques. The pap test was introduced in the late 1940s, it allowed for the identification of morphologic changes associated with cervical cancer. The pap smear was discovered by Dr. George Nicholas Papanicolaou when in 1928 he presented findings that uterine cancer could be identified by means of a vaginal smear. In 1941 Papanicolaou published a piece of work outlining the morphological characteristics of cancer cells from exfoliated cells of the superficial layer of the cervix. This led to the establishment of cervical screening by cervical cytology. Traditional screening was performed by conventional pap smear, this involved removing cells from the cervix using a spatula, which were then smeared/spread onto a glass slide. This was successful in reducing incidence of cervical cancer (Hakama et al., 1985). However, it was often limited by smear quality and difficult to interpret results. Liquid based cytology (LBC) was introduced as an alternative to conventional cytology in the 1990s. This involved making a suspension of cells, which allowed for the production of a monolayer of cells producing an evenly distributed cell layer void of obscuring factors such as blood and mucus. LBC demonstrated a number of advantages over conventional cytology, from increased detection of high grade abnormalities, reduction in unsatisfactory smears and provided residual material for subsequent molecular tests (Payne et al., 2000, Taylor et al., 2006). However, there remained issues over sensitivity, rate of false negatives in addition to individual subjectiveness and interpretation skills. To address poor inter-observer reproducibility automated screening was developed, this was found to have equivalent sensitivity and specificity to manual screening and was reported to be of benefit to increase laboratory productivity (Bolger et al., 2006, Quddus et al., 2009). In 1991, the association between HPV and cervical cancer was established. This led to the development of HPV detection techniques and subsequent introduction of HPV

testing into cervical screening. In 2003, the first HPV detection technology, Hybrid Capture 2 received FDA approval. There has since been an influx of detection assays developed, both HPV DNA and mRNA based, in addition to on-going research to identify novel biomarkers. This thesis examines the use of HPV DNA based technologies and recent, more novel, HPV mRNA based and biomarker assays.

1.3 The uterine cervix

The cervix is found at the lower portion of the uterus. It is a cylindrical shaped fibromuscle of 3-4 cm in length and 2.5 cm in diameter. The cervix is divided into two parts, the lower portion extends into the vagina through the upper anterior vaginal wall where approximately one half its length is visible. This portion is referred to as the portio vaginalis, it opens on to the vagina by the external oz (opening of the ectocervix). The internal oz forms the junction between the upper limit of the cervix and uterine fundus and cervical stroma. The outer portion of the cervix is called the ectocervix, while the endocervix forms the internal portion of the cervix. The endocervical canal opens onto the portio vaginalis at the external oz, from here it forms the passageway between the external oz and the endometrial cavity, it measures 6-8 mm wide and contains complex configuration of multiple longitudinal folds protruding into the lumen of the canal, giving rise to plicae (Sellors and Sankaranarayanan 2003, ASCCP 2012).

The ectocervix is covered with stratified, non-keratinizing glycogen containing squamous epithelium. The histological architecture reveals layers of cells, at the bottom, a single layer of basal cells is attached to the basement membrane. These cells have a large nucleus with little cytoplasm. Basal cells divide and mature to form parabasal cells, differentiation and maturation forms what are termed intermediate cells. These now have abundant basophilic cytoplasm and small nuclei. Further maturation forms the upper layer of superficial cells. These are large flat cells with small pyknotic nuclei and transparent cytoplasm (Sellors and Sankaranarayanan 2003, ASCCP 2012).

Glandular epithelium forms the endocervical canal. It is composed of a single layer of columnar cells with polar nuclei. The glandular epithelium of the

endocervical canal meets with the squamous epithelium of the ectocervix at the squamocolumnar junction (SCJ) where it forms a visible border. During perimenarche, the junction lies close to the external oz. At puberty, the cervix swells, under the influence of estrogen, the lower part of the endocervical canal becomes everted onto the ectocervix forming a large reddish area surrounding the external oz, this is known as extropion. As a result of this, the original SCJ moves further away from the external oz (Sellors and Sankaranarayanan 2003). As the columnar epithelium becomes exposed to the acidic vaginal environment cells undergo metaplasia, this involves the physiological replacement of the everted columnar epithelium to form squamous epithelium. A new SCJ is formed, this area between the new and the original SCJ is known as the transformation zone, shown in figure 1.1. This is an area of utmost importance; it is here that squamous neoplasia will almost exclusively arise. During reproductive life this is an area of constant cell turnover, at menopause the effect of prolonged exposure to progesterone causes atrophy and the SCJ recedes up the endocervical canal (ASCCP 2012).

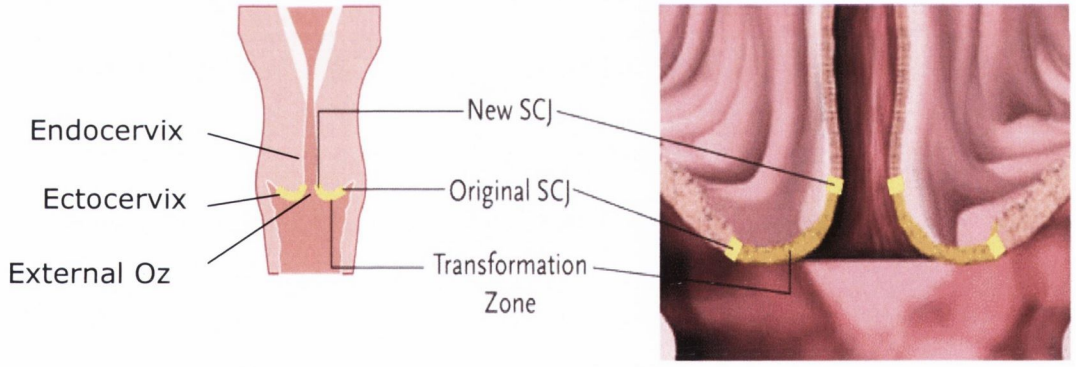

Figure 1-1 Cervical Squamocolumnar Junction and the Transformation Zone

The area where the squamous cells of the ectocervix meet the columnar cells of the endocervix is termed the Squamocolumnar Junction (SCJ). At puberty the SCJ is located at the external oz, following perimenarche the lower portion of the endocervical canal becomes everted and gradually undergoes metaplasia to form squamous epithelium. A new SCJ is formed; the area between the new and original (yellow) is the transformation zone. (Taken and Modified from Bratcher and Palefsky 2008)

1.4 Disease of the cervix

Cervical cancer is the third most frequent malignancy in women worldwide after breast and colorectal, with almost half a million cases diagnosed each year (Jemal et al., 2011). However, incidence rates of cervical cancer vary widely among different countries. In Ireland, rates were estimated for 2012 as 15.1 per 100,000 with a mortality rate of 4.3 per 100,000 making cervical cancer the 7th most commonly diagnosed cancer in women of the Irish population, ranking 12th highest among 30 European countries (NCRI, 2011, Ferlay et al., 2013). Data from the National Cancer Registry Ireland indicates that incidence rates of invasive cervical cancer in Ireland, showed a slight increase from 1994-2009 of 2.1% ±1.5%. Squamous cell carcinomas were the most common type detected representing 75% of invasive cancers, with the majority diagnosed at an early stage (stage 1) (NCRI, 2011). Adenocarcinoma followed at 14% then adeno-squamous 3%, sarcomas 1% and other/unspecified 7%. The incidence of invasive cervical cancer peak in women aged 30-49 years with 53.7% diagnosed in this age group (NCRI, 2011).

Up to 90% of invasive cervical cancer exists in either two histological forms depending on whether they originate in squamous or glandular epithelium (NCRI 2011). Squamous cell carcinomas (SCC) arise from immature cells of the transformation zone of the cervix, and can be visualised by speculum examination. Whereas adenocarcinomas arise at the glandular epithelium of the endocervical canal, their location makes them harder to detect than squamous carcinomas and they are often associated with larger infiltrative lesions with a greater rate of nodal metastases and lympho-vascular space leading to poorer prognosis (Sahdev, 2010). Of the two, SCC is the most common, accounting for over 75% of cases. However, the incidence of adenocarcinoma is increasing (Bray et al., 2005).

1.5 Traditional terminology

It has been previously described that SCC is preceded by a long phase of pre-invasive disease termed cervical intraepithelial neoplasia (CIN) (Richart et al., 1966). The pre-invasive phase begins as a slow progressive disruption of

normal epithelium surrounding the transformation zone. It involves progression from cellular atypia to various grades of dysplasia, before development of invasive cancer. Pre-cancerous lesions of squamous origin were characterised on histology as progressive lesions ranging from CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia). Traditionally it was considered that CIN represented a biological continuum and if left untreated, these lesions would develop in to invasive carcinoma (Richart et al., 1966). Grading is determined by the proportion of the epithelial thickness occupied by undifferentiated neoplastic cells, stratification and mitotic figures. CIN 1 represents dysplastic cells occupying the lower third of the cervical epithelium to CIN 3 where there is full thickness involvement of dysplastic cells and loss of differentiation in the cervical epithelium (figure 1.2 and 1.4) (Sellors and Sankaranarayanan 2003).

As our understanding of the pathogenesis of cervical cancer and its pre-cursor lesions improved, it became recognised that the human papillomavirus (HPV) plays an essential role in cervical cancer (Bosch et al.,2003). HPV interacts with squamous epithelia resulting in morphological changes which we identify as CIN on histology (figure 1.4) and squamous intraepithelial lesions (SIL) on cytology (figure 1.3). It is now known that the biology of pre-cancer does not necessarily follow what was previously described as a 3-tiered progression from CIN 1 to 3 but rather a dichotomous system reflecting transient (active) and transforming (persistent) HPV infections. Bearing this in mind, cervical pre-cancer can be classified as HPV associated lesions (Wright et al., 2006). A 2-tiered system has been recommended by the College of American Pathologists (CAP) and the American Society for Colposcopy and Cervical Pathology (ASCCP) as part of the Lower Anogenital Squamous Terminology (LAST) project (Darragh et al., 2013). This recommendation is supported by the fact that low grade lesions (CIN 1) are largely manifestations of self-limited transient HPV infections. Whereas high grade lesions (CIN 2+), represent transforming HPV infections, that have potential to progress to invasive cancer.

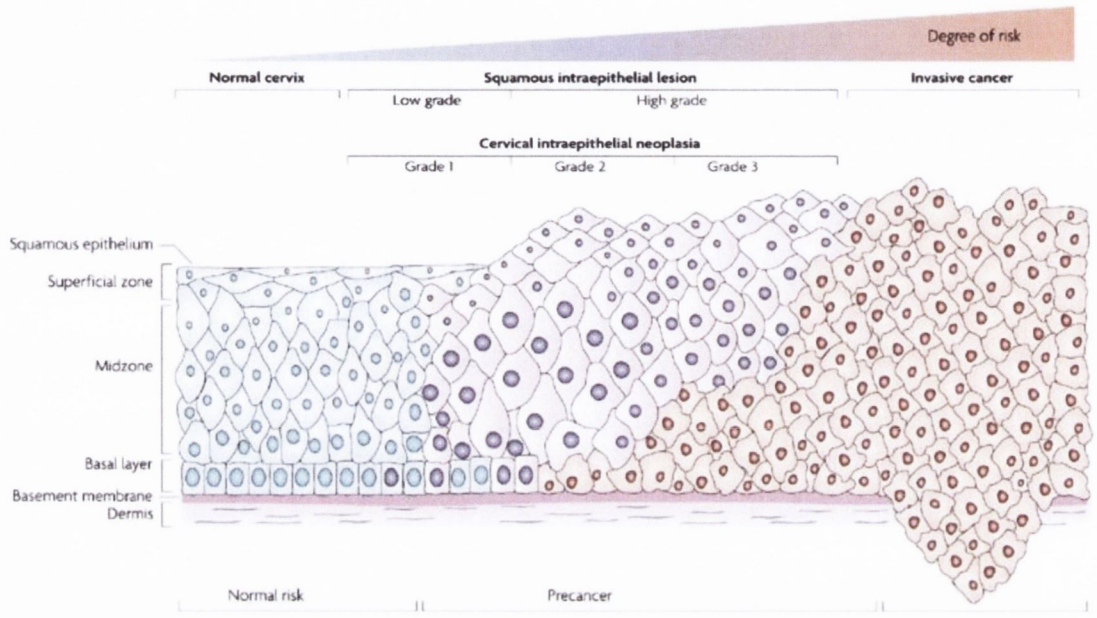

Figure 1-2 Progression of CIN

CIN is initiated as a slow progressive disruption of normal epithelium. It is graded CIN 1-CIN 3 in order of increasing severity and likelihood of progression to invasive cervical cancer. Typically the different grades are distinguished by the proportion of epithelium composed of undifferentiated cells. Cytological LSIL corresponds to CIN 1 and HSIL to CIN 2/CIN 3. Invasion occurs when the basement membrane is breeched as transformed cells enter the underlying stroma (Modified and taken from Kelloff and Sigman, 2007).

1.6 Human Papillomavirus

HPV is known to be causally related to cancer of the cervix, vagina, vulva, anal canal, penis and oropharynx (Parkin and Bray, 2006). Epidemiological data has clearly shown that persistent infection with HPV is associated with virtually all cervical cancers (Munoz et al., 1992, Ho et al., 1995, Walboomers et al., 1999). Collectively, genital HPV types are one of the most common sexually transmitted agents infecting up to 80% of women at some stage of their lives. There is a peak in prevalence in younger women between 18-30 years old. HPV infects squamous epithelial cells inducing proliferative lesions from benign warts to carcinomas. The association of HPV and CIN progression and cervical cancer was established by WHO in 1991. The idea that HPV was involved in cervical cancer was first suggested over 15 years prior to this in the 1970s by zur Hausen et al (zur Hausen et al., 1974, zur Hausen, 1977). This was closely followed by the identification of koilocytes, HPV infected cells, on cytology preparations (Meisels and Fortin, 1976). These findings were strengthened by zur Hausen and colleagues when they cloned and characterised two new HPV types HPV 16 and 18 from cervical biopsies (Durst et al., 1983, Boshart et al., 1984), now known to be the most common HPV types associated with cervical cancer. This work earned zur Hausen the Nobel Prize in 2008. The involvement of HPV was further confirmed by strong epidemiological evidence showing beyond reasonable doubt a strong and specific association of persistent HPV infection in cervical pre-cancer and cancer (Munoz et al., 1992, Walboomers et al., 1999).

1.6.1 Classification of HPV

Papillomavirus belong to the papovaviridae family. There have been over 120 different HPV types identified, more than 40 of which are known to infect the genital tract (Doorbar, 2006). The classification of different HPV genotypes is based on differences in open reading frames (ORF) of the L1, E6 and E7 regions of the genome (Schiffman et al., 2005). A difference of 2-10% in sequence homology in these regions define different subtypes, <2% define different variants (de Villiers et al., 2004). Papillomvirus have been classified in to phylogenetic clusters; alpha and beta are the main genera accounting for up to 90% of HPV types. The beta genesis are typically associated with

cutaneous infections which cause common warts (de Villiers et al., 2004). The alpha genera represent the largest group, containing the genital/mucosal HPV types and cutaneous HPV types. The remaining HPV types come from three genera, gamma, Mu and Nu and generally cause cutaneous papillomas and verruca.

It is recognised that cervical cancer is a rare outcome of HPV, the etiological association is restricted to a subset of high risk HPV (HR HPV) types. The alpha group contains over 60 members, the high risk types that cause cervical cancer. They predominately come from alpha 9 and to a lesser extent alpha 7. The International Agency for Cancer Research (IACR) has classified 12 HPV types as carcinogenic to humans (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), HPV 68 has been classified as probably carcinogenic and HPV 26, 53, 66, 67, 70, 73, 82 as possible carcinogenic (Schiffman et al., 2009). HPV 16 and 18 are recognised as the most important HR HPV types as they are found in over 70% of cervical cancers (Smith et al., 2007a). HPV types such as 6, 11, 42 and 44 are predominately associated with benign anogenital lesions and warts are aptly termed low risk HPV (LR HPV).

1.6.2 The genome and life cycle

The HPV virus is a double stranded circular DNA virus contained within an icosahedral capsid of 72 capsomers. The genome contains approximately 8000 basepairs and is divided into 9-10 ORF. The genome can be divided into three regions, the long control region, region of early proteins (E1, E2, E4, E5, E6 and E7) and region of late proteins (L1 and L2) (Figure 1.3A). The LCR varies from 500-1000 basepairs, it contains several control elements that regulate HPV DNA replication. The late proteins L1 (57kDa), which is the main structural element and L2 (43-53kDa) make up the virus coat. Both L1 and L2 are important for efficient virus infectivity (Doorbar 2006).

Infection requires access to the epithelial basal layer, this is thought to be achieved following microtrauma (Roberts et al., 2007) the exact mechanism of uptake is unknown but it is thought to be through heparin sulphate proteoglycans (HSPG) (Joyce et al., 1999). It is believed that L1 is the major determinant in attachment to HSPG. Binding induces a confirmation change to

the viral capsid exposing the N-terminus of L2 for cleavage by furin, this is necessary for entry (Richards et al., 2006). Together these two steps expose the binding site for a cell surface receptor, thought to be alpha 6 intergrin or syndecan-1 (Shafti-Karemat 2003), involved in internalization (Schiller et al., 2010). A number of methods have been proposed for cell entry, most commonly via clathrin mediated endocytosis (Day et al., 2003, Hindmarsh and Laimins, 2007) in which a carrier vesicle intermediate delivers virions to endosomes and lysosomes with uncoating of the virus beginning in late endosomes (Schmid, 1997). A caveolae-mediated pathway has also been suggested, describing transport of virions to the smooth endoplasmic reticulum through a pathway independent of endosomes and lysosomes (Smith et al., 2007a). Irrespective of the method used, it is established that internalisation occurs over several hours compared to most other virus types where it typically occurs within a matter of minutes (Day et al., 2003, Schiller et al., 2010). Once internalised, trafficking of the viral genome to the nucleus is poorly understood. One study reported transport of the genome along microtubules via the motor protein dynein (Florin et al., 2006).

Following entry to basal cells, genomes are established as an extrachromosmal element, an episome, in the nucleus. The life cycle of HPV is strongly related to the differentiation of host cells (zur Hausen, 2002). Initial infection is followed by a proliferative phase where the number of basal cells harbouring virus increases. This is a key stage of the HPV life cycle and necessary for successful onset of infection (Pyeon et al., 2009). It is thought that the viral genome is maintained in the basal layer at low copy numbers, around 10-200 copies per cell (De Geest et al., 1993). As a result HPV infected cells do not display HPV associated morphologic features, this is termed "latent" infection (Wright et al., 2006). As cells divide, daughter cells migrate away from the basal layer, they exit the cell cycle and initiate a process of terminal differentiation. This leads to amplification of viral DNA and expression of capsid proteins. Progeny virions are produced which are shed at the surface. The remaining daughter cell resides in the basal layer acting as a reservoir of viral DNA (Bosch and Iftner 2005).

There are eight early proteins, each serve a different function in viral replication. E1 and E2 play a role in initial infection in the basal cells, and maintain the virus as a stable episome (Wilson et al., 2002). E2 binds to a

non-coding up-stream regulatory region of the viral genome recruiting E1 helicase to bind to the viral origin of replication. E2 dissociates as host cell factors form a complex with E1 necessary for replication (Masterson et al., 1998). E2 can also regulate the early promoter and down regulate expression of E6 and E7 (Doorbar, 2006). As cells migrate though the epithelium copy number increases. There is up regulation of E6 and E7 as viral DNA replication occurs and viral copy number is amplified to at least 1000 copies per cell (Stanley, 2010). The difference with natural and oncogenic activity is the controlled expression of E6 and E7 by E2. High levels of E2 down regulate E6 and E7 via the early promoter (p97 in HPV 16 and HPV 31). E4 and E5 also contribute to viral amplification. E5 is associated with enhanced ligand-dependent activation of the epidermal growth factor receptor (EGFR), this is achieved by inhibiting endosome fusion, preventing endosome maturation (Suprynowicz et al., 2010). Consequently there of this is enhanced cell cycle entry (Pedroza-Saavedra et al., 2010). E4 is expressed in the intermediate and upper layers of infected epithelium. E4 can induce cell cycle arrest in the G2 phase which has been suggested to antagonise E7 mediated cell proliferation (Davy et al., 2002).

Structural proteins L1 and L2 are expressed only the terminally differentiated cells of the upper layers of the epithelium once amplification has been completed. Virus is packaged and released from infected cells. Papillomaviruses are non-lytic, and are not released until the infected cells reach the epithelial surface. Here they can go on to re-infect. As the epithelial supports virion production infections are productive or transient, and often represent the morphologic classification CIN 1 or LSIL. These productive HPV infections are often self-limited and cleared by the immune system in most women. The pattern of gene expression throughout the HPV life cycle is shown is figure 1.5B.

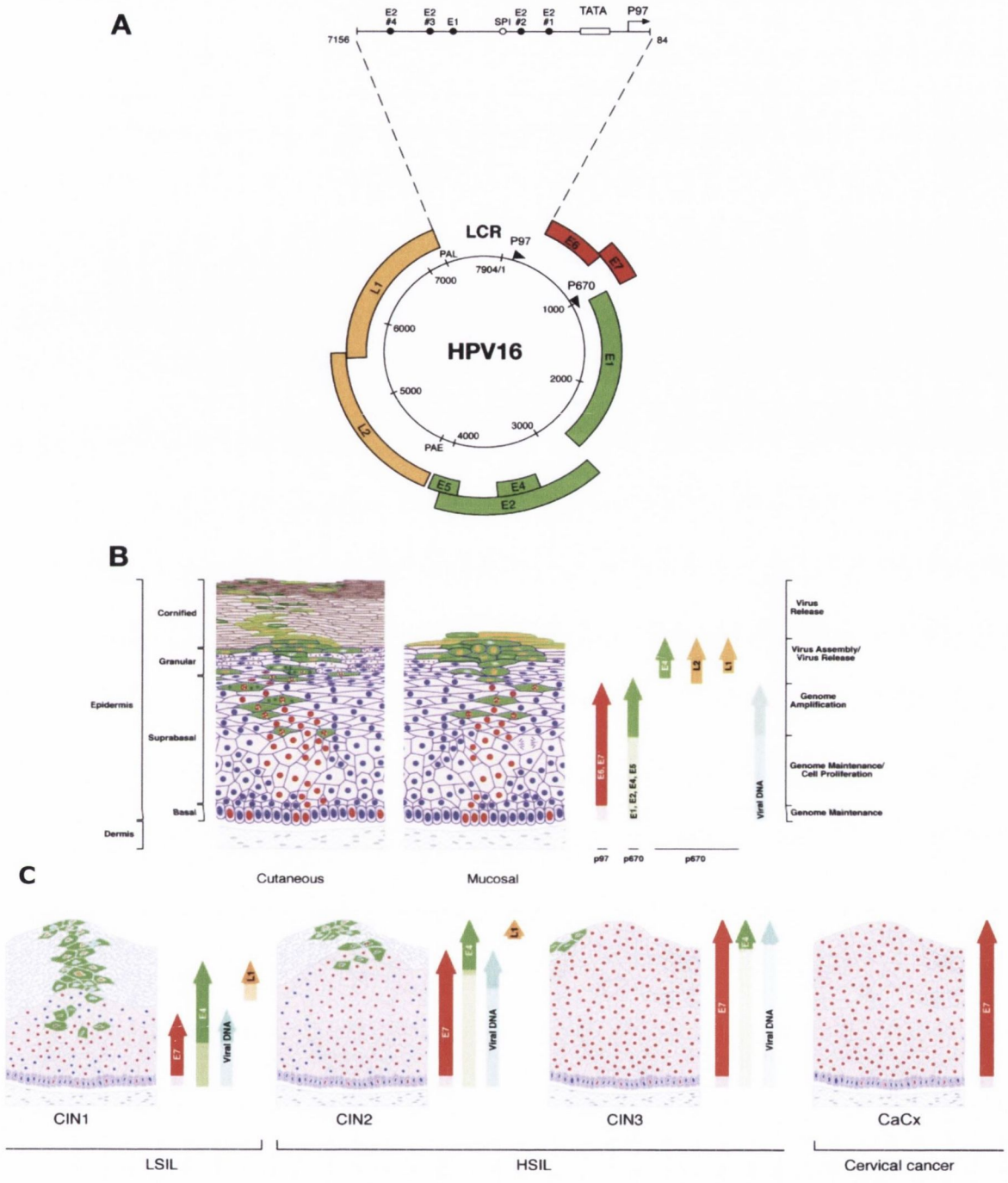

Figure 1-3 HPV gene expression

A) Genome of HPV 16 showing early and late region of the genome B) Upon cell division cells migrate away from the basal layer to begin differentiation. In order to maintain viral infection and replication E6 and E7 (red) are expressed, as they move towards the epithelial surface expression of L1 and L2 (yellow) for virus assembly occurs. Virons are shed form the surface. E7 is expressed in the lower epithelial layers, and is followed eventually by the expression of E4 and L1 closer to the epithelial surface. C) The pattern of gene expression changes following integration of the viral genome into the host genome leading to neoplastic progression. (Taken from Doorbar 2006)

1.6.3 Pathogenesis of HPV

Cervical malignancy is attributed to the increased expression of virus oncogenes E6 and E7, which together facilitate increased proliferation and cell immortalisation by interacting with cell cycle regulatory proteins (Figure 1.5C). The loss of cell cycle control can result in chromosomal instability. It has been suggested that during the repair process of chromosomal instability the HPV genome can become integrated into the host genome (Wentzensen et al., 2004). HPV DNA is found integrated in most invasive cancers (zur Hausen, 2002). It has been noted that much of the virus DNA can be lost during integration, in particular, the E2 region is often disrupted, as a result E6/E7 expression becomes deregulated a critical event in HPV induced carcinogenesis (Figure 1.5C)

The E6 ORF encodes several small proteins approximately 150 amino acids in size. E6 proteins interact with cellular factors resulting in deregulation of the cell cycle. The most important mechanism of E6 is the ability to interfere with the function of p53. The tumour suppressor p53 can induce growth arrest in the presence of DNA damage. It is stabilised post translationally to increase its half-life in case of DNA damage. E6 binds to p53 causing ubiquitin dependent degradation (Scheffner et al., 1990). This requires E6 association protein which acts as an E3 ubiquitin protein ligase generating a stable iospeptide bond via a lysine side chain forming an E6-p53-E6AP complex (Huibregtse et al., 1991). This leads to enhanced degradation of p53, shortening its half-life from 3 hours to 20 minutes (Scheffner et al., 1990). E6 activity has also been linked with degradation of other apoptotic regulatory proteins such as Bax and Bak (Thomas and Banks, 1998). In addition, E6 has been found to activate the cellular enzyme telomerase (Mantovani and Banks, 2001) which counteracts the shortening of chromosome telomeres, a process that naturally occurs with cell aging, increasing the life span of infected cells.

E7 is a small phosphoprotein of approximately 100 amino acids. E7 induces proliferation by binding cellular proteins of the pRB (retinoblastoma) family. In normal conditions pRB interacts with E2F transcription factor controlling transition of G1 to S phase by cyclin D (Dyson, 1998). E7 binds to functional pRB leading to its degradation and activation of E2F permitting cellular replication (Boyer et al., 1996, Chellappan et al., 1992). This forced entry into

the S phase is necessary for viral DNA amplification. Normally this would result in the stabilisation of p53 which would induce apoptosis however this is counteracted by E6 stimulated degradation of p53 by ubiquitin mediated proteolysis. E7 also binds inhibitory proteins p21 and p27, which inhibit the cyclin/cdk complex (Alberts, 2008) allowing re-entry into S phase of differentiating keratinocytes of the suprabasal layer (Longworth and Laimins, 2004). The E6 and E7 proteins have transformation and immortalisation properties. They are associated with the stimulation of quiescent cells of the suprabasal layer allowing them to enter the cell cycle and are consequently responsible for increased proliferation (Figure 1.5C).

1.6.4 Epidemiology

HR HPV types can be found widespread, they are typically transmitted by sexual contact (Kjaer et al., 2001). The prevalence of HPV is quite high, it is expected that over 80% of women will become infected with HPV at some stage of their life. Primary infection generally occurs in younger women under the age of 30 years (Jacobs et al., 2000). Infection is often asymptomatic and represents a transient event, some may present as an inconspicuous squamous intraepithelial lesion most of which will clear in within 6-14 months (Nobbenhuis et al., 1999, Woodman et al., 2001). The rate of clearance is high within the first few months following initial infection, approximately half clear within a year and more than 90% within a few years (Rodriguez et al., 2008). Clearance of HPV precedes regression of cervical abnormalities. An estimated 50% of women with LSIL can clear HR HPV infection within one year with HPV clearance preceding cytological regression by an average of 3 months (Nobbenhuis et al., 1999).

Epidemiological evidence based on the relationship between HPV infection and cervical cancer and cervical pre-cancer indicates that there is strong and specific association between HPV and cervical cancer. It has been recognised that this relationship is causal in nature and that while HPV is necessary it is not a sufficient cause of cervical cancer. Many HPV infections are not followed by cancer and represent productive infections where the expression of viral genes remains strictly regulated. The majority of CIN 1 lesions represent these types of infections, the virus is maintained as an episome with E6 and E7 gene

expression tightly regulated. The development of high grade neoplasia arises finally when HPV infection is not resolved by the host immune system and the infection remains persistent. Persistent infection can be long-lasting with initial HPV infection preceding the development of cervical cancer by a decade or more. Low grade cervical lesions can be caused by both low and high risk HPV types, here HPV gene expressions represent productive infection (Doorbar, 2006). During disease progression the pattern of viral gene expression changes, the expression of late proteins is reduced as production of infectious virons becomes restricted and eventually abolished in CIN 3+ lesions. Integration of the virus into the host genome leads to loss of E1 and E2 which is accompanied by deregulation of E6 and E7.

1.6.5 Histological and cytological features of HPV associated lesions

During productive infection, viral copy number increases as infected cells migrate through the epithelium to the surface where infectious virions are released. Morphologic features of productive HPV infections can be recognised as low grade lesions on histology and cytology. Whereas during transforming HPV infections, cell cycle control is lost and is characterised as high grade lesions on histology and cytology.

Currently in Ireland cervical screening is conducted using cytology. Signs of CIN can be identified on cytology by examining the exfoliated cells from the surface of the cervix. All degrees of neoplasia together with normal cells and once sampled correctly columnar cells from the endocervical canal coexist on a cervical smear. Cytological features of HPV induced lesions present as dyskaryosis which broadly correlates with CIN on histology. Dyskaryosis mirrors the maturity of the cells and is primarily recognised based on morphology of the nucleus. It is characterised by large hyperchromatic nucleus with irregular chromatin distribution and nuclear membrane. The degree of differentiation is an important consideration when determining grade, this is assessed on nuclear cytoplasmic ratio (N/C). For example, cells derived from low grade lesions resemble mature metaplastic, intermediate and superficial cells and have a lower N/C ratio (figure 1.3A) compared to high

grade lesions which reflect less mature cells such as parabasal and immature metaplastic cells and are expected to have a higher N/C ratio (figure 1.3B).

There are a number of cervical cytology classification systems which are sometimes used interchangeably, appendix table 2.0 outlines and compares the most frequently used classifications. The papanicolaou system uses a numeric structure, the British Society of Clinical Cytology (BSCC) classifies as low-grade dyskaryosis and high grade dyskaryosis. In Ireland, cervical cytology is classified according to the Bethesda System (TBS) as recommended by European guidelines for quality assurance in cervical cytology (Herbert et al., 2007). The Bethesda system classifies abnormalities as squamous intraepithelial lesion (SIL). Equivocal smears are classified as atypical squamous cells of undetermined significance (ASCUS) or atypical squamous cells can not excluded high grade (ASC-H). The 1988 TBS designated ASCUS to represent "*cellular abnormalities that were more marked than those attributed to reactive changes but that quantitatively or qualitatively fell short of a definite interpretation of SIL*" (Solomon et al., 2002). In 1988, TBS introduced a two tier nomenclature structure for SIL, low grade SIL (LSIL) and high grade SIL (HSIL) based on the knowledge that LSIL are likely to regress whereas HSIL have a higher risk of progression to invasive disease. Examples can be seen in figure 1.3. Glandular abnormalities are termed atypical glandular cells (AGC) and adenocarcinoma in situ (AIS).

Cells infected with HPV can be identified by cytological features in squamous cells, these are known as koilocytes. They are characterised by enlarged bi/multi-nucleated cells with a zone of peri-nuclear clearing surrounded by a dense peripheral cytoplasm (Meisels and Fortin, 1976). However, these cells may not always be present in cervical smears and are generally only identifiable in minor lesions. Molecular testing for HPV is a more sensitive method of detection.

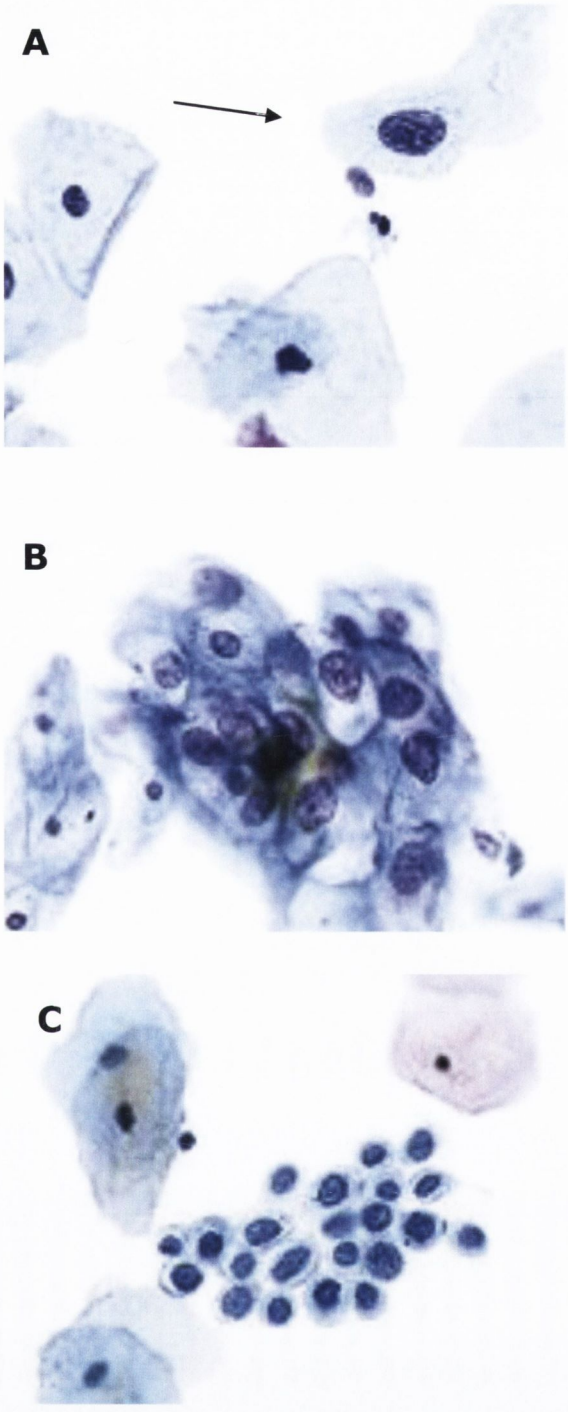

Figure 1-4 ThinPrep pap smears

(A) LSIL: Slightly increased N/C ratio, nucleus exhibit smooth to faintly irregular nuclear membranes (60X). **(B)** LSIL with HPV changes: well-defined cytoplasmic clearing with a dense cytoplasm at peripheral rim (60X). **(C)** HSIL: N/C ratio is markedly increased and asymmetrical structural abnormalities (60x). (www.cytologystuff.com)

Cytology is used as a screening tool, to help the identification of features of dysplasia induced by HPV. Following cytological identification, the next step is colposcopy. Referral criteria vary worldwide and will be discussed in section 1.7. Colposcopy involves magnified visual inspection of the cervix using a colposcope. Features of the cervical epithelium are examined following the application of 3-5% acetic acid followed by lugols iodine solution. Acetic acid dehydrates the cells, reversibly coagulating nuclear proteins, this produces what is known as acetowhitening (AWE). The intensity of AWE depends on the extent of nuclear activity and hence the degree of abnormal changes. Margins and surface contours are also taken in to account when grading. Lugols iodine may be applied to further inspect the cervix. Normal intermediate and superficial cells are glycogen rich and stain brown/black with lugols iodine. This is a sign of normal maturation, however dysplastic epithelium contains little or no glycogen and does not take up iodine, and these cells appear mustard yellow/saffron following the application of iodine. Glycogen is lacking in endocervial cells and does not change colour with application of lugols iodine (Buckely and Bulter 1982, Sellors and Sankaranarayanan 2003).

Final diagnosis is only confirmed by histopathological examination of biopsies or excised tissue specimens. Grades CIN 1, CIN 2 and CIN 3 can be seen in figure 1.4. For CIN 1, nuclear abnormalities can be seen throughout the lower region of the epithelium, cells in the middle and upper thirds of the epithelium demonstrate cytoplasmic differentiation. These cells are pleomorphic with prominent nucleoli and coarse granular chromatin. Cells of the lower third of the epithelium show no differentiation or stratification. They lack clearly defined borders and have a high nuclear cytoplasmic ratio and nuclear crowding. The upper layers show decreased nuclear cytoplasmic ratio with cytoplasmic maturation and stratification. In CIN 1, cells tend to show normal maturation but differ from normal cells by their abnormal nuclei (figure 1.4A). Aberrant keratinisation can be seen in some cases. Mitotic figures are usually absent. CIN 2, similar to CIN 1 with abnormal cells characterised as before have more marked nuclear abnormalities, they are undifferentiated, non-stratified cells with high nuclear cytoplasmic ratio and pleomorphic nuclei are now extended in to the middle portion of the epithelium (figure 1.4B). Mitotic figures are also now present. In CIN 3 the abnormal cells are extended into the upper layer of the cervix. Enhanced undifferentiated, non-stratified pleomorphic crowded nuclei occupy the full thickness of the epithelium. Mitotic

figures can be seen throughout (figure 1.4C) (Sellors and Sankaranarayanan, 2003).

Based on what we know of the biology of HPV infection it is recognised that cervical neoplasia is capable of spontaneous regression, this is particularly the case for low grade lesions CIN 1 which often represent transient HPV infections. It has been estimated that 70-80% of CIN 1 will regress with only 20% likely to progress to CIN 2+ (Cox et al., 2003, Moscicki et al., 2010). Progression of high grade lesions is thought to be in the region of 5% for CIN 2 and 12-30% CIN 3 (Ostor, 1993, McCredie et al., 2008). The progression rate of CIN will be discussed further in chapter 3.

Histology is considered the gold standard for diagnosis of cervical neoplasia. Accurate grading of CIN is of utmost importance as this determines clinical management. CIN 2 and CIN 3 are considered high grade lesions and represent transforming HPV infections.

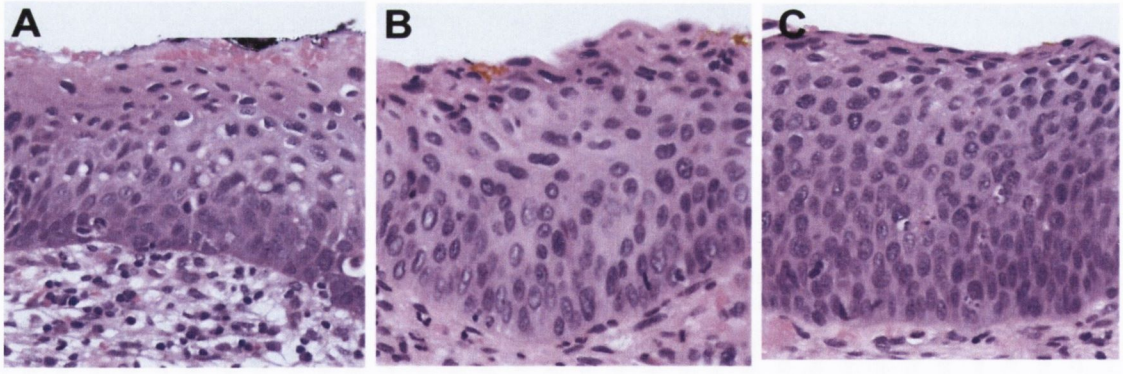

Figure 1-5 Cervical intraepithelial neoplasia (CIN)

(**A**) CIN 1: dysplastic squamous cells in the lower one-third of the epithelium with HPV changes in the rest of the superficial epithelium. (**B**) CIN 2: dysplastic squamous cells in two-thirds of the epithelium, the upper half of the epithelium shows HPV changes; (**C**) CIN 3: dysplastic squamous cells marked throughout the full thickness of the epithelium. HPV changes are confined to the superficial layer. (Taken from Wei 2009).

1.7 Cervical cancer screening

According to "A strategy for Cancer Control in Ireland 2006," screening is a means of detecting disease before it has developed to a point where it results in symptoms. It does not guarantee a diagnosis but rather highlights those who require further investigation with the aim to improve survival, limit morbidity and improve quality of life.

In Ireland, up until 2008 cervical screening was opportunistic. An organised national cervical screening program, CervicalCheck was introduced in late 2008, and provides free smear tests for detecting pre-invasive neoplasia to women aged 25 to 60 years. It is based on a "call, re-call" system of invitation over three to five year screening intervals depending on age. Women with cytological high grade abnormalities and repeat minor abnormalities are referred to colposcopy, this is based on the knowledge that minor abnormalities are likely to regress which is the rationale for repeat cytological surveillance, whereas women with cytology showing HSIL have a higher risk of progression to invasive disease. The generic classification minor abnormalities encompass both ASCUS and LSIL. Currently, guidelines recommend cytology indicating LSIL needs to be repeated after 6 months (Jordan et al, 2008). Similarly, with ASCUS, however three repeat abnormal smears are required before referral to colposcopy. Patients with smears more severe than LSIL are referred immediately for colposcopy (Jordan et al, 2008). The CervicalCheck screening protocol can be seen in appendix table 1.0.

The role of colposcopy is to determine the source of abnormal cells and make an informed decision whether or not treatment is required. Appropriate treatment and follow up is based on cytology, colposcopy and histology results taking individual clinical situations into account for each individual patient. This is in line with the recommendations from the European Guidelines for quality assurance in cervical screening (Jordan et al., 2008).

1.7.1 HPV testing in cervical screening

Despite challenges, the use of cervical pap cytology has greatly reduced the incidence of cervical cancer. The classification for reporting cervical pap smears has evolved since its implementation in the 1940s. Management of

cytological abnormalities continues to be refined but there remain challenges in this area. The pap test is a subjective test, with many limitations, these are largely due to minor abnormalities. One of the most commonly reported smear results in most screening programs is one of ambiguity, ASCUS. ASCUS smears accounted for 30,964 (9.3%) smears in Ireland from 2010-2011, representing 59.7% of abnormal smears (CervicalCheck, 2010-2011). This was closely followed by LSIL, accounting for 13,102 (3.9%) smears, representing 25.3% of abnormal smears. Collectively, approximately 85% of abnormal cervical smears in Ireland represented minor abnormalities. These figures are higher than what is seen in other countries with organised cervical screening programs, in particular the reported rate of ASCUS. For example, in England, the NHSCSP reported an ASCUS rate of 3.2% and LSIL 1.9% for the same period (NHSCSP 2011). In The Netherlands, <2.5% of smears are minor abnormalities (Health Council of the Netherlands 2011), in France ASCUS is reported in the region of 3% (Bergeron, 2012). In fact, it has been recommended that rates of ASCUS should not be above 3% (Davey et al., 2000). We recognise that Ireland is reporting a figure of 9.3%, three times what is recommended. There are several reasons to explain the high rates of ASCUS seen in Ireland. Firstly, it is important to note that our screening program only began in 2008, prior to 2008 ASCUS was reported in the region of 3% (ICSP Program Report 2000-2008). In 2008, CervicalCheck went nationwide, as the entire population was screened rates of ASCUS increased to over 9%. This coincided with the out sourcing if cytology services to the United States based company Quest Diagnostics where a different screening protocol in place. In the United States women are screened by cytology on an annual basis (Wright et al., 2006) compared to 3 year intervals in Ireland. This may explain divergences in the rate of minor cytological abnormalities in Ireland compared to other countries. A consequence of this is an increased burden on our screening program, more women are brought back for repeat smears which ultimately results in excessive colposcopy referrals. CervicalCheck has recognised this issue and made a number of efforts to improve screening such as extending screening intervals for ASCUS abnormalities from 6 months to 12 months. In addition, efforts to bring cytology screening back to Ireland have been made, in 2012, MedLab Pathology Ldt (Dublin), were awarded a contract along with Quest Diagnostics Inc. to provide cytology services. Plans for a designated National

Cytopathology Training Centre at the Coombe Women's and Infants University Hospital, Dublin are also underway. Finally, to further stratify minor abnormalities HPV triage due to be introduced in autumn 2013. However, due to the nature HPV, additional methods will be required to managed transient HPV infections to avoid over referral to colposcopy.

While the management of HSIL is well defined due to the knowledge that these abnormalities are more likely associated with transforming HPV infections and at a higher risk of progression to cervical cancer. The management of minor abnormalities remains a challenge. Many minor cytological abnormalities are unlikely to progress to a more severe lesion and become cancerous.

The recognition of the association between specific HR HPV types and cervical cancer and its pre-cursor lesions has been enormously important in the context of cervical screening. It has allowed for the development of new methods in screening, aiding in early detection of cervical neoplasia by identifying the presence of HR HPV. This will instigate further investigation to identify infections that are persistent, a risk factor in the development of progressive lesions (Ho et al., 1995). HPV testing has been found to predict the risk of cervical cancer and its pre-cursors better than cytology or colposcopy which simply highlights signs of HPV infection. In the absence of HR HPV, the risk of cervical cancer is extremely low. This is an important attribute as a high negative predictive value (NPV) permits extension of screening intervals (Dillner et al., 2008). Evidence from randomised control trials (RCT) supports the incorporation of HPV based methods into screening programs (Bulkmans et al., 2007, Kitchener et al., 2009, Ronco et al., 2010, Rijkaart et al., 2012a). HPV DNA testing can provide an automated, objective and sensitive test, allowing for longer screening intervals, improve costs, in addition to convenience for women taking part in screening.

HPV DNA testing can be applied to the three basic levels of cervical screening:

- Primary Screening
- Management and triage
- Test of cure

1.7.1.1 Primary Screening

A number of Randomised Controlled Trials (RCT) have shown that the sensitivity of HPV DNA testing exceeds cytology for the detection of CIN 2+ (Mesher et al., 2010, Kitchener et al., 2009 , Ronco et al., 2010 Rijkaart et al., 2012a). In addition, the NPV of a single HPV test is extremely high, 99.7% (95% CI 99.6-99.9) and greater than that shown for cytology 99.0% (95% CI 98.7 -99.5) (Dillner et al., 2008). The specificity of HPV DNA detection has consistently scored lower than cytology, however this loss in specificity can be minimised by avoiding HPV-based screening in younger women and applying appropriate triage algorithms (Arbyn 2012). Results from a large Dutch RCT, POBASCAM (Population Based Screening study Amsterdam) reported a reduction in the incidence of CIN 3+ over two screening rounds using a validated HPV DNA test compared to cytology alone (relative risk 0.73 95% CI 0.55-0.96). The knowledge gained from this RCT has led to The Netherlands to officially introducing HPV DNA primary screening in women over 30 years. The management of screen positive women will be crucial as many positive results are representing transient infection that will not develop into a high grade lesion. Results to date indicate that reflex cytology would provide optimal triage for HPV positive cases compared to a repeat HPV test (Cuzick et al., 2003). Other markers are currently being investigated such as restricted genotyping (HPV 16 and 18), HPV mRNA detection and $p16^{INK4A}$ immunocytochemistry in order to minimise the number of women in short term follow up due to a HPV positive result. Further cross-sectional and longitudinal research is necessary in order to identify a suitable candidate test.

1.7.1.2 Management and triage (cytology based screening)

There is on-going debate over what is considered optimal management with respect to minor abnormalities LSIL and ASCUS. An ideal triage test will identify which cases of minor abnormal smears are masking high grade disease and/or are likely to progress. It is reasonable that HPV DNA testing would be a likely candidate test for triage due to its high sensitivity and NPV. This has been proven in a number of studies showing that HPV triage is accurate in detection of CIN 2+ in patients with minor smears (Arbyn et al., 2004, Moss et al., 2006, Ronco et al., 2007) The ASCUS-LSIL Triage Study (ALTS) study was initiated to seek the comparison between three main

management strategies for LSIL and ASCUS: repeat cytology, immediate colposcopy and HPV triage. Women were followed at 6 month intervals over a 2 year period. Findings concluded that HPV could be approved for use as triage to ASCUS cytology results (ASCUS-LSIL Triage Study group, 2003). Data relating to triage of LSIL is mixed, some report that its use is limited due to the high prevalence of HPV in LSIL (ASCUS-LSIL Triage Study group 2003). Others have found HPV triage of LSIL to be successful (Kelly et al., 2011). Mixed reports on the value of HPV testing in LSIL is mainly due to variation in how women are managed by HPV triage. For example, triage of repeat minor cytology or triage of initial minor cytology (Cuzick et al., 2008). Triage of LSIL and ASCUS will be discussed further in chapter 3.

1.7.1.3 Test of cure

Treatment of CIN by excision procedures such as LLETZ is successful at eradicating disease. Treatment failure can occur and has been reported in the region of 10%, re-occurrence most likely occurs during the first 2 years following treatment (Soutter et al., 1997, Baldauf et al., 1998, Aerssens et al., 2008). In the past, standard practice for post treatment follow up involves surveillance of women by cytology at 6 months followed by annual smears for 10 years (Leusley and Leeson 2004). Follow up over long term periods is associated with cost and risk of non-compliance. In addition, anatomical changes post treatment can result in sampling issues for cytology testing (CervicalCheck 2012). The knowledge that in the absence of HPV the risk of CIN 2+ is very low has led to the approach of HPV DNA testing for post treatment management of women. This will help identify if women who have been treated are harbouring residual disease. The multicentre HART study examined the role of HPV testing post treatment (Mesher et al., 2010). Findings concluded that HPV testing offered improved protection from CIN 2+ after a negative test result compared with the protection afforded from a normal cytology result. Women with both cytology and HPV negative results at the baseline only 0.2% had CIN 2+ identified during five years follow up (Mesher et al., 2010). Therefore a negative HPV test can safely avoid unnecessary colposcopy and reduce long term follow up by cytology alone.

In 2012, HPV testing in "test of cure" was introduced in Ireland as an adjunct to cytology. The CervicalCheck algorithm for HPV testing post treatment involves HPV/cytology test at 6 months and 18 months. Women with repeat negative tests are returned to routine screening. Women positive for cytology or HPV test at 6 or 18 months undergo further colposcopic investigations.

1.8 HPV Vaccination

Vaccines have been developed in an attempt to prevent both cervical cancer and genital warts. They are made from highly-immunogenic virus-like particles (VLPs) of the capsid L1 protein (Barr et al., 2008). Immunization with these VLPs should induce high titres of antibodies protecting against persistent HPV infection and CIN lesions. There are currently two licensed HPV prophylactic vaccines. Cervarix® (GlaxoSmithKline) is a bivalent vaccine that is available for protection against HPV types 16 and 18 (Pomfret et al., 2011). Gardasil® (MERCK) is a quadrivalent vaccine which was developed to protect against two non-oncogenic HPV types (HPV 6 and 11) and two oncogenic HPV types (HPV 16 and 18). Both vaccines may also offer some cross-protection against other types, HPV 31 (related to HPV 16) and 45 (related to HPV 18) (Pomfret et al., 2011). Vaccination has been proven to be effective if administered before infection with HPV, and is therefore recommended for adolescent girls, aged 12, to protect them from exposure to HPV before they become sexually active. Vaccination programs have been implemented worldwide. In September 2011 the Irish National Vaccination Programme was launched in schools to provide all girls entering first and second year in the secondary school system, the course of HPV vaccine.

HPV types 16 and 18 are responsible for roughly 70% of cervical cancers (Bosch and de Sanjose, 2003, Smith et al., 2007a). As vaccination will not completely protect against all HR HPV types, it is recommended that women continue to undergo cervical cancer screening. Investigation is underway for an L2 based prophylactic vaccine that will offer increased cross-protective neutralizing antibodies protecting against a broader spectrum of HPV types (Schellenbacher et al., 2012). However, L2 shows weaker immunogenicity, the TA-CIN vaccine is currently in early clinical studies, it includes L2, E6 and E7

and is believed to increase therapeutic response (Hibbitts, 2010, Stern et al., 2012).

It is estimated that the introduction of prophylactic HPV vaccines will reduce the incidence of cervical cancer and precursors and therefore will reduce the PPV of abnormal smears from currently 50–70% down to 10–20% in regions with high vaccination coverage (Franco et al., 2006). Australia was the first country to introduce vaccination. Early effects observed included a reduction in genital warts in sentinel STD clinics of 77%, this was matched with a 44% reduction in age matched heterosexual males, indicating herd immunity (Donovan et al., 2011). In addition, studies from the Victorian Cervical Cytology Registry have shown a decrease in high grade cervical abnormalities in young women under the age of 18 years after the implementation of the vaccination programme (Brotherton et al., 2011).

As the vaccinated adolescent generation reach the appropriate age for cervical screening, the rate of screen-positive for cervical lesions should decline, as such carcinogenic HPV infections and their associated CIN 3+ lesions will become less frequent (Schiffman et al., 2011). Leading to a reduction in the number of women referred for colposcopy (Ault, 2007). This will further strengthen the argument for less frequent screening and the switch from cytology to molecular based methods as cytological abnormalities will become less common and the need for a more objective test will be essential. Overall this should translate to reduced costs to the healthcare system, or at least cover the cost of vaccination.

1.9 Cervical cancer biomarkers

Currently appropriate management of minor cytological abnormalities involves a complex work up of repeat tests and possible colposcopy and biopsy. Associated with this are high costs in addition to the psychological effect on the women involved. Despite the evident success of HPV DNA testing, specificity for detection of high grade disease is low resulting in a number of false positives. This is due to the fact that a majority of HPV infections that induce minor abnormalities are acute infections that are cleared spontaneously followed by regression of cellular lesions (Nobbenhuis et al., 1999). Our

knowledge of the pathogenesis of HPV and understanding of the pattern of gene expression that leads to neoplastic progression has developed a foundation for the selection of biomarkers that may be used to predict disease outcome. The identification of biomarkers, both viral and cellular, will play a role in increasing the overall efficiency of cervical screening.

Several novel biomarkers such as HPV mRNA and $p16^{INK4A}$/Ki-67 have been evaluated in initial studies and have shown promising results. In the context of triage an appropriate management strategy is yet to be identified that will deliver high sensitivity and specificity for detection of underlying CIN 3+.

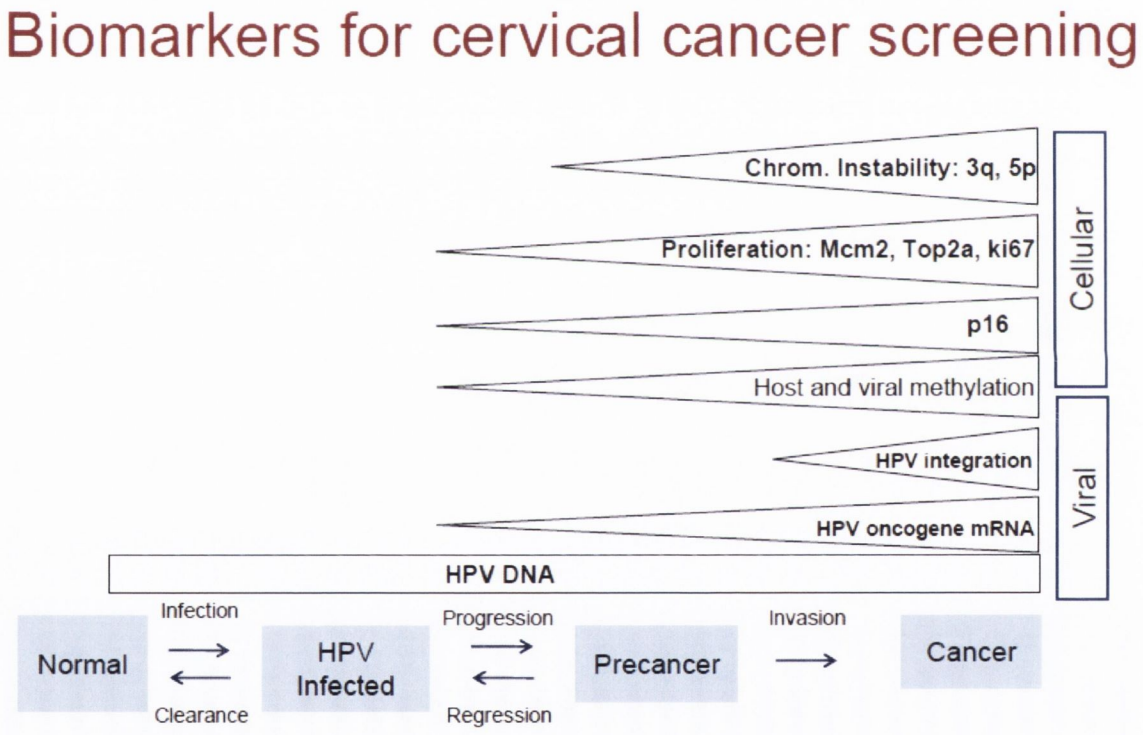

Figure 1-6 Biomarkers for cervical cancer screening

During progression from transient HPV infection to transforming infection and progression of cervical lesions, HPV E6/E7 oncogene expression is substantially increased. Deregulated expression of HPV oncogenes induces chromosomal abnormalities, expression of proliferation markers and expression of $p16^{INK4A}$. HPV integration is a rather late event following the chromosomal instability. (Taken from Wentzensenand and Knebel Doeberitz 2007).

1.9.1 HPV mRNA

The understanding of the major molecular events involved in transformation and malignancy has led to the identification of a number of HPV and host cell biomarkers. Progressive lesions are characterised by the deregulated expression of viral oncogenes exemplifying HPV transforming infections. The two early genes, E6 and E7, are the most important agents in cellular transformation induced by HPV. Their increased expression is a hallmark of progression from transient HPV infection to transforming infection and development high grade disease. Their over expression can be monitored directly by viral markers or indirectly by cellular markers. Over expression of HPV E6/E7 can be measured directly by detecting the presence of mRNA transcripts in cervical specimens. Several commercially available assays have been developed for the specific detection of HR HPV E6/E7 mRNA. The PreTect™ HPV Proofer assay (Norchip, AS) and the related NucliSENS Easy Q HPV v1 (Biomerieux) make use of NASBA (Nucleic Acid Sequenced Based Amplification), an isothermal mRNA amplification method for the detection and genotyping of 5 HR HPV types (16, 18, 31, 33 and 45) (Molden et al., 2007, Jeantet et al., 2009). More recently The APTIMA assay (Gen-Probe, CA) was developed. This makes use of Transcription Mediated Amplification (TMA) technology to amplify and detect E6/E7 mRNA from 14 HR HPV types (Castle et al., 2007). Due to the difference in the number of genotypes detected by each assay, clinical sensitivity and specificity differs between NASBA based assays and APTIMA. A meta-analysis by Burger *et al* made a direct comparison between DNA based and mRNA based HPV detection. Findings indicate that mRNA tests have fewer false positives than DNA tests leading to higher specificities. Overall APTIMA mRNA test consistently has a higher sensitivity, in the range 91%-95%, compared to PreTect™ Proofer/Easy Q which ranged from 41%-86%. While APTIMA had slightly lower specificities (42%-56%) when compared to the PreTect™ Proofer/Easy Q (63%-85%) (Burger et al., 2010). The use of HPV mRNA detection based methods will be discussed in chapter 5.

1.9.2 $p16^{INK4A}$

Over expression of $p16^{INK4A}$ is a direct consequence of deregulated HPV oncogene expression. The $p16^{INK4A}$ protein is a cyclin dependent kinase inhibitor that blocks CDK 4 and CDK 6 mediated pRB phosphorylation to inhibit E2F dependent transcription and cell cycle progression (Zhang et al., 1999). However, in HPV infected cells, under the control of E7, phosphorylation mediated control of pRB is lost resulting in the over expression and accumulation of $p16^{INK4A}$ in cells (Figure 1.6) (Sano et al., 1998, Klaes et al., 2001, Wentzensen and von Knebel Doeberitz, 2007). Hence, increased $p16^{INK4A}$ expression acts as a surrogate marker of transforming HPV infection. $p16^{INK4A}$ staining by immunocytochemistry has been applied to cervical biopsies demonstrating strong diffuse staining in almost all high grade lesions (Sano et al., 1998, Klaes et al., 2001, Murphy et al., 2005). It has been identified as a useful marker to distinguish between atypical non-HPV changes from HPV-related changes (Redman et al., 2008) in differential diagnosis of low and high grade CIN, and improving accuracy in diagnosis of high grade lesions (Bergeron et al., 2010, Tsoumpou et al., 2009). It has also been suggested as a potential prognostic marker of high grade disease (Queiroz et al., 2006, Carozzi et al., 2008).

Over expression of $p16^{INK4A}$ has been analysed in cytological preparations, evidence from a number of studies has shown that the use of $p16^{INK4A}$ immunocytochemistry has a high sensitivity and specificity for detection of underlying high grade disease on cytology (Denton et al., 2010, Wentzensen and von Knebel Doeberitz 2007, Monsonego et al., 2007). One limitation with the analysis of $p16^{INK4A}$ as a marker is that expression can be seen in non-neoplasic cells such as endocervical, squamous metaplastic, or atrophic cells (Trunk et al., 2004). Wentzensen et al proposed a nuclear scoring system to avoid miss-classification of $p16^{INK4A}$ expression positive cells. This score was based on a four-tiered classification system of nuclear abnormalities including increase size, granular/hyperchromatic chromatin, irregular shape or variable morphology. $p16^{INK4A}$ positive squamous cells were scored as 1, no abnormal nuclei, from 2-4 representing increased degree of nuclear abnormality (Wentzensen et al., 2005). Several studies have examined the use of $p16^{INK4A}$ immunocytochemistry to as a tool in the triage of LSIL/ASCUS. A systematic

review by Roelens *et al* compared $p16^{INK4A}$ and HPV DNA testing in minor cytology. The authors reported pooled sensitivity of $p16^{INK4A}$ for detection of CIN 2+ was 83.2% (95% CI 76.8-88.2) and 83.8% (95% CI 73.5-90.6%) and a pooled specificity of 71.0% (95% CI 65.0-76.4) and 65.7% (95% 54.2-75.6) for ASCUS and LSIL respectively (Roelens et al., 2012). Reported data on a subset of 8 studies comparing $p16^{INK4A}$ and hc2 concluded both tests had a similar sensitivity in detection of CIN 2+ in ASCUS, $p16^{INK4A}$ demonstrated a lower sensitivity compared to hc2 in LSIL. $p16^{INK4A}$ had significantly higher specificity in LSIL and ASCUS for the detection of CIN 2+ (Roelens et al., 2012).

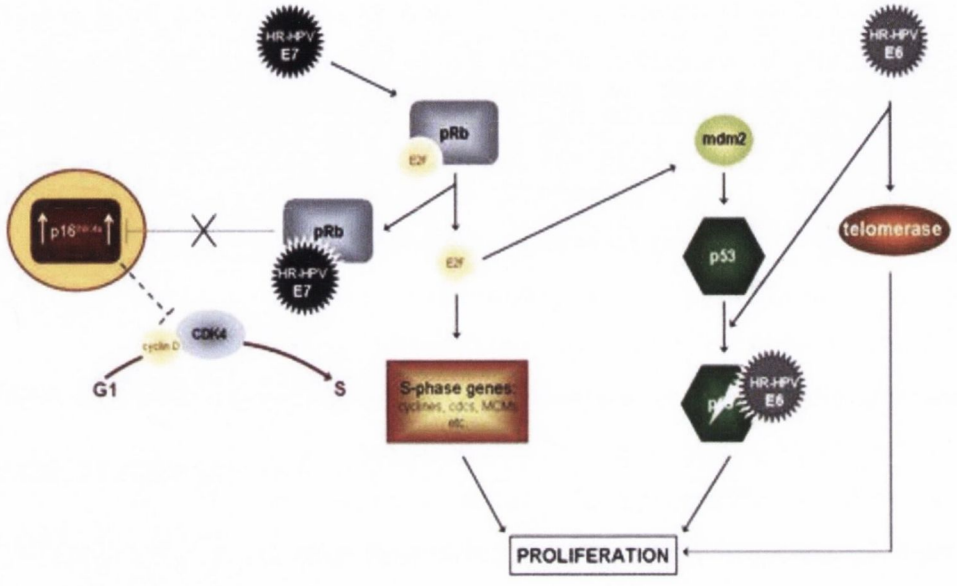

Figure 1-7 Interference of HPV oncogenes on cell cycle regulatory proteins

In normal cells phosphorylation of pRb by Cdk4/6 releases E2F from its binding to pRb and leads to the expression of S-phase genes. Cdk4/6 activity is blocked by $p16^{INK4A}$ that is activated by E2F and blocked by pRb/E2F complexes. E7 leads to disruption of E2F-pRb binding. $p16^{INK4A}$ is strongly overexpressed due to the loss of pRb/E2F repression and the strong activation by free E2F. However, S-phase genes are continuously activated since the $p16^{INK4A}$ mediated repression of Cdk4/6 has no downstream effect on pRb. Deregulated S-phase activation in normal cells would lead to apoptosis. Apoptosis is abrogated by E6 mediated degradation of p53. (http://www.klinikum.uni-heidelberg.de/HPV-pathogenesis.atb)

1.9.3 Ki-67

Ki-67 is a proliferation marker that is normally confined to the parabasal layer of cervical squamous epithelium (Bulten et al., 1996). It has a very complex and specific localization pattern within the nucleus, which changes during the cell cycle when it is relocated from the nucleus to the perichromosomal layer (Endl and Gerdes, 2000). It has been identified in HPV transformed cells, dysplastic lesions and carcinoma of the cervix (Kruse et al., 2001) and has been found to have a good prognostic and diagnostic value in a number of cancers (Kuo et al., 2006, Brown and Gatter, 2002). Kruse and colleagues have proposed a number of criteria in staging Ki-67 positive cells including Ki-67 cell clusters to discriminate low grade CIN lesions from normal and reactive epithelia (Kruse et al., 2002). Another classification model based on the stratification index and the percentage Ki-67 positive nuclei in the middle third layer of the epithelium distinguishes between low and high risk of progression (Kruse et al., 2004). This qualitative Ki-67 model was found to have a good prognostic value (Kruse et al., 2004). Its use has been examined in cytology specimens and has been found to be an accurate predictor of high grade disease (Sahebali et al., 2003, Dunton et al., 1997, Zeng et al., 2002). However, there is variation in interpretation with some studies reporting based on number of positive cells (Sahebali et al., 2003) others based on morphology score (Dunton et al., 1997). Ki-67 positive staining can be seen in metaplastic cells (Dunton et al., 1997) and so interpreting staining is morphology dependent.

1.9.4 $p16^{INK4A}$ and Ki-67 dual testing

$p16^{INK4A}$ and Ki-67 have recently been combined in a dual staining kit (CINtec PLUS) that simultaneously detects both proteins in cervical smears. The combined expression of $p16^{INK4A}$ and Ki-67 should be indicative of HPV mediated deregulation of the cell cycle and likelihood of progression to high grade CIN. Hence, co-expression is morphology independent, overcoming a key disadvantage that limits the use of either biomarker as standalone test. Initial studies showed promising results as a triage tool for LSIL/ASCUS. Schmidt et al reported sensitivity for CIN 2+ as 92.2% (95% CI 83.3-97.1) and 94.2% (95% CI 88.8-97.4) for ASCUS and LSIL respectively and

specificity 80.6% (95% CI 75.6-85.1) and 68.0% (95% CI 62.2-73.4) (Schmidt et al., 2011). Petry et al investigated the use of p16 ^{INK4A}/Ki-67 in the triage of HPV positive, pap negative women over the age of 30 reporting high sensitivity, 91.9% (95% CI 78.1-98.3) and specificity 96.4% (95% CI 81.7-99.9) for detection of CIN 2+ (Petry et al., 2011).

1.9.5 TOP2α and MCM

The nuclear enzyme Topoisomerase II-α (TOP2α) is a regulator of DNA topology. It functions by un-knotting DNA for DNA replication, and plays an important role in chromosome organization and segregation (Wang 2002). Increased expression of TOP2α has be identified on histological specimens of CIN and cervical carcinoma (Davidson et al., 2000) and is associated with progression from CIN 2 to CIN 3 (Branca et al., 2006). Minichromosome maintenance proteins 2-7 (MCM) are DNA replication licensing proteins. Together with cell cycle division protein (CDC6) they form a pre-replication complex that is required for DNA replication (Shin et al., 2003). During replication, the complex is phosphorylated and disassembled to prevent reinitiating of DNA replication per cell cycle (Li et al., 2011a, Ibarra et al., 2008). In dysplastic cervical cells, there is a continuous activation of the replication complex. Increased expression of MCMs has been identified in dysplastic cervical cells (Williams et al., 1998, Murphy et al., 2003). MCM2 and TOP2α, have been made commercially available in the ProEx C Kit. Preliminary studies have shown ProEx C score was consistently negative in Normal cases and positive in HSIL cases (Shroyer et al., 2006). Compared with p16^{INK4A} staining it has shown to be less sensitive but more specific for detection of CIN 3+ (Guo et al., 2011).

1.9.6 Methylation

As part of the search for novel biomarkers in cervical cancer attention has been directed to methylation. Methylation is an epigenetic modification of gene expression. It involves the addition of a methyl group, usually at cytosine-guanine dinucleotide (CpG) islands resulting in transcriptional repression. Methylation is a necessary process within the cell for maintaining genomic

stability. However, in many cancers tumour suppressor genes are inactivated by methylation. Methylation alterations to gene expression have been studied in cervical cancer and pre-cancer, a number of genes have been identified to be consistently methylated. Figure 1.8 shows a number of genes which have been shown to have distinct hypermethylation patterns in both squamous cell carcinoma and adenocarcinoma of the cervix.

Cervical Cancer compared to Normal Control	**Squamous Cell Carcinoma compared to Adenocarcinoma**	**Adenocarcinoma compared to Squamous Cell Carcinoma**
DAPK, HIC1	p16, DAPK	HIC1, APC
	DAPK, CDH1	HTLF, TIMP3
p16 ^{INK4a}, DAPK	CDH1	
CDH1, DAPK, RARB, HIC1	CDH1, RARB, FHIT, MGMT	HIC1, APC, BRCA1
DAPK, MGMT, p16, PTEN	DAPK	PTEN
CALCA, DAPK, ESR1, TIMP3, APC, RARβ2		APC, TIMP3, RASSF1A

Figure 1-8 Hypermethylation pattern of genes detected in squamous cell carcinoma and adenocarcinoma of the cervix. (Taken from Martin et al 2007)

Methylation patterns identified in numerous genes have been linked with high grade CIN. Examples include death-associated protein kinase 1 (DAPK1), retinoic acid receptor (RARB) and TWIST1 which were used as a panel of targets in exfoliated cell samples by Feng *et al.* At least one of the three genes was hypermethylated in 57% of samples with CIN 3+ with a reported sensitivity of 60% and a specificity of 95% (Feng et al., 2005). Recently, interest has been focused on cell adhesion molecule (CADM) and T-lymphocyte maturation associated protein (MAL). These genes were frequently silenced by methylation in cervical cancer cell lines and appeared to possess tumour suppressor activity when their expression was reconstituted, indicating a functional contribution to cervical cancer development (Overmeer et al., 2009, Steenbergen et al., 2004). Hesselink *et al* investigated a methylation marker panel including CADM1-m12, CADM1-m18, MAL-m1, MAL-m2 on cervical scrapings. The authors reported CADM1-m18 combined with MAL-m1 was the best panel. For detection of CIN 3+, sensitivities range from 100% (95% CI 92.4–100) to 60.5% (95% CI 47.1–74.6), with corresponding specificities ranging from 22.7% (95% CI 20.2–25.2) to 83.3% (95% CI 78.4–87.4) depending on C_t ratio threshold (Hesselink et al., 2011). CADM1 and MAL were suggested as a possible triage tool for HPV positive women, however further studies are needed (Overmeer et al., 2011). Eijsink *et al* reported a methylation panel consisting of JAM3, EPB41L3, TERT and C13ORF18. This panel was detected in 94% of cervical cancers, 82% CIN 3+ and 65% CIN 2+ (Eijsink et al., 2012). Methylation markers have also been investigated in viral genes. Strong methylation has been identified in E2, L2 and L1 regions of HR HPV (types 16, 18, 31, 45) over 80-120 CpG sites. Methylation was found to be enhanced in CIN 3 compared to normal controls (Wentzensen et al., 2012). A possible combination panel of both viral and host methylation sites have been suggested (Wentzensen et al., 2012).

Methylation of $p16^{INK4A}$ has been identified in many cancers and is considered important in the development of squamous cell carcinoma (Lea et al., 2004, Nuovo et al., 1999, Belinsky et al., 1998). Some studies have reported methylation of the promoter region of the $p16^{INK4A}$ gene (Lea et al., 2004, Huang et al., 2011). However, conflicting data exists on the methylation of $p16^{INK4A}$ with respect to cervical cancer (Martin et al., 2007). Some studies report hypermethylation of the $p16^{INK4A}$ promoter region is associated with loss of $p16^{INK4A}$ expression (Kang et al 2006). Methylation data is often obtained

using methylation specific PCR (MSP), which reports methylation based on 7 CpG islands (Herman et al., 1996). Nehls et al analysed methylation status by novel bisulphite sequencing PCR, allowing analysis of 28 CpGs (Nehls et al., 2008). They reported methylation of $p16^{INK4A}$ in 18/70 cervical cancers but not in any pre-cancers, methylation did not have any influence on $p16^{INK4A}$ expression levels (Nehls et al., 2008). While silencing of the $p16^{INK4A}$ gene by methylation has been suggested (Nuovo et al., 1999, Ferreux et al 2003) over expression of $p16^{INK4A}$ is consistently found in high grade cervical pre-cancer and cancer (Murphy et al., 2003, Murphy et al., 2005).

1.9.7 HPV integration

Cancer tissues may contain both episomal and integrated HPV DNAs at the same time. During HPV DNA integration, the viral genome usually breaks in the E1/E2 region, leading to disruption in E1 and E2. Loss of E2 results in uncontrolled and increased expression of E6 and E7 oncogenic proteins. HPV integration into the host genome plays a fundamental role in the progression from low to high grade cervical neoplasia (Klaes et al., 1999, Wentzensen et al., 2004). Integration events have been related to common fragile sites (CFSs) (Thorland et al., 2003). A number of chromosomal abnormalities have been associated with these fragile sites. Martin *et al* described a number of chromosomal abnormalities associated with cervical cancer, most commonly gain of chromosomal region 3q. Other gains have also been found including 1q, 3q, 8q, 11q and 20q and loss of chromosomal regions 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q and 18q (Martin et al.,2007). Disruptions in cellular genes, such as Notch 1, by HPV 16 integration, have been found which may contribute to a malignant phenotype (Thorland et al., 2003). Methods for detection of viral integration were reviewed by Wentzensen and Knebel Doeberitz concluding that while HPV integration points to advanced lesions with a very high progression potential, detection methods can be challenging. HPV integration detection is highly specific for advanced lesions, but lacks sensitivity since all studies measuring HPV integration directly, have not shown more than 80–90% integration positive cervical cancers (Wentzensen and Knebel Doeberitz, 2007).

1.10 Co-factors to HPV and cervical cancer

A number of important risk factors have been proposed for the acquisition of HPV. The number of sexual partners and age of first sexual intercourse are key factors (Bosch and Ifner 2005). HPV detection has been found to increase with increasing number of lifetime sexual partners and with decreasing age at sexual debut (IACR). HPV infection is undoubtedly the main ethological factor in the development of cervical cancer. However, while it is necessary it is not a sufficient cause given the high prevalence of transient infections that do not result in cervical disease (Walboomers et al 1999). Risk factors linked to sexual behaviour merely reflect the probability of HPV exposure and acquisition and not disease progression. Several environmental co-factors have been suggested to also play a role in the progression of a transient HPV infection to a transforming infection and malignancy.

1.10.1 Role of smoking in cervical pre-cancer

Cigarette smoking remains to be one the main preventable causes of disease. It is estimated that smoking is responsible for around 6,500 deaths in Ireland each year costing the state €1 billion a year to provide health care for smokers. Approximately 29% of our population are smokers, 35% of which aged between 18 and 29 years (Brugha et al 2009).

The role of smoking in the development of cervical cancer was first suggested by Winkelstein in 1977 (Winkelstein, 1977). The relationship between smoking and cervical cancer was questioned over the effects of other confounding factors such as sexual behaviour (IARC, 1986). In a review by Swarezski and Cuzick 1998, they reported the effect of smoking remained after adjustment for other sexual variables (Swarerski and Cuzick 1998). In 2003, an International Agency for Research on Cancer (IARC) multicenter study by Plummer *et al* reported that smoking increases risk of cervical cancer in HPV positive women (OR for ever vs. never, 2.2, 95% CI: 1.5–3.2) (Plummer et al. 2003). This is consistent with other studies that reported an increased risk of cervical cancer in women who smoke (Kruger-Kjaer et al., 1998, Hildesheim et al., 2001, McIntyre-Seltman et al., 2005). The IACR went on to list cervical cancer as causally related to smoking (IARC, 2004).

Evidence is based on a number of factors including the presence of tobacco specific compounds such as nicotine, cotinine (McCann et al., 1992) and nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a known carcinogen (Parkin and Bray, 2006, Prokopczyk et al., 1997) in cervical mucus. The knowledge that cervical epithelium is exposed to components of tobacco smoke led to investigations to identify if tobacco-related carcinogens may exert direct mitogenic effects promoting DNA alterations in the cervix. Increased presence of DNA adducts in cervical epithelium of smokers has been reported (Simons et al., 1995). In addition, smoking has been found to cause local immunosuppression in the cervix due to reducing the number of Langerhan cells (Poppe et al., 1995, Szarewski et al., 2001). The quality of the immune response is a critical step in defence against HPV infection. Langerhan cells (LC) are a type of dendritic cell, they are responsible for antigen presentation to naïve T cells in lymph nodes. T cells provide cell mediated response when activated by viral antigens. LCs have been shown to be directly cytotoxic towards HPV 16 E6/E7 immortalised cells (Poole et al., 2008). Their suppression could favour a reduction in generating an adequate immune response, promoting persistent HPV infection facilitating carcinogenesis. More recently, nicotine has been found to directly interact with HPV. It has been shown to up regulate transcription of E6 through interaction with the transcription factor Brn-3a in HR HPV 16 variants (Ndisang et al., 2010).

Self-reporting is the cheapest and easiest way to collect information of a person's smoking status. However, self-reported measures of cigarette smoking are imprecise due to under reporting and variability in how cigarettes are smoked. Smokers adjust smoking behaviour to achieve the amount of desired dose of nicotine per drag, in addition to variation in exposure to environmental smoke from person to person (Benowitz et al., 1999). Biological biomarkers for exposure to tobacco smoke offer an improved measurement of exposure and an objective method to identify relationships and risks associated with smoking. There are a number of biomarkers that can be used to assess exposure to tobacco smoke. The most commonly used are carbon monoxide in expired air, thiocyanate and nicotine in saliva, plasma and urine, carboxyhemoglobin in blood and cotinine saliva, plasma and urine. However some markers may be influenced by other environmental factors such as diet and air pollution.

A cigarette on average contains 8.4mg of nicotine. Nicotine is inhaled to the lungs where is enters the bloodstream via alveoli. Once in the bloodstream it circulates in the body reaching the heart, central nervous system and brain where the positive pharmacological response is achieved (Moyer et al., 2002). It is the liver that is the main site of metabolism where it is metabolised to several different metabolites by cytochrome P450 isoenzymes (Moyer et al., 2002). 70-80% of nicotine is converted to cotinine. Cotinine is further metabolised to *trans*-3'-hydroxycotinine and both are excreted in urine. Cotinine is the main metabolite of nicotine, it has been recognised as the most selective biomarker for exposure to tobacco smoke (Benowitz et al., 1999). It is specific to tobacco smoke and can be measured easily in a urine sample. It is a more favourable marker as it has a longer half-life of 19 hours compared to 2 hours for nicotine. Whereas carbon monoxide has a shorter half-life of 2-5 hours, it can also be influenced by respiration.

Numerous studies have reported that cotinine is a sensitive marker of exposure tobacco smoke (Javis, 1984). Elevated levels of cotinine can be found several days after cessation of smoking (Woodword 1992). Cotinine can be measured in blood, urine, saliva, serum, semen and hair. All fluids have been shown to correlate well but urine may be the marker of choice due to the non-invasive nature of collection. Saliva and blood cotinine levels are highly correlated, with a saliva-to-blood ratio of 1 to 1.4. Therefore, saliva and blood cotinine levels can be used interchangeably. Urine concentrations are also highly correlated with blood concentrations, with urine levels about 6 times higher than those for blood (Benowitz et al., 1999).

Cotinine, a metabolite of nicotine has been established as an accurate biomarker for detection of exposure to tobacco smoke. Besides tobacco exposure some foods including aubergine, potato, tomato and black tea contain small amount of nicotine (Davis et al., 1991). However, it has been reported that the average daily urinary cotinine concentrations from intake of food products would be 0.6ng/ml, and would be highly insignificant compared to cigarette smoke (Davis et al., 1991). The use of cotinine has been used to validate self-reported smoking habit in a number of studies (Lindquvist et al., 2002, Holl., 1998, Swamy et al., 2011, Boffetta et al., 2006) but has rarely been used in studies related to cervical cancer and pre-cancer. One study used urinary cotinine concentrations to examine the presence of DNA adducts in

cervical epithelial tissue. The authors reported a significant increase in DNA adducts in reported smokers, significance increased when analysis was based on urinary cotinine concentration (Simons et al., 1993). Another study by Szarewski et al, investigated the effect of smoking cessation on Langerhan cells of the cervix. Salivary cotinine concentration was used to confirm non-smokers but not to assess risk based on cotinine concentration (Szarewski et al., 2001).

In this study, for the first time urinary cotinine concentrations will be used to assess the risk of HR HPV infection through the presence of HR HPV DNA and transforming HR HPV infection through the presence of HR HPV E6/E7 mRNA in cervical smears. Urinary cotinine concentrations will also be used to examine the risk of detecting underlying CIN 2+ in women referred to colposcopy with minor cytological abnormalities.

1.10.2 Contraceptive Pill

In 2005, a Working Group for the International Agency for Research on Cancer classified combined oral contraceptives as carcinogenic to the human uterine cervix (Cogliano et al., 2005). The nature of the association between combined oral contraceptives and cervical cancer was further investigated by an International Collaboration of Epidemiological Studies of cervical cancer based on pooled analysis from 24 studies. Findings concluded the relative risk of cervical cancer is increased in long term oral contraceptive use (5+years) (International Collaboration of Epidemiological Studies 2007).

Studies have suggested several methods for the mechanism for the association of hormonal contraception and cervical cancer. Estrogens and progesterone could enhance expression of certain human papillomavirus genes and stimulate cell proliferation in the human cervix. B-estradiol has been found to up regulate HPV 16 gene expression via LCR (Chen et al., 1996). Some suggest that estrogens may act synergistically with HPV and enhance transcription of E6/E7 HPV mRNA (Mitrani-Rosenbaum et al., 1989). Furthermore, estrogen and progesterone have been linked with a reduction in cell-mediated immunity (Queiroz et al., 2006 Stopinska-Gluszak et al., 2006) potentially effecting clearance of HPV and thus neoplastic diseases.

Caution must be made when considering risk, women using oral contraceptives are more likely to be exposed to HPV than are those using barrier methods of contraception (Winer et al., 2006). It has been found that the use of condoms is associated with regression of CIN and clearance of HPV (Hogewoning et al., 2003, Tokudome et al., 2004). Thus, even if oral contraceptives are not causally associated with cervical cancer, women positive for HPV who use them, instead of barrier methods, might be at increased risk of progression of cervical lesions.

1.11 CERVIVA

This study stems from work carried out under CERVIVA, the Irish cervical screening research consortium. CERVIVA is a multi-investigator, multi-institutional consortium which was established in 2005 by Prof. John O'Leary and Dr. Cara Martin. This collaborative research effort between various academic institutions, hospitals and industry partners is pursuing a number of research projects which will aid cervical screening services in Ireland and provide information and guidance on its delivery. CERVIVA has focused on the development of biochip technologies, virtual slide technologies and quality assurance systems, the impact of vaccination, social acceptance of HPV testing, education and outreach programmes, HPV in HIV infected women, the development of automated screening technologies. Phase 2 (CERVICA 2) was recently initiated through funding under the Health Research Board's Interdisciplinary Capacity Enhancement (ICE) Awards scheme. The funding will allow the CERVIVA consortium to build upon its existing capacities to further develop and expand emerging research topics of interest to cancer screening in Ireland. CERVIVA 2 will focus on the areas of health economics, molecular biology/epidemiology, and health psychology.

1.12 Current challenges

Cervical cancer is a preventable disease. Current strategies involve screening by pap smear to identify pre-cancerous changes. However, from a clinical performance perspective, the pap smear is relativity insensitive and must be

repeated over frequent intervals in order to achieve benefit in screening. Cytology is often associated with low sensitivity and many equivocal results. In addition, it is unable to distinguish progressive disease, a significant limitation. As discussed in section 1.6.5, many precancerous stages can regress, in particular low grade lesions. In section 1.7.1 different approaches and the benefit of using HPV was discussed. HPV testing can be accompanied with, or used in replacement of cytology, to overcome some of the issues associated with cytology-based screening. However, there are is still challenges with respect to minor abnormalities, where HPV infection is highly prevalent. Current management options of minor abnormalities includes, repeat cytology in 6 months, reflex HPV DNA test or immediate referral to colposcopy (Jordan et al., 2008). All of these methods are successful at identifying underlying or progressive high grade disease however, the proportion of women attending colposcopy with whom no high grade disease if found is high. Further research is needed to distinguish women with minor cytological abnormalities who are truly at high risk. Advances in the detection and utility of molecular biomarkers may ameliorate this situation. A number of biomarkers were described in section 1.9, commercially available kits have been developed for some markers such as HPV mRNA and $p16^{INK4A}$/Ki-67 which show promise as specific markers for high grade disease.

1.13 Hypothesis

Combined testing with HPV mRNA and $p16^{INK4A}$/Ki-67 will be more specific and have a higher PPV than HPV DNA testing and/or cytology alone for detecting high grade disease in women referred to colposcopy with minor abnormal smears, LSIL and ASCUS. In addition, women who smoke are more likely to harbour an integrated HPV infection due to the local immunosuppressive effect smoking has on the cervix. Consequently, women with high levels of urinary cotinines are more likely to harbour integrated HPV infection and therefore at increased risk to developing high grade cervical pre-cancer.

1.14 Aims

1 To evaluate HPV DNA, HPV mRNA and $p16^{INK4A}$/Ki-67 expression as an approach for detecting high grade pre-cancer in women referred to colpsocopy with minor cytological abnormalities.

2 To evaluate a combined HPV mRNA and $p16^{INK4A}$/Ki-67 detection as a more specific approach compared to HPV DNA testing for management of women with minor cytological abnormalities.

3 To define the role of smoking in HPV infections and cervical pre-cancer, in order to highlight the effects of smoking in young women.

4 To correlate urinary cotinines with HPV DNA, HPV mRNA and disease status in women referred to colposcopy with minor cytological abnormalities to better understand the role of smoking in progression of cervical pre-cancer.

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was transferred into the bottom of the 10ml conical Sarstedt tube. 400µl of conversion buffer was added to each specimen. The samples were placed in the centrifuge and spun at 3800rpm for 12 minutes. Samples were removed from the centrifuge, a pink/orange pellet was identified in each tube. Individually the supernatant was carefully poured into a waste container containing 10% Virkon. Maintaining an inverted position the tube was gently blotted on to a low lint paper towel to remove maximum amount of PreservCyt®. Three drops of indicator dye were added to vial labelled DNR (denaturation reagent) from the sample conversion kit and mixed well to form a uniform dark colour. DNR reagent was stored at 4°C for 3 months. Specimen transport medium/DNR was prepared in a 2:1 ratio using the guidelines shown in Table 2.1. 150µl of STM/DNR was added to cell pellets. Samples were vortexed thoroughly and placed into 65°C ±2°C waterbath for 15 ±2 minutes. After 15 minutes the rack was removed and samples vortexed for 10-15 seconds. Incubation was continued in waterbath at 65°C ±2°C for a further 30 ±3 minutes.

Table 2.1 Volume requirements: STM/DNR

Number of tests	PresevrCyt® Volume	STM volume	DNR volume	STM/DNR added to sample
1-2	4ml	120µl	60µl	150µl
3	6ml	170µl	85µl	225µl
4	8ml	220µl	110µl	300µl

2.2.2 Calibrator and Quality Controls

Calibrators and Quality Controls are provided with hc2 High-Risk HPV DNA Test® kit. Caps were removed and discarded, denaturation reagent containing indicator dye was added according to the volumes shown in Table 2.2. Specimens were recapped with clean labelled collection tube screw caps and mixed thoroughly by vortexing for 5 seconds. Specimens were incubated in a water bath set at 65°C ±2°C for 45 ±5 minutes.

Table 2.2 Calibrators and Controls

Calibrator, Quality Control	Volume of Denaturation Reagent
Negative Calibrator	1000µl
High-Risk HPV Calibrator	500µl
Low-Risk Quality Control	500µl
High-Risk Quality Control	500µl

Samples were stored at -20°C for no longer than 3 months with no more than 3 freeze/thaw cycles.

2.2.3 Hybridization

Specimens were allowed to thaw to 20-25°C. While specimens were thawing a microplate heater was preheated to 65°C. Plate layout was set up on the Digene Micoplate System 2.0 software. All samples, calibrators and quality controls were vortexed thoroughly before 75µl was pipetted into the bottom of an empty hybridisation microplate well. To avoid cross-contamination extra-long pipette tips were used for transfer. Once specimen transfer was complete the microplate was covered with a lid and incubated at 20-25°C for 10 minutes. In this time the High-Risk HPV Probe mix was prepared by making a 1:25 dilution of High-Risk HPV Probe in Probe diluent. Probe mix was thoroughly mixed and added to a disposable reagent reservoir. 25µl of probe mix was added to each well containing calibrators, quality controls and samples using an 8-channel pipettor. The Hybridisation Microplate was covered with lid and placed on Hybrid Capture System Rotary Shaker I at 1100 ±100rpm for 3 ±2 minutes. A colour change from purple to yellow was identified in all wells containing calibrators, quality controls and from purple to pink for PreservCyt® samples. If no colour changed was observed, wells remaining purple had an additional 25µl of probe mixed added and the microplate was shaken again. If wells remained purple following this procedure, specimens were retested. The Hybridization Microplate was incubated on Microplate Heater I at 65°C ±2°C for 60 ±5 minutes.

2.2.4 Hybrid Capture

The Hybridization Microplate was removed from the Microplate Heater I, the lid was removed immediately to avoid condensation. The entire contents approximately 100µl was transferred from the Hybridization Microplate wells to the bottom of the corresponding Capture Microplate using an 8-channel pipettor. The Microplate was covered with lid and shaken on the Rotary Shaker I at 1100 ±100 rpm for 60 ±5 minutes. During this incubation Wash Buffer was prepared for Automated Plate Washer I by adding 966.7ml deionized water to 33.3ml wash buffer concentrate. The waste reservoir was emptied and the Rinse reservoir filled with deionized water. Once the capture step was complete the plate was removed from the Rotary Shaker I. The liquid was removed from the wells by discarding in to a sink, taking care to avoid backsplash by not decanting too closely to the bottom of the sink. Keeping the plate fully inverted it was blotted to remove remaining liquid by tapping firmly on clean lint free paper towels.

2.2.5 Hybrid Detection

Detection Reagent 1 was aliquoted into a disposable reagent container. 75µl was pipetted into each well of the Capture Microplate using an 8-channel pipettor. As recommended by the manufacturer, reverse pipetting was used to improve consistency of reagent delivery. The pipette tips were initially over-filled by using the second stop on the pipettor's dispense control, 75µl of reagent was dispensed into microplate wells by depressing the plunger to the first stop. The plunger was not released until tips had been re-immersed into Detection Reagent 1 to refill the tips. Once all wells had been filled they were examined to ensure similar pink colour intensity. The Capture Microplate was covered with plate lid and incubated at 20-25°C for 30-45 minutes.

2.2.6 Washing

The washing steps were performed on Automated Plate Washer I. Prior to use it was verified that Wash Reservoir and Rinse Reservoir were filled to 1 litre and that the Waste Reservoir was empty and cap securely tightened. The

plate lid was removed and Capture Microplate placed on Automated Plate Washer 1 platform. Firstly it was verified that plate washer display read "Digene Wash Ready". The number of rows to be washed was selected before starting the wash. The washer performs six fill and aspirate cycles taking approximately 10 minutes. The plate was removed once the display read "Digene Wash Ready" again. The plate was examined to ensure all wells appeared white and no residual pink liquid remained as this could result in false positive results.

2.2.7 Signal Amplification

Detection Reagent 2 was aliquoted into a disposable reagent reservoir. Addition of 75µl of Detection Reagent 2 was made swiftly without interruption into each well using an 8-channel pipette. It was verified that all wells on the Capture Mircoplate turned yellow. The plate was covered with plate lid and lint free paper towel to avoid direct sunlight and incubated for 15 minutes at 20-25°C. Following incubation the microplate was read on the Digene Microplate Luminometer 2000 (DML 2000™).

2.2.8 Assay Calibration and Verification Criteria

The hc2 High-Risk HPV DNA Test® requires calibration with each assay, both the negative and high-risk calibrators are run in triplicate. Verification according to the manufacturer is based on the criteria shown below. Verification is calculated automatically by the Digene Qualitative Software and printed on the data analysis report.

1. Negative Calibrator

The negative calibrator mean must be ≥ 10 and ≤ 250 RLU's, in addition to this they must show a coefficient of variation (%CV) of ≤25%. In cases where the %CV was >25% the value with a RLU furthest from the mean was discarded as an outlier and the remaining two used to recalculate the mean. If the difference between the mean and each two values is ≤25% the assay negative calibration is valid. If the difference between the mean and two values is >25% the assay calibration is invalid and the assay was be repeated.

2. High-Risk HPV Calibrator

The High-Risk HPV Calibrator must show a %CV of ≤15%. If the %CV is >15% the value with a RLU furthest from the mean was discarded as an outlier and the remaining two used to recalculate the mean. If the difference between the mean and each two values is ≤15% the assay calibration is valid. If the difference between the mean and two values is >15% the assay calibration is invalid and the assay was be repeated.

3. The High-Risk HPV Calibrator mean ($\overline{HRCx}$) and Negative Calibrator mean ($\overline{NCx}$) are used to calculate the $\overline{HRCx}/\overline{NCx}$ ratio. Assay calibration verification acceptable ranges $2.0 \leq \overline{HRCx}/\overline{NCx} \leq 15$

4. Calculate the $\overline{HRCx}$ / $\overline{NCx}$ ratio. If the ratio is <2.0 or >15 the assay is invalid and must be repeated.

2.2.9 Quality Control

Quality control samples are supplied with the hc2 High-Risk HPV DNA test. These are cloned HPV DNA targets and not derived from wild-type HPV. Quality controls were included with each assay. Quality control RLU/CO must fall within acceptable ranges outlined by the manufacturer (Table 2.3). If the quality controls do not fall within these ranges the assay is invalid and must be repeated.

Table 2.3 Expected ranges for Quality Controls

Quality Control	HPV Type	Expected Result (RLU/Cutoff Value) High-Risk HPV Probe			
		Minimum	Maximum	Average	%CV
QC1-LR	Low-Risk (HPV 6)	0.001	0.999	0.5	25
QC2-HR	High-Risk (HPV 16)	2	8	5.0	25

2.2.10 Interpretation of specimen results

The hc2 High-Risk HPV DNA test cutoff is 1pg/ml which is equivalent to 100,000 copies/ml or 5,000 HPV copies per assay.

1. Specimens with RLU/Cutoff Value (RLU/CO) ratios ≥1.0 are considered "Positive"

2. Specimens with RLU/Cut off ratios <1.0 are considered "Negative" for the 13 High Risk HPV types tested. HPV DNA sequences are either absent or below the detection limit of the assay.

3. If the RLU/CO ratio of a specimen is ≥1.0 and <2.5 QIAGEN recommends the specimen be retested. If the initial retest is ≥1.0 the specimen is reported as positive. However if the first retest is <1.0 then an additional retest is required to generate a final result. Due to limitation in sample availability in this study if a sample required a second retest is was excluded from the study.

2.3 Cobas® 4800 HPV

The cobas® 4800 HPV test is a PCR based test for detection of 14 HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. Individual detection of HPV 16 and HPV 18 occurs while concurrently detecting the 12 remaining HR HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). It is a fully automated system that involves two major processes, DNA extraction and real-time PCR amplification.

Cervical specimens are lysed to release HPV DNA and human β-globin DNA which acts as a process control. Nucleic acids are purified and washed through absorption to magnetic glass particles.

The cobas® 4800 HPV test utilizes a pool of HPV primers and probes specific for a 200 base pair target within the polymorphic L1 region of the HPV genome and an additional primer/probe set to target human β-globin gene (330bp amplicon). The β-globin gene provides a control for cell adequacy, extraction and amplification. The cobas 4800® HPV test has a limit of detection of 600 copies/ml. Detection is based on four different fluorescent dyes, HPV 16, HPV

18, HR HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and β-globin amplicons are labelled by their own fluorescent dye allowing each to be measured independently (Figure 2.2). The test was performed following the manufacturer's instructions for the cobas x480 for DNA extraction and cobas z480 for real-time PCR.

TaqMan Probes:

Channel 1 (FAM) – "other" high risk HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68

Channel 2 (HEX) - HPV 16 only

Channel 3 (JA270) - HPV 18 only

Channel 4 (Cy5.5) - ß-globin Target

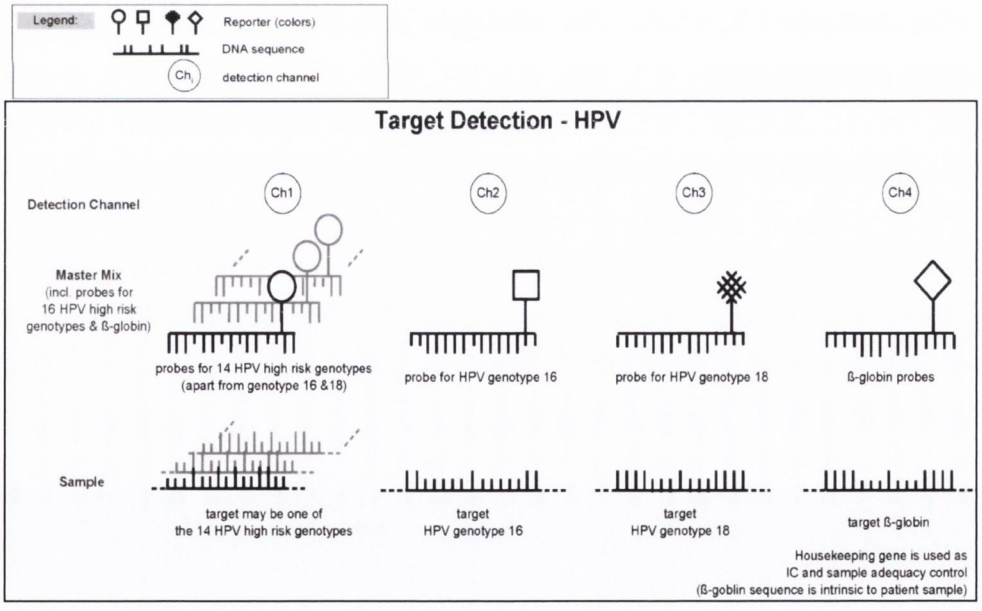

Figure 2-2 cobas® 4800 HPV test - amplification and detection

2.3.1 Sample Preparation

The cobas z480, heater shaker unit, cobas x480 and cobas 4800 software were turned on. Periodic maintenance was performed on the cobas x480 instrument, daily and weekly maintenance is mandatory. To perform maintenance the "Run Daily/Weekly Maintenance" icon was clicked under maintenance status in the cobas x480 tab. All reagents were removed from the fridge and allowed to come to room temperature. A work order file was created form the cobas 4800 work order editor. This creates an ordered list of the samples to be tested.

To start a new run HPV workflow is selected. An on screen wizard guides you through the loading of the deck (Figure 2.3). First bar coded samples were loaded in the same order as they appear on the work order file. Controls were not loaded with samples, they were loaded with reagents on reagent carrier. Samples were vortexed on Multi-Tube Vortexer, caps removed and placed into the appropriate carrier ensuring barcodes were facing out. Sample carriers were inserted into designated tract positions on the auto load tray indicated by blinking LEDs. Sample carriers were loaded automatically onto the cobas x480 instrument scanning the barcodes. Following successful loading the wizards requests the corresponding work order file. This is cross checked against the loaded samples. Consumables including deepwell plate, microplate and two full tip rack carriers (960 tips) were loaded into their designated track positions. The wizard then guides the loading of reagents, each reagent and reagent reservoir is scanned to track their use. Wash Buffer, SDS, Lysis Buffer and Elution Buffer are supplied with kit ready for use. Magnetic Glass Particles must be thoroughly vortexed before use. Each reagent was scanned, poured into reagent reservoirs and loaded into their designated track positions. Positive and Negative controls, Master Mix, Magnesium/Manganese Ions, proteinase K were loaded in their original vial into the designated track position. Once loading was complete "Start Run" was clicked to begin sample preparation.

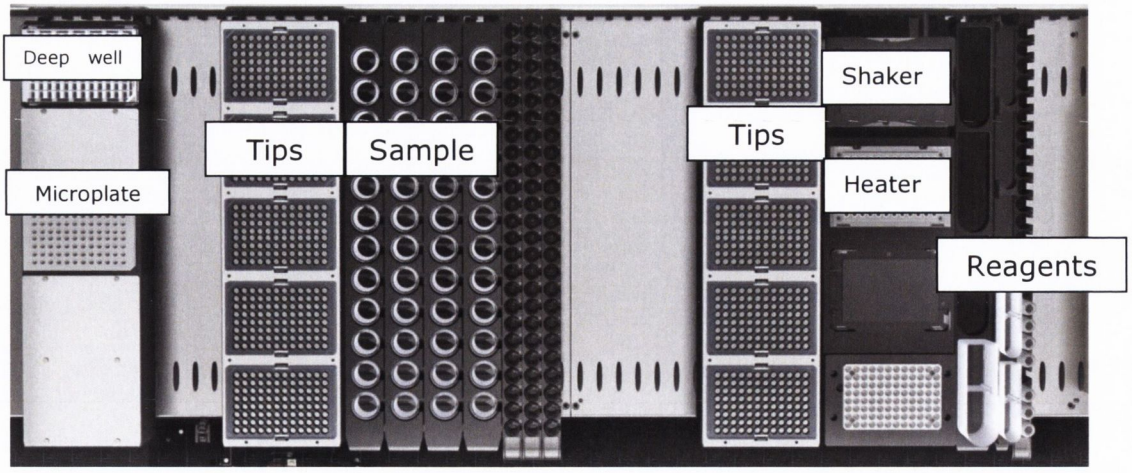

Figure 2-3 Cobas x480 deck layout

Once sample preparation was complete the microwell plate was unloaded form the deck and sealed with sealing film.

2.3.2 Amplification and Detection Run

The sealed microwell plate was transferred to the Cobas z480 analyser for amplification and detection. Once the plate was loaded the run started immediately.

A growth curve of positive and negative controls of all channels is displayed on the screen. All assay and run validation is performed by the Cobas 4800 software. Sample results were viewed under the results tab.

2.4 HPV genotyping

The Linear Array HPV Genotyping Test can detect 37 different high and low-risk HPV genotypes (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 , 81, 82, 83, 84, IS39 (HPV 82 sub-type), and CP6108 (HPV 89 sub-type)). There are 4 steps to the Linear Array HPV Genotyping Test procedure; 1. DNA Extraction, 2. PCR amplification, 3: Hybridisation, 4. Detection.

2.4.1 DNA extraction

DNA is extracted from a 250µl aliquot of PreservCyt sample, using the Ampilute Media Extraction Kit (Roche Diagnostics, Germany) along with a supplied positive (HPV16 Plasmid) and a negative controls.

A dry heat block was set to 56°C to equilibrate for an upcoming incubation. The reagents, specimens and controls were allowed to equilibrate to room temperature for at least 15 minutes. The Tissue Lysis (ATL) and Lysis (AL) buffers were pre heated to 70°C and gently agitated to dissolve any precipitate that had formed. The Lyophilised carrier RNA (CAR) was dissolved by the addition of 310µl of AVE in a nucleic acid extraction hood and the solution was vortexed for 10 seconds. At this point to prevent freeze thaw cycles the AVE was aliquoted into 4 equal stocks (~77µl) frozen at -20°C for up to 2 months for use in subsequent extractions. 30ml of 100% molecular grade ethanol was added to the supplied wash buffer 2 (AW2) (Tris-HCL buffer <0.09% Sodium azide), the solution was mixed, dated, and stored at room temperature for up to 2 months. A working AL solution was prepared in a 10ml Sarstedt centrifuge tube by adding the appropriate volume of dissolved CAR to appropriate volume of AL as shown in Table 2.4. The solution was mixed by gently inverting the tube 10 times, as vortexing could have resulted in foaming of the solution.

Table 2.4 Volume of reagents required to make working AL solution.

	Number of Specimens/Controls to be processed	
Reagents	12	24
CAR (ml)	0.04	0.7
AL (ml)	4.0	7.0

2ml screw cap Sarstedt tubes for each specimen and control to be extracted were labelled clearly using a solvent resistant marker. An 80µl aliqout of ATL was added to each labelled 2ml Sarstedt tube. Each specimen and control was vortexed individually for at least 10 seconds before 250µl of the PreServCyt

specimen was added to the appropriately labelled 2ml tube. 20µl of Proteinase K was added to each 2ml tube, the tubes were capped and vortexed for 10 seconds. The tubes were then placed on the dry heat block and incubated at 56°C ±2°C for 30 minutes. During the incubation, QIAamp MinElute® Columns (CLM) with waste collection tubes were removed for each specimen and control from sealed blister packages and labelled as before. Following incubation the tubes were removed from the dry heat block and the temperature of the block was set to 70°C ±2°C. 250µl of working AL was added to each tube, the tubes were capped and vortexed for 10 seconds. The tubes were placed on the heat block and incubated at 70°C ±2°C for 15 minutes. During the incubation the tubes were vortexed occasionally throughout the incubation period. When the incubation was completed 300µl of 100% molecular grade ethanol was added to each tube. The tubes were capped, vortexed for 15 seconds incubated at room temperature for 5 minutes. The tubes were then pulse centrifuged for 3-5 seconds at maximum relative centrifugal force (RCF). The lysate from each tube was transferred in to a corresponding labelled CLM; the CLM was capped and left to incubate at room temperature for 1 minute. The CLM and collection tubes were centrifuged at 16,000 RCF for 3 minutes. The waste collection tube was discarded and the CLM was placed into a new waste collection tube. 750µl of AW2 was added to each CLM; the CLM was capped and left to incubate for 1 minute at room temperature. The CLM and waste collection tubes were centrifuged at 16,000 RCF for 3 minutes. The waste collection tube was discarded and the CLM was placed into a new waste collection tube. 750µl of 100% molecular grade ethanol was added to each CLM; the CLM was capped and left to incubate for 1 minute at room temperature. The CLM and collection tubes were centrifuged at 16,000 RCF for 3 minutes. The collection tube was discarded and the CLM was placed into a new collection tube. The CLM and collection tubes were centrifuged at maximum speed for 3 minutes to ensure all ethanol was removed from the silica membrane prior to elution. An individual 1.5ml Elution Tubes (ETL) was labelled for each specimen and control. The waste collection tube was discarded and the CLM was placed into the corresponding labelled 1.5ml ETL. 120µl of AVE was added to each CLM and the CLM were left to incubate for 5 minutes at room temperature. The CLM and ETL were centrifuged at maximum speed for 1 minute. The CLM was discarded and the elute was visually identified at the bottom of the 1.5ml ETL.

The 1.5ml ETL were capped and the stored, at room temperature for a maximum of 6 hours if amplification was carried out on the same day, at 2-8°C if amplification was carried out within 7 days of extraction, or at -20°C for a maximum of 8 weeks with no more than 1 freeze-thaw cycle.

2.4.2 Amplification

DNA was amplified in a multiplex PCR capable of detecting 37 HPV genotypes along with β-globin controls. The Applied Biosystems Gold-plated 96-well GeneAmp PCR System 9700 (AB 9700) (Applied Biosystems Inc., USA) was used to perform the PCR protocol with the following thermocyling conditions. Hold programme 2 minutes at 50°C, hold programme 9 minutes 95°C, cycle programme of 40 cycles (30 seconds at 95°C, 1 minute at 55°C, and 1 minute at 72°C), hold programme 5 minutes at 72°C and a final hold at 72°C indefinitely. The ramp rate was set to 50% in the cycle programs, with the ramp speed in method options set to "MAX". The working PCR master mix was prepared in a dedicated pre-amplification room with a laminar flow hood for amplification plate set-up. The master mix for the Linear Array HPV Genotyping Test contains a pool of HPV primers designed to amplify HPV DNA from 37 HPV genotypes. An additional primer pair targeted the human β-globin gene to provide a control for cell adequacy, extraction and amplification. The master mix was prepared in the hood by adding 125µl of vortexed LINEAR ARRAY HPV Magnesium Solution (Mg^{2+}) (<1% Magnesium chloride, Amaranth dye and 0.05% Sodium azide) to one vial (580µl) of LINEAR ARRAY HPV Master Mix (MMX) (Tris buffer, Potassium chloride, <0.02% AmpliTaq® Gold DNA Polymerase (microbial), <0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial), <0.001% (dATP, dCTP, dUTP, dGTP, dTTP), <0.001% each of the upstream and downstream primers and 0.06% Sodium azide). Approximately two vials of HPV MMX mixed with 125µl of MG2+ (1400µl) were required for a full run of 24 amplification reactions. The DNA extracts were removed from storage and brought up to room temperature. A plate plan was prepared to outline the position of the appropriate number of samples on a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems Inc., USA). A 50µl aliquot of working master mix was added to and individual well on the 96 well plate for each specimen and

control. The plate was removed from the hood and 50µl of extracted DNA was added to the wells on the plate containing the master mix according to the plate plan. The plate was placed into the AB 9700 (Applied Biosystems Inc., USA). The programme described above was selected and the reaction volume set to 100µl. The programme was started, with a completion time of approximately 3 hours and 15 minutes. AmpliTaq® Gold DNA Polymerase was used for "hot start" amplification of the HPV target DNA and the β-globin control. The PCR reaction was heated; the AmpliTaq® Gold DNA Polymerase became activate and denatured the viral DNA and genomic DNA, exposing the primer target sequences. The primers annealed to the DNA and in the presence of Mg^{2+} and excess dNTPs, the AmpliTaq® Gold DNA Polymerase extended the primers along the target templates producing double stranded HPV DNA and β-Globin DNA. The process was repeated for 40 cycles with each cycle effectively doubling the amount of amplicon. Amplification only occurred in the region of the HPV genome or β-globin gene between the appropriate primer pairs, the entire genome was not amplified.

AmpErase enzyme contained within the master mix solution ensured that selective amplification of the target nucleic acid from the specimen occurred and any carry over amplicon is destroyed. The AmpErase enzyme recognised and catalysed the destruction of amplified DNA strands containing which contained deoxyuiridine which is always found in amplified DNA and never in natural DNA. The AmpErase enzyme only acts at the beginning of the reactions and once the temperature is above 55° C the enzyme becomes inactive. Therefore throughout the amplification cycles AmpErase does not destroy any target amplicon.

Following amplification the tray was removed from the thermal cycler within 4 hours of the start of the final HOLD programme (∞ 72°C). Denaturation solution was immediately added to the amplicons when removed from the thermocycler to denature any residual enzyme, thereby preventing the degradation of any target amplicon. The denaturation solution also chemically denatured the HPV and β-globin amplicons to form single-stranded DNA. The denatured amplicon was stored at 2-8 °C for up to 7 days. Amplified specimen was considered to be a major source of potential contamination and therefore amplified specimen was kept separate from the pre-amplification areas and equipment.

2.4.3 HPV genotype detection

HPV detection was carried out within the 7 day period in a typing tray containing 24 wells. The Linear Array HPV Genotyping Test detection kit uses reverse hybridisation technology whereby labelled target DNA is hybridised to immobilised oligonucleotide probes captured onto a test strip supplied with the kit.

The HPV hybridisation and detection kit, specimens and controls were removed from storage at 2°C-8°C and allowed to equilibrate to room temperature. Two water baths were pre-warmed for use in the detection protocol, a non-shaking water bath was warmed to 53°C ±2°C and a shaking water bath was pre-warmed to 53°C ±2°C at a shaking speed of approximately 60 rpm. The shaking water bath was checked to ensure that the water level was sufficient to cover approximately a quarter (0.5cm) of the outside of the tray to ensure sufficient heating of the tray but to prevent water from splashing into the tray. The SSPE Concentrate (SSPE) (Sodium phosphate solution, sodium chloride, EDTA and 1% ProClin® 150 preservative), the SDS Concentrate (SDS) (20% Sodium lauryl sulphate and 1% ProClin® 150) and the Citrate Concentrate (CIT) were checked for signs of precipitation. If precipitation was seen the reagents were warmed to 53°C in the pre-warmed water bath until the precipitate was dissolved. The working hybridisation buffer was prepared for 1 tray of 24 tests by adding 25ml of SSPE to 97ml of distilled water. The solution was mixed well and 3.125ml of SDS was added and mixed well. The Working Hybridisation Buffer was stored at room temperature for no more than 30 days. The Working Ambient Wash Buffer was prepared for 1 tray of 24 tests by adding 33.25ml of SSPE to 630ml of distilled water. The solution was mixed well and 3.325ml of SDS was added and mixed well. The Working Ambient Wash Buffer was stored at room temperature for no more than 30 days. The Working Stringent Wash Buffer was prepared fresh on the day of detection by removing 5ml of Working Ambient Wash Buffer to a clean media bottle (120ml for 24 tests). The Working Hybridisation Buffer and the Working Ambient Wash Buffer were placed in the 53°C ±2°C water bath for a minimum of 15 minutes, and were not removed from the water bath until required. The Working Citrate Buffer was prepared by adding 6.25ml of CIT to 118.75ml of distilled water and mixed well. The Working Citrate Buffer was stored at room temperature for no more than 30 days.

The required number of Linear Array HPV Genotyping Strips (Strip) were removed from their bag using a pre-sterilized (washed, and stored in 100% Ethanol) forceps. Each Strip was labelled using a 2B pencil with the appropriate specimen or control identification for the assay. The Strips were placed in the 24-well tray with the probe lines facing upward. The pre-warmed Working Hybridisation Buffer was removed from the 53°C ±2°C water bath and 4ml aliquots were dispensed into each well of the 24-well tray containing a Strip. Each sample and control was vortexed immediately before 75µl of denatured amplicon was carefully dispensed into the appropriate well of the 24-well tray using a pipettor with an aerosol barrier tip. When all samples and controls were added to the 24-well tray the tray was covered with the accompanying lid and placed in the 53°C ±2°C shaking water bath for 30 minutes at approximately 60 rpm. A weight was placed on the tray lid in order to hold the tray in place throughout the incubation.

During the hybridisation the Working Conjugate solution was prepared by adding 15µl of Streptavidin-Horseradish Peroxidase Conjugate (SA-HRP) (Streptavidin-horseradish peroxidase conjugate, ACES Buffer, sodium chloride and 1% ProClin® 150 preservative) to 5ml of Working Ambient Wash Buffer for each strip being tested and the solution was thoroughly mixed. For a full run of 24 samples 360µl of SA-HRP was added to 120ml of Working Ambient Wash Buffer. Working Conjugate was stored at room temperature for no more than 3 hours.

Following hybridisation the 24-well tray was removed from the shaking water bath and the Working Hybridisation Buffer was aspirated from the wells using a separate 3.5ml transfer pipette (Sarstedt AG & Co., Germany) for each well. 4ml of Working Ambient Wash Buffer was added to each well containing a test strip. The tray was gently rocked 3-4 times to rinse the strips. The Working Ambient Wash Buffer was then aspirated from the wells using a separate 3.5ml transfer pipette for each well. The Working Stringent Wash Buffer was removed from the 53°C ±2°C water bath and immediately 4ml of pre-warmed Working Stringent Wash Buffer was added to each well containing a test strip to remove any unbound material. Any condensation was removed from the lid of the tray using lint free paper towels, the tray was covered and placed in the 53°C ±2°C shaking water bath for 15 minutes at approximately 60 rpm and a weight was placed on the tray lid in order to hold the tray in place throughout

the incubation. Following the incubation the 24-well tray was removed from the shaking water bath and the Working Stringent Wash Buffer was aspirated from the wells using a separate 3.5ml transfer pipette. 4ml of Working Conjugate was added to each well containing a test strip to detect the biotin-labelled amplicon now bound to the strip. The condensation was removed from the lid of the tray using lint free paper towels. The tray was covered and placed at room temperature on an orbital shaker at approximately 60 rpm for 30 minutes. Following incubation, the tray was removed from the orbital shaker and the Working Conjugate was aspirated from the wells using a separate 3.5ml transfer pipette.

4ml of Working Ambient Wash Buffer was added to each well containing a test strip. The tray was gently rocked 3-4 times to rinse the strips and the Working Ambient Wash Buffer was immediately aspirated from the wells using a separate 3.5ml transfer. 4ml of Working Ambient Wash Buffer was added to each well containing a test strip. The tray was covered and placed at room temperature on an orbital shaker for 10 minutes at approximately 60 rpm. The tray was removed from the orbital shaker and the Working Ambient Wash Buffer was aspirated from the wells using a separate 3.5ml transfer pipette. 4ml of Working Ambient Wash Buffer was again added to each well containing a test strip. The tray was covered and placed at room temperature on an orbital shaker for 10 minutes at approximately 60 rpm. The wash steps were to ensure that any unbound Working Conjugate was removed from the strips. The tray was removed from the orbital shaker and the Working Ambient Wash Buffer was aspirated from the wells using a separate 3.5ml transfer pipette.

4ml of Working Citrate Buffer was added to each well containing a test strip. The tray was covered and placed at room temperature on an orbital shaker for 5 minutes at approximately 60 rpm. During the incubation the Working Substrate was prepared by adding 4ml of Substrate A to 1ml of Substrate B per strip. The Working Substrate, a substrate solution containing hydrogen peroxide and 3,3',5,5'-TMB was stored at room temperature in a blacked out container to prevent exposure to direct sunlight for no longer than 3 hours. The tray was removed from the orbital shaker and the Working Citrate Buffer was aspirated from the wells using a separate 3.5ml transfer pipette. 4ml of Working Substrate was added to each well containing a test strip. The tray was covered and placed at room temperature on an orbital shaker for 5

minutes at approximately 60 rpm. In the presence of the hydrogen peroxide and TMB found in the Working Substrate, the bound SHR-P catalyses the oxidation of the TMB to form a blue coloured complex which was precipitated at the probe positions where hybridisation had occurred. The tray was removed from the orbital shaker and the Working Substrate was aspirated from the wells using a separate 3.5ml transfer pipette. 4ml of distilled water was added to each well containing a test strip. The tray was gently rocked 3-4 times to rinse the strips and the strips were removed using a clean forceps. The strips were placed on a clean dry lint free paper towel and allowed to air dry for 24-72 hours at room temperature prior to interpretation. A 10% RBS35 Tray Cleaning Solution (RBS35) was prepared by adding 1 part RBS35 to 9 parts distilled water. The wells of the 24-well tray were filled with the 10% solution and let soak overnight at room temperature. The tray was rinsed thoroughly with distilled water and dried completely before being re-used.

2.4.4 Interpretation of results

The results were read from the individual strips by comparing each strip with the Linear Array HPV Genotyping Test Reference Guide. The Linear Array HPV Genotyping Test Reference Guide was placed over the strip, with the strip in the cut out section of the guide so that the HPV genotype reference lines appeared on both sides of the individual strip Figure 2.4. The black ink reference line on the guide was aligned with the solid black line on the Strip. The positive and negative control for each run was checked to determine the validity of each individual run. The negative control was checked to ensure that there was no visible band on the strip. If any bands were visible the entire run was declared invalid and the entire process (Specimen and Control Preparation, Amplification and Genotype Detection) was repeated. The positive control was checked to ensure that the HPV 16, β-Globin high, and β-Globin low bands were visible on the strip. The β-Globin low band appeared faint relative to the β-Globin high band, but had to be visible to validate the assay. If the positive control did not yield this exact result the entire run was declared invalid and the entire process (Specimen and Control Preparation, Amplification and Genotype Detection) was repeated. If the controls validated the assay, the positive visible bands were recorded for each specimen and the

HPV and β-Globin results for each strip were interpreted as shown in Table 2.5. The Linear Array HPV Genotyping Test test strips contain a cross reactive probe that hybridises with HPV genotypes 33, 35, 52, and 58. Therefore any samples that were positive for the cross reactive band have to be interpreted against the 33, 35, and 58 individual bands as shown in Table 2.6 HPV 52 prevalence therefore could only be displayed in the results as a range due to the limitations of the Linear Array HPV Genotyping Test as described.

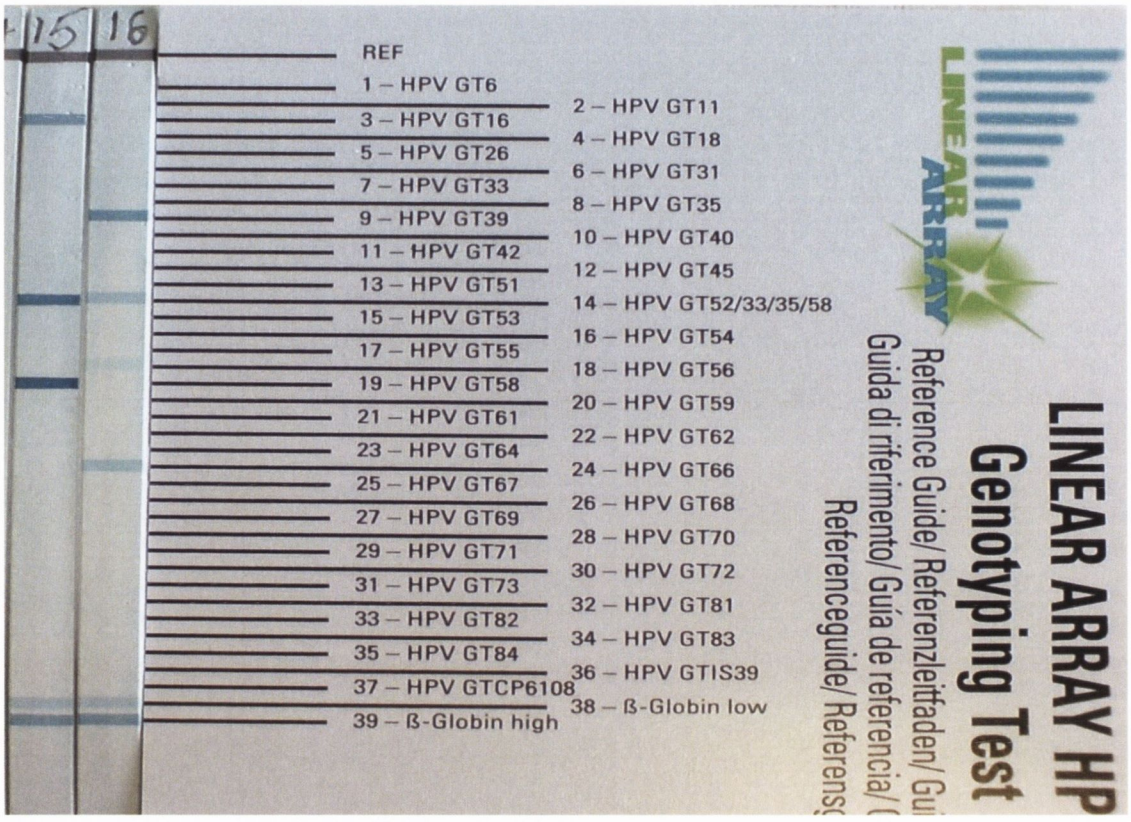

Figure 2-4 HPV Linear Array HPV Genotyping Test Strips and Reference Card.

The Strips (left) are lined up so that the black strip at the top is lined up with the REF line on the genotyping card. The HPV genotypes found in the sample is then read from the reference card. Sample 15 above contains positive bands for HPV 16, 52*, and 58; *HPV 52 presence cannot be ruled out in this sample as the sample is also positive for HPV 58, one of the samples included in the HPV 52 band. Sample 15 represents a valid genotyping result as the bands for low and high concentrations of β-glogin. Sample 16 was contains positive bands for HPV 39, 52*, 56 and 66. In this example HPV 52 infection was detected in sample 16. Sample 16 is also representative of a valid result as the strip contains bands for both control concentrations.

Table 2.5 Interpretation of genotyping results.

HPV Result	BG Low Result	BG High Result	Interpretation
-	-	-	Result Invalid: HPV DNA, if present could not be detected. The absence of BG bands indicated inadequate specimen, processing, or the presence of inhibitors. Another aliquot of the original specimen was processed and the test repeated.
-	-	+	Result Invalid: HPV DNA, if present could not be detected. The absence of BG Low indicated inadequate specimen, processing, or the presence of inhibitors. Another aliquot of the original specimen should be processed and the test repeated.
+	-	-	HPV DNA detected, Invalid Result: Specimen was positive, but must be retested as the absence of BG bands indicated inadequate specimen, processing, or the presence of inhibitors. Another aliquot of the original specimen should be processed and the test repeated.
+	-	+	HPV DNA detected, Invalid Result: Specimen was positive for HPV DNA, but must be retested as the absence of the BG low band indicated inadequate specimen, processing, or the presence of inhibitors. Another aliquot of the original specimen should be processed and the test repeated.
-	+	+	HPV DNA not detected, Valid Result: Sample negative for HPV DNA and the result was entered in to the database.
+	+	+	HPV DNA detected, Genotypes reported: Specimen was positive for HPV DNA and the genotypes were entered in to the database.

Table 2.6 Interpretation of genotyping results positive for the cross reactive band

HPV positive Bands	Interpretation
HPV 33 and HPV 52/33/35/58	HPV 33 positive *
HPV 35 and HPV 52/33/35/58	HPV 35 positive *
HPV 58 and HPV 52/33/35/58	HPV 58 positive *
HPV 52/33/35/58	HPV 52 positive

* Co-infection with HPV 52 cannot be ruled out.

2.5 Detection of HPV E6/E7 mRNA

The HPV PreTect™ Proofer kit was chosen for detection of HPV E6/E7, at the time this was the only commercially available kit for the detecting expression of HPV oncogenes. The HPV PreTect™ Proofer is a real time nucleic acid amplification based, qualitative assay for the detection of oncogenic expression of HR HPV types. The assay detects E6/E7 mRNA from five high risk HPV types HPV 16, 18, 31, 33 and 45 in addition to mRNA from the human U1 small nuclear ribonucleoprotein specific protein A (U1A) to monitor sample mRNA integrity. It is based on NASBA (Nucleic Acid Sequence Based Amplification) technology, an enzymatic amplification process that can amplify RNA under isothermal (41°C) conditions. Figure 2.5 shows how the reaction involves three enzymes and DNA intermediary from which many copies of antisense RNA are made and detected using molecular beacon probes (figure 2.5).

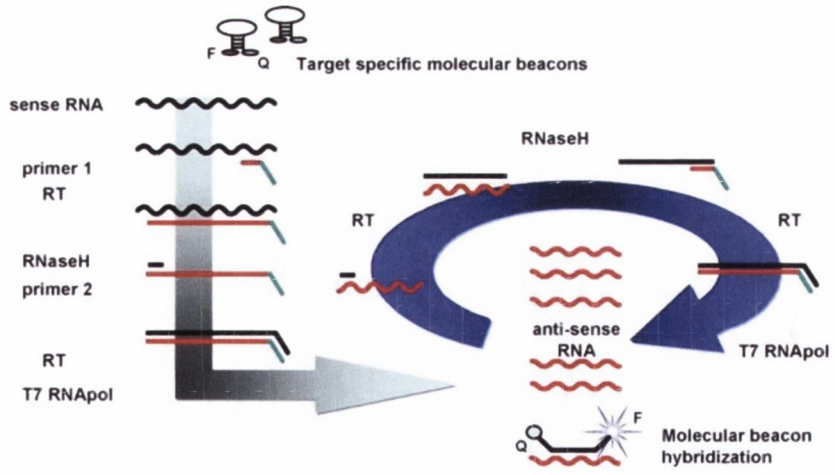

Figure 2-5 NASBA reaction with molecular beacon probe detection

2.5.1 NASBA

The PreTect™ HPV Proofer involves two procedures as shown in figure 2.5; nucleic acid amplification and nucleic acid real-time detection with molecular beacon probes. The first phase of NASBA is "non-cyclic". Primer 1, which contains 5'-terminal T7 RNA polymerase promoter sequence, anneals to the sense RNA target sequence. Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) extends the 3' end of primer 1 forming a cDNA copy of the RNA template resulting the formation of a RNA:DNA hybrid. Next, RNA is hydrolysed from the hybrid by the enzyme RNase H resulting in single stranded DNA molecule. Primer 2 anneals to the single strand DNA and reserve transcriptase synthesises a second DNA strand and a promoter region creating a transcriptionally active double stranded DNA molecule. T7 RNA polymerase can now transcribe antisene RNA copies based on the original sense RNA sequence. Each new antisense RNA can act as a template for further reactions. Primer 2 can bind to antisense RNA, the action of reverse transcriptase elongates the strand generating RNA:DNA hybrid as before. RNase H hydrolyses the RNA strand leaving the single stand DNA molecule. Primer 1 binds to the DNA strand and AMV-RT again yielding a transcriptionally-active promoter allowing more anti-sense RNA to be produced by T7 RNA polymerase. As reaction occurs in a self sustained manor more anti-sense RNA becomes available for molecular beacons to bind.

2.5.2 Molecular Beacon Probes

Detection is based on binding of molecular beacon probes in real-time to antisense RNA. Molecular beacon probes are single stranded oligonucleotides with a stem loop structure containing a sequence complementary to the target sequence, the amplified anti-sense RNA, on the loop region. The stem is double stranded, the 5' arm contains a fluorphore which is captured by the presence of a quencher on the 3' arm when in a non-hybridised state. Molecular beacons remain in the quenched state in the presence of sense RNA, on hybridization with the target sequence, anti-sense RNA the beacon undergoes a conformational change resulting in the separation of the fluorophore and quencher emitting fluorescence signal. The fluorescence intensity is proportionate to amplicon concentration, real time measurement of

fluorescence results in generation of an exponential curve with an eventual plateau occurring.

2.5.3 Sample preparation

Extraction of Total Nucleic Acid (TNA) from PreservCyt Specimens was required in order to perform PreTect™ HPV Proofer. 10ml conical sarstedt tubes were labelled with sample study identification number. Each PreserCyt™ vial was individually vortexed until cells were homogenously dispersed. Immediately a 5mls aliquot was transferred into the bottom of 10ml conical Sarstedt tube. Remaining sample was stored at room temperature. Specimens were centrifuged at 3800rpm for 12 minutes. The supernatant was discarded with a 3.5ml Pasteur pipette and washed with 1ml of 96-100% ethanol (prepared in RNase free H_2O). The pellets were resuspended by pipetting up and down. Specimens were centrifuged at 3800rpm for 12 minutes. The supernatant was removed using a 1ml pipette. and 400µl of buffer RLT (lysis buffer) from M48 RNA Cell mini kit (Qiagen, UK) containing 1% ß-mercaptoethanol was added to cell pellets (Lysis buffer was prepared fresh in sufficient volumes for the preparation of the number of samples being processed). The cell pellet was broken up by pipetting up and down ensuring no visible pellet could be seen. Each sample was vortexed for 1minute and incubated at room temperature for 10 minutes. All samples were checked to ensure full lysis of cell pellet. Samples were stored at -80°C until further use or used directly for TNA extraction.

2.5.4 Extraction of Total Nucleic acids by M48 Qiagen BioRobot™

TNA extraction was performed using the MagAttract M48 Nucleic Acid Extraction kit (Qiagen, UK) for the BioRobot® M48 (Qiagen). The TNA extraction was performed using customised TNA extraction protocol designed by Norchip for the use with the HPV PreTect Assays. Lysed samples were removed -80°C and allowed to thaw on ice. The M48 Qiagen BioRobot™ was turned on and GenoM48 software opened. From the drop down menu "custom norchip" and cross tab "total nucleic acid" was selected. An elution tube volume of 1.5ml was selected and the number of samples selected (multiple of

6 up to 48). Sample volume was set at 400µl and an elution volume of 50µl was selected. The M48 BioRobot was set up by following the on screen wizard. First the tip guard and waste chute were checked to ensure they were clean and waste bag was attached to the waste shoot. The required number of M48 filter tips (Qiagen, UK), reagent reservoirs, sample prep plates, 1.5 ml caped tubes, 1.5 ml uncapped tubes were added to the instrument deck. Using the volumes described on screen, reagents were loaded from the MagAttract RNA Cell Mini M48 kit; (RNase free H_2O, buffer RPE, buffer MW from the Magattract M48 RNA Cell mini kit (Qiagen, UK) and 96% Ethanol (made up in RNase free H_2O). Reagents were dispensed in the nucleic acid extraction hood into large reagent containers to the required volumes and loaded in the positions indicated. Suspension E/F magnetic beads were vortexed vigorously at max speed for 5 minutes when opening a new vial and 1 minute in all other cases. The required amount was dispensed into a small reagent container (Qiagen, UK) in the nucleic acid hood and placed in the appropriate position in the M48. Lysed samples were vortexed, 400 uL of each lysed sample was placed in to labelled 1.5 ml microfuge tube with no cap and into the sample racks. The samples were then loaded into the sample racks in sequence from left to right, with the positions from 1-46 matching the position of the corresponding labelled 1.5ml elution tubes in heating block 2. A visual inspection of all the settings, positions of plastics, reagents and samples was made. When all required elements were confirmed the extraction was started. Upon completion of the extraction protocol the lids were replaced on the 1.5 ml screw cap tubes, the extracted sample was removed from the M48 and the was stored at -80°C for HPV mRNA detection by the PreTect™ HPV Proofer assay (NorChip AS, Norway).

2.5.5 Detection of HPV16, 18, 31, 33 and 45 E6/E7 mRNA by Pretect™ HPV Proofer

Each PreTect™ HPV Proofer kit supplies enough reagents to test 30 samples with 6 supplied controls on a 96 well plate. Positive controls are synthetic oligonucleotides for respective HPV types. On delivery the PreTect™ HPV Proofer kits were stored at -20°C. Prior to testing a kits were thawed at room

temperature. TNA samples were removed from the freezer and allowed to thaw on ice.

The PreTect™ Analyser and accompanying PC were turned on. The Proofer Plate Design software (PPD) was started. The sample name, run name, operator and lot number were entered as required and the file was saved and exported. The KC4 software was opened and the PreTect™ analyser protocol was selected. Via the control function, a preheating temperature of 41°C was set. To ensure correct performance of the Analyser an Optics test was performed at this stage before every test.

Mastermix was prepared in the hood. Enzymes were provided as lyophilised spheres containing AMV-RT, RNase H and RNA polymerase. Firstly 171µl enzyme sphere diluent was added to each of three tubes containing three enzyme spheres and mixed by flicking, spun briefly and left in the hood to dissolve for at least 20 minutes and no longer than 60 minutes. Lyophilised reagent spheres containing nucleotides, dithiotretiol and Magnesium chloride were reconstituted with 240µl of reagent diluents. The reagent solution was vortex immediately until spheres fully dissolved. Mastermix for each reaction (U1A/HPV 16, HPV 18/HPV 33 and HPV 33/HPV 45) was prepared in three separate 1.5ml caped tubes by aliquoting 240µl of reagent solution in to each tube. 30µl of primer/molecular beacon probe was added to each corresponding tube. 42µl of NASBA H_2O and 48µl of potassium chloride was added to each tube. Mastermix was mixed thoroughly by pipetting up and down. The assay was set on in a 96 well non-skirted plate (ABgene) as shown in figure 2.6. The plate set up was performed on the QIAgility™ (Qiagen, UK), a benchtop instrument for the set up of PCR plates. The plate was set up using a custom protocol for addition of mastermix and samples to a 96 well plate (figure 2.6). Controls were added manually. The QIAgility and the accompanying pc were turned on. QIAgility software was launched for plate set up of 30 samples. The waste chute and tip collection box were checked to ensure clean and empty. The necessary tips were added to appropriate positions. Tips were selected as available on screening by right clicking tip tray and selecting "select whole tip rack as available". Prepared mastermix was mixed before loading on to the QIAgility by pipetting up and down, the caps were removed and tubes placed in the appropriate positions as indicated on screen. An empty 96 well non-skirted plate was place on the plate rack. Samples were flicked to mix and

briefly spun, the caps were removed and samples loaded on the sample rack in positions 1-30.

On completion the plate containing mastermix and samples was removed from the QIAgility. The NucliSens EasyQ (Biomerieux S.A., France) thermocycler was turned on and a cycle program of 2 minutes at 65°C followed by 2 minutes at 41°C was selected. 10µl of U1A/HPV 16 mastermix was added to wells G4 and H4, 10µl of HPV 18/HPV 31 mastermix was added to wells G8 and H8, 10µl of HPV 33/HPV 45 mastermix was added to wells G12 and H12. Positive controls were provided in the kit, 5µl of U1A/HPV 16 positive control was added to G4. 5µl of HPV 18/HPV 31 positive control was added to G8. 5µl of HPV 33/HPV 45 positive control was added to G12. NASBA H_2 O was used as negative control as recommended by the manufacturer, 5µl NASBA H_2 O was added to wells H4, H8 and H12. Cap strips (ABgene) were placed onto the plate and the plate was placed on the thermocycler. The reaction was heated to 65°C to destabilise secondary structure of RNA, the reaction was then cooled down to 41°C for 2 minutes. While the thermocycler program was running enzyme solution was mixed by pipetting up and down and 5µl was added to new cap strips. On completion of the thermocycler incubation the lid was opened and the cap strips were removed from the plate and replaced with the cap strips containing added enzyme while the temperature was held at 41°C. The plate was removed from the thermocycler and centrifuged at 1500 rpm at 20°C for approximately 30 seconds to spin the enzyme into wells. It was important to ensure that the centrifuge was set to between RT-41°C as a lower temperature could detrimentally affect the enzyme. The plate was placed into the PreTect™ HPV Proofer Analyser and the Analyser was started from the computer terminal. The reaction was run at 41°C for 2 hours and 30 minutes. A measurement was taken every 1.5 minutes.

FAM type	HPV 16				HPV 31				HPV 33			
TxR type	U1A				HPV 18				HPV 45			
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G				Pos.ctrl				Pos.ctrl				Pos.ctrl
H				Neg.ctrl				Neg.ctrl				Neg.ctrl

Figure 2-6 Plate set up

Results are computed from Relative Fluorescence Units and reported as a ratio along with graphs FAM (HPV 16, HPV 31 and HPV 33) and TxR (U1A, HPV 18, HPV 45) for each sample. Ratios are calculated once necessary conditions are satisfied for each curve. The linear regression slope for the curve must be positive, the start RFU must be less than the total mean RFU, the minimum RFU must be greater than 0. Ratios are calculated from the highest RFU and start RFU. Calculation of the relative fluorescence signal increase and qualitative interpretation of the results (positive, negative, undetermined) was automatically performed by the PreTect Analysis Software. Results were regarded as positive when the relative fluorescence signal increase during the amplification process was at least 1.7 (≥1.7) and the curve S-shaped. Results were regarded as indeterminate when the relative signal increase was in the range 1.4 –1.7. These samples were retested by re-extracting from the original sample. Two indeterminate results from the same sample were considered as a positive result. Results were regarded as negative when the relative signal increase was less than 1.4 (<1.4).

2.5.6 Assay controls

The kit positive controls were expected to have a relative signal increase during the amplification process of at least 1.7 and an S-shaped curve. If positive control was negative (the relative fluorescence signal increase was less than 1.4 all results for the corresponding primer and probe set were considered invalid and all samples retested were possible. A negative control was regarded negative when the relative fluorescence signal increase during the amplification process was below 1.4 (<1.4). If a negative control is

positive (≥1.4) all results for the corresponding primer and probe set were considered invalid and all samples retested were possible. A positive result for U1A was required for a negative HPV test result to be valid. If the U1A was negative and the sample was negative for all 5 HPV types the entire run was declared invalid for the sample in question and the sample was re-tested where possible. A negative U1A result together with a positive HPV test result did occur in some cases. In these cases a valid and positive HPV result was recorded as recommended by the manufacturer.

2.6 Nicotine Metabolite Assay

The nicotine metabolite cotinine, the principle metabolite of nicotine was measured using Nicotine Metabolite Immunoassay on Siemens Immulite 2000 analyser. The assay is a solid phase competitive chemoiluminescent immunoassay that makes use of the highly specific polyclonal rabbit anti-cotinine antibody and alkaline phosphatase conjugate. The IMMULITE 2000 is a continuous random-access instrument that performs chemiluminesent immunoassays. The instrument uses antibody/antigen coated polystyrene beads as the solid phase. The bead is dispensed in to a reaction tube where it undergoes incubation, washing and signal development process. An alkaline phosphatase-labeled reagent and dioxetene substrate emit a light signal quantifying target analyte in the sample. Light emission is measured by Photomultiplier Tube. The nicotine metabolite assay ranges 10ng-500ng/ml, samples measuring outside the assay range are diluted using IMMULITE® 2000 multi-diluent 2.

2.6.1 Sample preparation

Upon arrival to the molecular pathology laboratory each urine sample label was cross checked with sample submission form. A 5ml aliquot of sample was transferred to a 10ml conical Sarstedt tube and stored at -80°C. Samples were removed from -80°C and allowed to thaw at room temperature. Barcoded labels were attached to each sample. Individual barcode identifier number and sample number were recorded.

Periodic maintenance was performed on the IMMULITE 2000 instrument by the biochemistry laboratory staff, daily and weekly maintenance is mandatory. The Nicotine Metabolite kit supplies enough reagents for 200 tests. Prior to use of a new kit lot adjustment was run followed by a recommended adjustment interval of 2 weeks with two adjustment reagent supplied in the kit. Adjustment allows for any difference between in instruments from the manufacturer and the laboratory. Two levels of adjusters were received with each nicotine metabolite kit. 500µl aliqutes were transferred to tubes labelled with provided barcodes. Adjustor A and adjustor B were placed in to sample rack and loaded on to sample carousel. Once the carousel was fully interrogated within the worklist tab on screen the the information for each adjustor was reviewed, lot number and expiration date was up dated where necessary. Before processing any samples adjustment must be rendered "complete" by the instrument under the following criteria. 1. The slope does not exceed rejection limits of ±20% of instrument slope or ±10% of instrument readjustment. The coefficient of variance must be <10%. Control samples must be run to validate adjustment and with each batch of 100 samples. Serum Drug Control Module, an assayed human serum based, bi-level control containing drugs and metabolites is recommended for use with Nicotine Metabolite assay. The module contains two levels of control containing different concentrations of selected drugs and metabolites. At least 30 minutes prior to use each control was reconstituted with 5ml deionized water. Reconstituted controls were mixed gently by swirling and inversion. Controls were stable at 2-8°C for 30 days after reconstitution. Each quality control must be entered on the instrument before use. Under data entry new control was selected. The control name, lot number and expiration date were entered. The mean standard deviation and control ranges (provided in package insert) was entered for each new control lot. Once the kit was adjusted and validated by controls patient samples can be tested. Urine samples were removed form -80°C, allowed to thaw and a barcoded label was attached to each sample. Samples were centrifuged for 10 minutes at 3800rpm. Samples and diluent were placed in sample racks and loaded on the sample carousel. Once the carousel was fully interrogated Nicotine Metabolite test was ordered for each sample by entering the worklist tab, clicking tests and selecting NM. Samples from women reporting as active smokers were diluted on board, with the worklist tab arrow buttons were used to scroll

through samples by clicking dilutions and selecting x40 (recommended by the manufacturer). Results were displayed on screen as ng/ml.

2.7 Immunocytochemistry-CINtec PLUS

The CINtec® PLUS kit (Roche) was chosen for $p16^{INK4a}$/Ki-67 expression. This is the only commercially available kit for detection of over expression of $p16^{INK4a}$/Ki-67. The CINtec® PLUS kit is an immunocytochemistry assay for the simultaneous qualitative detection of $p16^{INK4a}$ and Ki-67 antigens in cervical cytology preparations.

The assay involves a two-step immunocytochemical staining procedure. Antigens are detected by a primary monoclonal mouse antibody clone E6H4™ directed at human $p16^{INK4a}$ protein and a primary monoclonal rabbit clone 274-11 AC3 directed at human Ki-67 protein. Visualization is based on a polymer reagent conjugated to horseradish peroxidase and goat anti-mouse Fab antibody fragments and alkaline phosphatase and goat anti-rabbit Fab antibody fragments for $p16^{INK4a}$ and Ki-67 respectively. Chromogen reactions are based on horseradish peroxidase mediated conversion of a DAB chromogen and phosphatase-mediated conversion of Fast Red chromogen. Specimens were evaluated by light microscopy.

2.7.1 Validation Samples

A collection of 20 residual ThinPrep cervical smear samples were anonymised and donated from the cytology laboratory at the Coombe Women's and Infants University Hospital including 7 Negative, 6 LSIL and 7 HSIL. In addition, as a positive control cytospin preparation of HeLa cells were stained using the CINtec® PLUS kit.

2.7.2 Patient Samples

Following testing for HPV DNA and HPV mRNA residual PreservCyt® material was used for immunocytochemistry. Samples were processed on the ThinPrep 2000 Processor (T2) (Hologic). The T2 is an automated slide preparation unit,

a filter membrane controls dispersion, collection and transfer of cells from the sample to the slide.

The lid is removed from the sample the vial is placed into the instrument. The filter is placed into the vial where it rotates separating debris and dispersing mucus. Negative pressure pulses draws liquid through the filter collecting a thin even layer of cells on the exterior surface of the filter membrane. The rate of flow through the filter is monitored ensuring an appropriate amount of material is collected. Once cells are collected the filter inverts and is gently pressed against the ThinPrep® slide. Surface tension and positive air pressure cause the cells to adhere to the slide giving an even distribution of cells over a circular area. The slide is then ejected into an alcohol fixative bath.

Slides were fixed in 99% ethanol for at least 20 minutes and maximum 24 hours. Epitope retrieval solution was prepared by making a 1:10 dilution with diH_2O, poured in to coplin jars and placed in a waterbath set to 95°C. While the water bath was heating slides were removed from ethanol and air dried for a minimum of 20 minutes (maximum 16 hours). Specimens were then rehydrated in deionised water for 10 minutes. A thermometer was used to ensure the temperature of the epitope retrieval solution was at 95-100°C before slides were added. Addition of slides caused a drop in temperature, epitope retrieval solution was allowed to reached 95°C again was and the slides incubated for a further 10 minutes. The coplin jars were then removed from the waterbath, lids removed and allowed to stand at room temperature for 20 minutes or until the liquid inside reached 50°C. Slides were then removed and placed in wash buffer solution (1:10 dilution with diH_2O) for 5 minutes. Slides were removed and tapped to remove excess liquid. Each specimen was covered with 200µl of peroxidase-blocking reagent and incubated for 5 (±1) minutes followed by 5 minutes in wash buffer solution. Slides were removed and tapped to remove excess liquid. The specimen was covered with 200µl of primary antibody and incubated for 30 (±1) minutes followed by 5 minutes in wash buffer. Slides were removed and tapped to remove excess liquid. The specimen was covered with 200µl of visualization reagent HRP and incubated for 15 (±1) minutes followed by three washes in wash buffer for 5 minutes. Slides were removed and tapped to remove excess liquid. The specimen was covered with 200µl of visualization reagent AP and incubated for 15 (±1) minutes followed by three washes in wash buffer for 5

minutes. The specimen was covered with 200µl of Substrate-Chromogen Solution (DAB) prepared fresh by adding 1 drop of DAB chromogen per 1ml of DAB substrate solution and incubated for 10 (±1) minutes followed by incubation in diH_2O for 5 minutes then 5 minutes in fresh wash buffer. The specimen was covered with 200µl of substrate chromogen solution fast red and incubated for 15 (±1) minutes followed by wash buffer for 5 minutes. Slides were rinsed in diH_2O for 5 minutes. Slides were countered stained in filtered Harris Hematoxlyin for 3 minutes and blued in tap water for 30 seconds. Slides were briefly rinsed in diH_2O. A two-step mounting procedure is recommended by the manufacturer involving first liquid cover slipping with 4 drops of CINtec PLUS mount. Slides are allowed to dry overnight. After drying of the aqueous mount slides were incubated in xylene for 1-20 minutes followed by glass cover slipping using Pertex mounting medium.

2.7.3 Controls

Positive controls: a pooled group of known HSIL/dual positive cervical smears samples (provided by cytology laboratory at the Coombe) were used as positive control. Samples were stained in batches of 20, each batch included a positive and negative control. Controls were processed in the same manner as the patient samples. To validate performance of test reagents positive controls had to demonstrate dual positive staining. Negative controls verified specificity of staining procedure and provided indication of background staining. Negative controls were expected to demonstrate no positive staining. In addition the presence of known negative cell types in representative cervical cytology specimens (eg superficial cells) served as an internal negative control.

2.7.4 Interpretation of results

The CINtec® PLUS procedure produces two distinct coloured reaction products, a brown precipitate at the $p16^{INK4A}$ antigen sites, and a red precipitate at the Ki-67 antigen sites. $p16^{INK4A}$ overexpression was indicated by brown staining of cytoplasm and/or nuclei. Ki-67 overexpression was indicated by red nuclear staining. Cell staining positive for both antigens displayed brown staining over the cell with pronounced red nuclei. The presence of one

or more dual stained cell was regarded as a positive result. A qualified pathologist evaluated all samples blind to HPV test results and histology results.

2.8 Statistical Analysis

The final outcome was based on the histological grade taken as the worse of the histological findings on punch or large loop excision during a patient's time at colposcopy. CIN 2 and CIN 3 were grouped as high grade disease (CIN 2+) and CIN 1 and Normal taken as no high grade disease (<CIN2). Data was analysed using Minitab statistical software version 16. Comparisons on independent group proportions were calculated using the Pearson's chi-squared test. Confidence intervals were calculated where appropriate. The clinical performance of the HPV tests was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the relative 95% confidence intervals (CI) for detecting CIN2+ and CIN 3. The McNemar's test was used to compare differences in rates of disease detection between HPV tests. The Cohen's kappa coefficient was used to ascertain the agreement between two tests. Binary logistic regression was used to calculate odds ratio and 95% confidence intervals. A p value < 0.05 was considered statistically significant.

-Chapter 3-

The prevalence of high grade cervical intra epithelial neoplasia (CIN 2+) in women presenting to colposcopy with low grade cervical abnormalities LSIL and ASCUS

Overview

- Low grade smears LSIL and ASCUS represent a high proportion of referrals to colposcopy.
- Not all women with low grade smears develop high grade cervical disease.
- Debate remains over the appropriate management of women with low grade smears LSIL and ASCUS.
- Decisions regarding triage, follow up and treatment of low grade cervical lesions have implications with respect to patient's anxiety in addition to health services planning and allocation of resources.
- The main objective of the chapter is to identify the overall prevalence of high grade disease (CIN 2+) in a population of Irish women referred to colposcopy with LSIL/ASCUS.

3 The prevalence CIN 2+ in LSIL and ASCUS

3.1 Introduction

Cervical screening programs aim to reduce the incidence and mortality of cervical cancer by the detection and effective treatment of pre-cancerous lesions. Cytological screening has proven to be very successful in reducing incidence of cervical cancer. Its use is based on the premise that cervical cancer develops gradually through well defined pre-malignant stages.

Pre-cancerous lesions of squamous origin are characterised on histology as progressive lesions ranging from CIN 1 - CIN 3, depending on degree of involvement of the epithelial thickness by dysplastic cells. Cytological squamous intraepithelial lesion (SIL) is suggestive of a CIN lesion and is recognised from atypical and low grade SIL to high grade SIL. Morphology of the various grades of SIL are shown in figure 3.1. These changes are graded according to the Bethesda system (appendix 1) as Negative, ASCUS (abnormal squamous cells of undetermined significance), LSIL (low grade squamous intraepithelial lesion), ASC-H (abnormal squamous cells cannot exclude high grade) and HSIL (high grade squamous intraepithelial lesion). The classification ASCUS represents abnormal cellular changes that although are not negative do not meet the criteria for a squamous intraepithelial lesion. They are considered minor abnormalities and are managed similarly to other low grade abnormal smears, LSIL. LSIL represents the BSCC classification of mild dyskaryosis and corresponds to histological diagnosis of CIN 1. While HSIL represents moderate and server dyskaryosis and corresponds to CIN 2/CIN 3 histological diagnosis. Currently all women with cytological evidence of HSIL and ASC-H and those with repeat LSIL and ASCUS smears are referred to colposcopy. In Ireland, two consecutive LSIL smears and three consecutive ASCUS smears warrants referral to colposcopy.

Colposcopic abnormalities are diagnosed by a process of pattern recognition and can be graded according to Reids colposcopic index, this takes into consideration four signs, intensity of acetowhitening, lesion margin and surface contour of acetowhitening, vascular features and iodine staining (Ferris and Greenberg, 1994). Recently a new scoring system was suggested, the Swede score takes in to account lesion size for a more accurate prediction of CIN 2+ (Bowring et al., 2010). Colposcopy directed biopsies can be obtained

and interpreted by histopathology which has traditionally been considered to be the "gold standard" for diagnosis of cervical disease.

It is well known that infection with human papillomavirus (HPV) is the main causative agent in the development of cervical cancer and pre-cancer. In the mildest scenario, CIN 1, a low progression rate is seen and is often just the manifestation of a transient HPV infection. As shown in figure 3.1, it is persistent infection that can lead to the risk of high grade lesions. Transition from initial infection to cancer does not always occur, if malignancy ensues it is a slow progression taking up to 10-15 years before invasive cervical cancer develops.

While HPV is necessary it is not a sufficient cause of cervical cancer. It is believed that other risk factors are involved. The International Association for Research on Cancer (IARC) has listed long term (five or more years) use of the combined oral contraceptive and smoking to be key carcinogenic factors in the development of cervical cancer (Plummer et al., 2003, Cogliano et al., 2005).

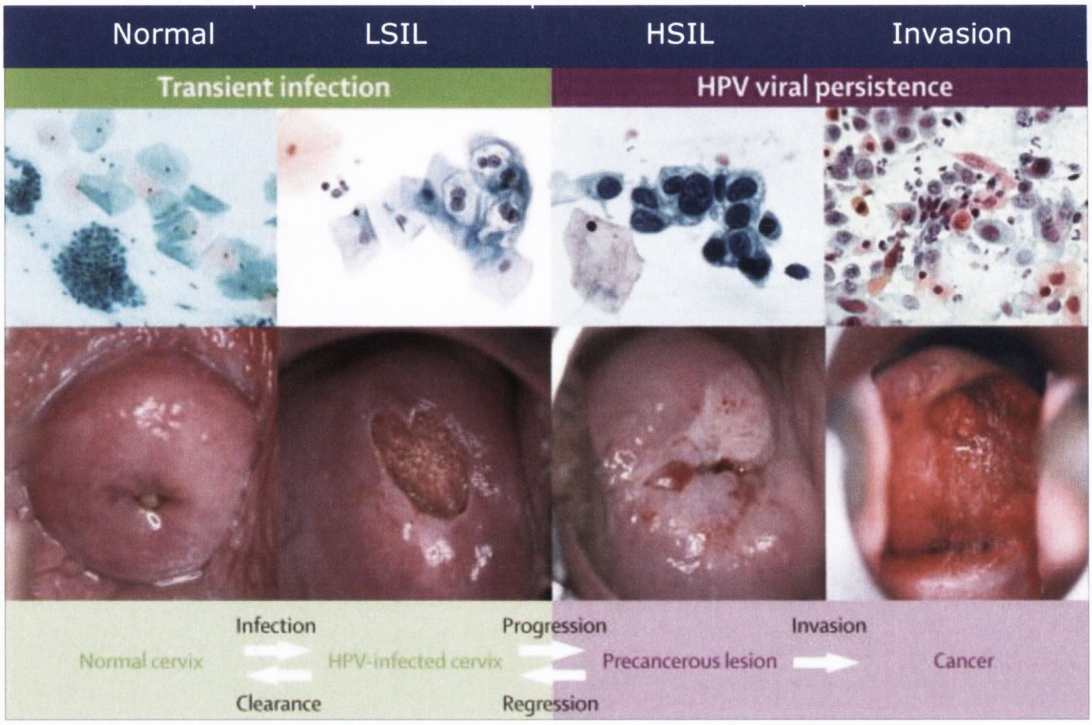

Figure 3-1 Progression of cervical cancer

Progression of cervical cancer by cytology (top) and colposcopy (bottom) in relation to HPV infection. Cervical cancer develops by four main steps, HPV transmission, viral persistence, progression to pre-cancer and invasion. However this is a two way system, clearance of HPV infection can occur and regression to normality of pre-cancerous lesions. Cytologically squamous abnormalities are classified by the Bethesda system as low and high squamous intraepithelial lesions distinguished primarily by increasing nuclear cytolplasmic ratio, hyperchromasia, irregular nuclear membrane and chromatin distribution (National Cancer Institute Workshop, 1989). Colposcopally, abnormalities are diagnosed by a process of pattern recognition and can be graded according to Reids colposcopic index, this takes into consideration four signs, intensity of acetowhitening, lesion margin and surface contour of acetowhitening, vascular features, and iodine staining (Ferris and Greenberg, 1994). Adapted from Schiffman et al 2005.

It is widely accepted that CIN 1-3 represent successive stages in the development of cervical cancer. These precursors of invasive disease can be easily treated to prevent development of cancer. General consensus of excisional treatment exists regarding the management of women with high grade cervical lesions. However, for over the last two decades debate continues over the appropriate management of minor abnormalities (Ahmed et al., 2008, Flannelly et al., 1994, Rana et al., 2004). Cytological surveillance is the current management option for single minor abnormal smears, alternative strategies have been suggested such as immediate colposcopy for LSIL and HPV triage for ASCUS (ASCUS/LSIL triage group, 2003). In 2011, the NHS introduced HPV DNA triage for LSIL and ASCUS. Studies from sentinel sites found that although the negative predictive value of HPV testing was high (96%) a number of women positive for HPV were found to be negative at colposcopy (Kelly et al., 2011). Thus, controversy remains regarding appropriate management with concerns with respect to overtreatment following immediate referral (Cotton et al., 2010, Ahmed et al., 2008). Within colposcopy, guidelines for management of women with colposcopic/histological evaluation of less than CIN 2 recommend repeat cytology at 6, 12 and up to 18 months. Further repeat colposcopic examination may follow if minor abnormalities persist after 18 months (CervicalCheck, 2009). The rationale for repeat follow up and treatment of CIN 1 is in the knowledge that while many of these abnormalities will spontaneously revert to normal over time, there remains potential risk of progression to more server disease. Thus, colposcopy is a critical modality in the triage of minor cervical lesions.

Reported regression rates and outcome of cytological minor abnormal smears LSIL and ASCUS vary. This may be due to the subjective nature of diagnosis of low grade and atypical abnormalities (Stoler and Schiffman, 2001). In addition, there is variety in the type of outcomes measured, the number of smears taken and variable lengths of follow up periods. Progression from low to high grade CIN has been reported in the range of 10-30% (Emerson et al., 2002, Rana et al., 2004, Giannopoulos et al., 2005, Ahmed et al., 2008, Jones et al., 1992). Histologically the regression rates of CIN 1 have been found to be in the region of 60-80% (Cox et al., 2003, Moscicki et al., 2004, Nasiell et al., 1986) with approximately only 10% progressing to CIN3. The progression rate to invasive cervical cancer is reported as 0.1-0.2% (Melnikow et al., 1998). As disease severity increases the rate of regression decreases. As high

grade lesions are normally treated little is known about the progression rate to invasive disease however it is thought to be in the region of 5% for CIN 2 and 12-30% CIN 3 (Ostor, 1993, McCredie et al., 2008).

Treatment for cervical abnormalities has evolved, we have moved forward from the invasive procedures such as hysterectomy and cold knife to the safer excisional procedures that can be performed in an outpatient clinic. Large Loop Excision of the Transformation Zone (LLETZ) is the method of choice in most colposcopy clinics, it has been found to be successful in eradicating disease however it is not without its problems. While obstetrical morbidity is reduced compared with past methods, excisional treatments remain associated with obstetrical morbidity and high costs. Another important cause of concern is the possibility of over treatment (Kyrgiou et al., 2006, Shafi, 1994, Ferris et al., 1996) especially in women with minor abnormalities.

In Ireland, a national cervical screening program CervicalCheck was introduced in late 2008, and provides free pap smear tests for detecting pre-cancerous lesions to women aged 25 to 60 years. Since its launch, the programme has proved very successful with an average of 1,000 women availing of free smear test per day. The latest statistics available from the programme indicate that in excess of 280,000 women were screened during the second year, the majority of whom (85%) had a negative smear test, of the remaining 15%, minor abnormalities accounted for 12.5% and high grade abnormalities for 1.7% of smears. This disproportionate number of minor abnormal smears is largely due to smears reported as ASCUS. As discussed in section 1.6.1, there has been a dramatic increase in the number of reported ASCUS smears in recent years following the outsourcing of cytology services to the US. In total, 16,811 women attended a colposcopy clinic for the first time, with 7,546 receiving treatment (CervicalCheck, 2009-2010).

From 2009-2010, 9,552 women in total were referred to colposcopy on the basis of having a minor abnormality. This represents 67% of overall referrals to colposcopy. It is important to note that organised cervical screening begin in late 2008. It would be expected that the rate of abnormalities would be quite high as many women would never have been screened before. However minor abnormalities are a global issue, in England 48% of referrals are on the basis of a minor abnormal smear LSIL or ASCUS (NHSSCP, 2011). While many low grade abnormal and equivocal smears regress spontaneously a

small proportion will go on to develop CIN 2 and CIN 3. This chapter examines a population of women enrolled at their first visit to colposcopy, their initial colposcopic evaluation and what happens to these women during the course of their follow up in colposcopy. A subgroup of women, who have to date completed an 18 month follow up is further evaluated to identify the cumulative incidence of CIN 2+.

3.2 Aims

- To determine the mean time to identification of CIN 2+ in women referred to colposcopy with LSIL/ASCUS.

- To identify the 18 month cumulative rate of CIN 2+ in women referred to colposcopy with initial persistent LSIL/ASCUS smears and further examine if referral smear affects outcome.

- To identify the 18 month cumulative rate of CIN 2+ in women referred to colposcopy with LSIL/ASCUS with a normal colposcopic impression at first visit.

- To identify risk factors including contraceptive use and smoking in development of CIN 2+.

3.3 Materials and Methods

3.3.1 Study population

Women were recruited into this study through the colposcopy clinics at the National Maternity Hospital, Holles Street, Dublin from October 2008 to July 2011 as described in section 2.1. Enrolment was on the basis of being referred for the first time to colposcopy with two or more minor abnormal smears including LSIL or ASCUS. The women's informed consent was obtained and all clinical and demographic details were collected from the hospital's Mediscan system for each patient. At their first visit, all women were assessed by colposcopy, if an abnormal area was seen a biopsy was taken during colposcopy for histological diagnosis of disease. Women were followed over subsequent visits where they had a cervical smear test or were examined colposcopically if cytology was HSIL or persistent minor abnormal cytology. Women were followed until they reached defined study endpoints which included having two consecutive normal smears and discharge from the clinic without treatment or alternatively having a LLETZ (Large Loop Excision of the Transformation Zone) treatment for presence of high grade pre-cancer.

3.3.2 Statistical analysis

The final outcome was based on the histological grade taken as the worse of the histological findings on punch or large loop excision during a patient's time at colposcopy. CIN 2 and CIN 3 were grouped as high grade and CIN 1 and Normal taken as no high grade disease. Data was analysed using Minitab statistical software version 16. Comparisons on the prevalence of CIN 2 and CIN 3 in LSIL and ASCUS referrals were calculated using the chi-squared test. Confidence intervals were calculated where appropriate. A p value <0.05 was considered statistically significant. Binary logistic regression was used to calculate odds ratio and 95% confidence intervals as a measure of association of cigarette smoking and hormonal contraception use with histological diagnosis of CIN 2+.

3.4 Results

3.4.1 Study Population

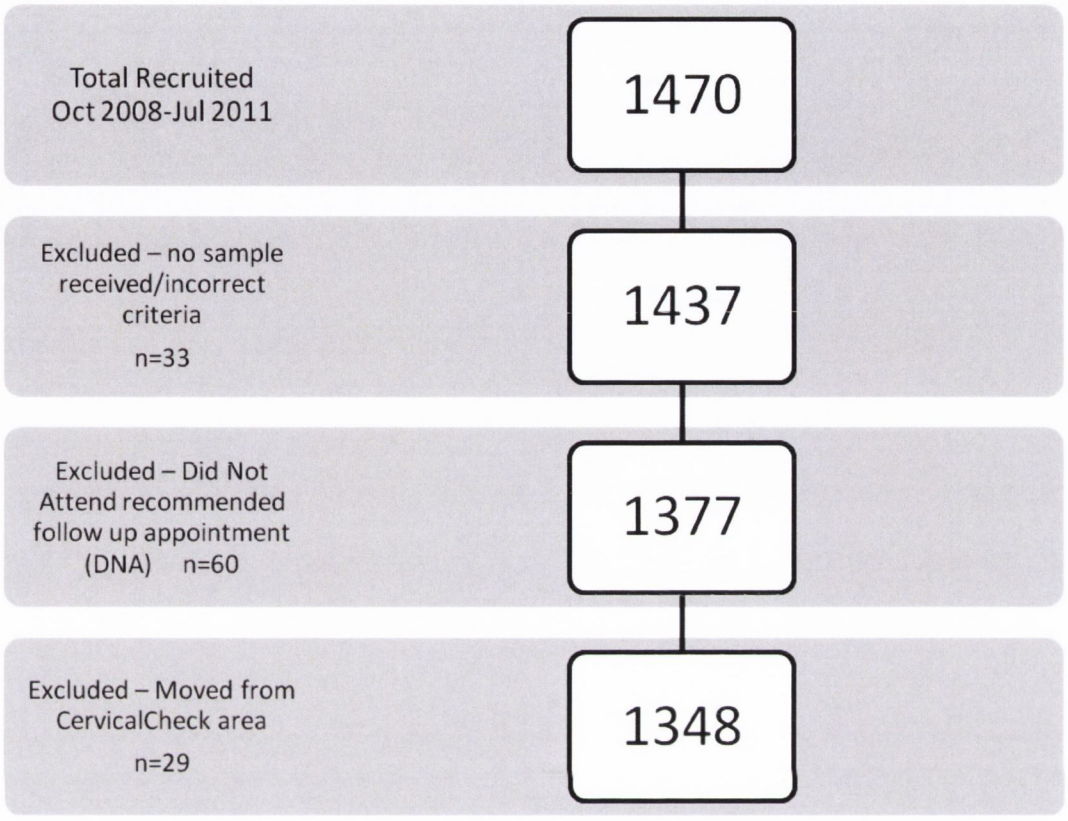

Figure 3-2 Breakdown of study population

Figure 3.2 details the breakdown of the study population. In total 1470 women were enrolled in the study. Due to not meeting the correct criteria or failing to obtain a sample, 33 patients were excluded. An additional 60 patients were lost due to lack of compliance with recommended follow up appointments. The majority of these, 78% (47/60), occurred following the first visit. The overall rate of women defaulting from follow up appointments in this study population is 4% (60/1437), which is within the limits set out in the CervicalCheck Guidelines in Quality Assurance in Cervical Screening document which states that the rate of defaulted appointments without any prior notice given should be maintained below 15%. A further 29 patients were lost from the study due to moving out of the CervicalCheck area during their follow up time. This results in 1348 patients eligible for analysis.

3.4.2 Socio-demographic characteristics

Demographics were collected from women at their first visit to colposcopy (Time 0) including referral smear diagnosis, age, smoking behaviour, contraceptive use and parity. Table 3.1 outlines the socio-demographic characteristics of the study population. The majority of women, approximately 80%, were under the age of 40 years, with 42.2% (570/1348) under the age of 30 years. LSIL referral was more common representing 59.1% (798/1348) of referrals compared to ASCUS at 41.0% (550/1348), while 15.4% (207/1348) of women were referred with a history of both LSIL and ASCUS cervical smears. Smoking, contraceptive use and parity data was available on a subset of patients. Smoking behaviour, available from 1286 patients indicated that 37.5% (483/1286) were reported as active smokers (table 3.2). Oral contraception was the most common form of contraception used, 37.3% (459/1232) of women were currently on oral contraception at time of enrolment. Further details of contraception use are outlined in figure 3.3.

Table 3.1 Socio-demographic characteristics for 1348 women referred to colposcopy with LSIL/ASCUS at baseline

Characteristic	Subjects n=1348 (%)
Age	18-65 y
Median	31y (interquartile range 27-38)
18-29y	42.3% (570/1348)
30-39y	35.8% (483/1348)
>40 y	21.9% (295/1348)
Referral smear	
LSIL	59.0% (798/1348)
ASCUS	41.0% (550/1348)
Active smoker	37.6% (483/1286)
Oral Contraceptive Pill	37.2% (458/1232)
Full term pregnancies	
0	60.2% (598/993)
1	15.6% (155/993)
2	12.1% (120/993)
3	7.6% (75/993)
4	2.4% (24/993)
>5	1.7% (17/993)

3.4.3 Contraceptive use

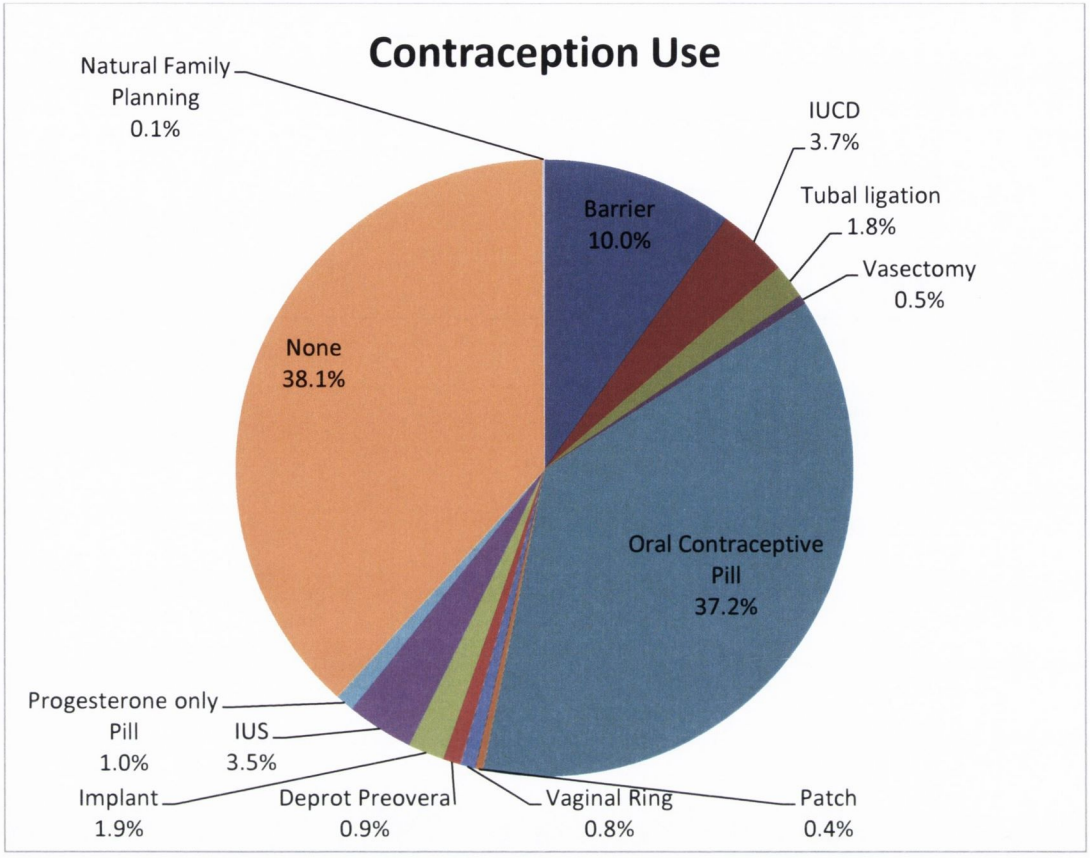

Figure 3-3 Reported contraceptive use at enrolment

From the 1348 patient population, data on contraceptive use was available for 1230. At enrolment a number of women 38.1% (469/1230) reported using no contraceptives; however, this figure must be taken with caution as details on sexual activity were not collected. Of those reporting use of contraceptives at enrolment, the oral contraceptive pill was the most common with 37.2% (458/1230) of the cohort currently taking oral contraceptives. In total, combined hormonal (oestrogen and progesterone) contraception including the oral contraceptive pill, patch, vaginal ring represented 38.5% (473/1230) of women. Other hormonal (progesterone only) based contraceptives including depot provera, progesterone only pill, implant and IUS (intrauterine system) accounted for 7.4% (91/1230). Barrier method represented the next most commonly used at 10.0% (123/1230) within the population. Other non-hormonal methods including barrier, IUCD (intrauterine contraceptive device), vasectomy, tubal ligation and natural family planning represented 16.0% (197/1230) in total.

3.4.4 Colposcopic findings - first presentation in colposcopy

On entering the study all women were assessed by colposcopy. The cervix was examined by a colposcopist and graded following the application of dilute acetic acid. Acetic acid dehydrates cells and causes coagulation of nuclear proteins identifying areas of increased nuclear activity. Lugol's iodine detects dyskaryotic regions by the absence of glycogen in the cells. Digital images were available for each patient, figure 3.4 show images for common colposcopy findings. A colposcopic impression was available for 1318 women. Non-neoplastic reports included normal, atrophic cervicitis, viral changes, and unsatisfactory (where the transformation zone was not identified). Squamous neoplasia was identified as Low Grade CIN, High grade CIN or ?microinvasion, when a range was given or multiple findings reported, the highest grade was recorded. Lesion severity is based on four main attributes, intensity of acetowhitening, lesion margin and surface contour of acetowhiting, vascular features, and iodine staining (figure 3.4).

Of the 1318 patients 22.1% (291/1318) appeared normal on colposcopy, 56.6% (746/1318) appeared as low grade CIN, 17.1% (226/1318) had a colposcopic impression of high grade CIN and there was 1 case of ?microinvasion (table 3.4). There were 6 cases of atrophic cervicitis, 9 viral, 5 not applicable. There were 21 cases where visualisation of the squamocolumnar junction was unsatisfactory. As the squamocolumnar junction tends to recede into the endocervical canal as a woman ages it is not surprising that cases of unsatisfactory colposcopy were often found in women over the age of 40 years (62%). Table 3.4 outlines the colposcopic impression in relation to referral smears LSIL and ASCUS. There was no significant difference in the presence of CIN based on colposcopy findings between LSIL and ASCUS referrals ($p=0.08$).

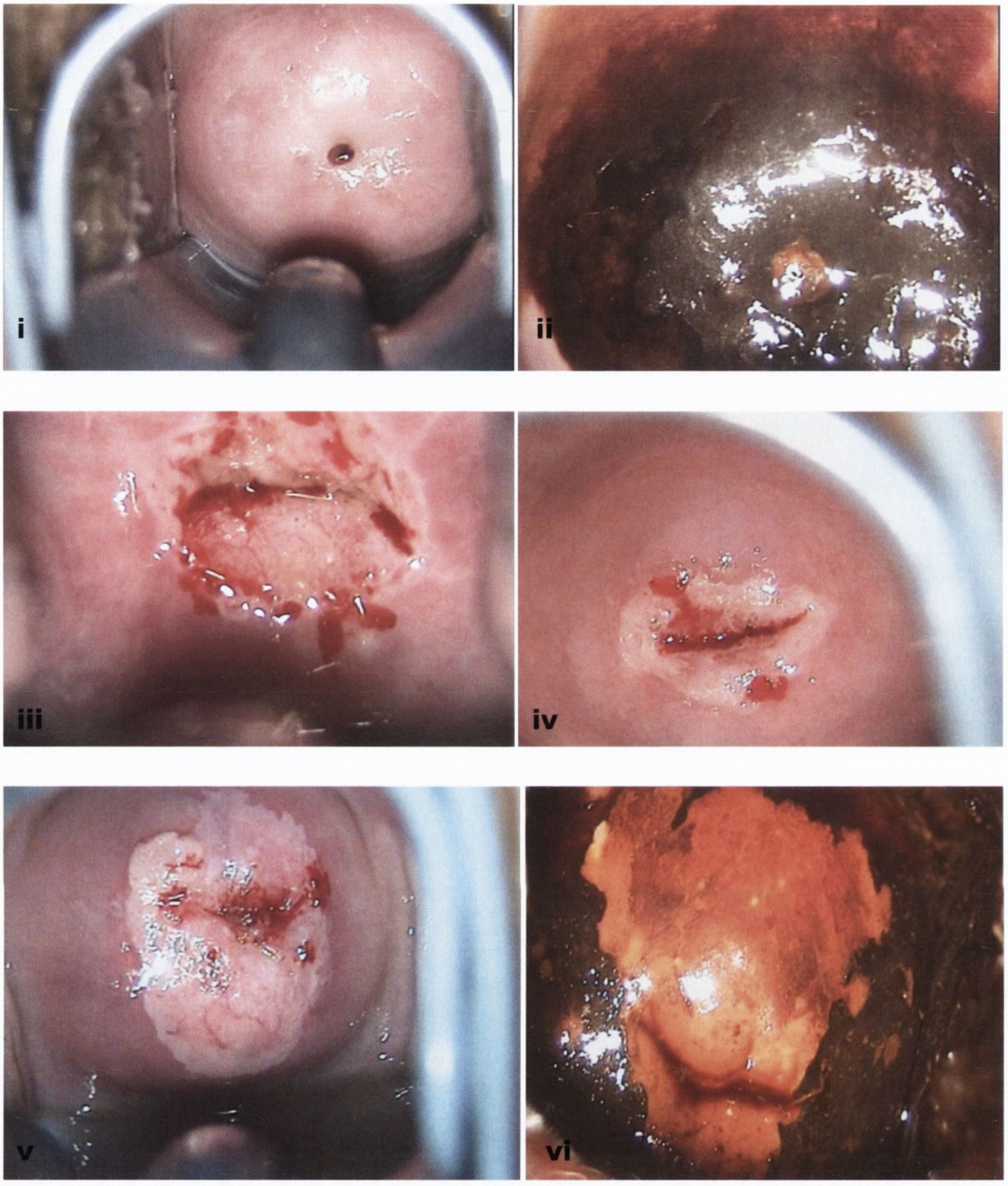

Figure 3-4 Colposcopic impression at first visit

i.) Normal cervix: cervix appears a dull pink the transformation zone can be identified a well demarcated, opaque acetowhite area. ii) Normal cervix: lugols iodine showing normal cervical epithelium positive black/brown staining, columnar epithelium negative mustard staining. iii and iv) Low grade CIN: the density of the acetowhitening is increased with irregular margins. v) High grade CIN: Dense acetowhitening with regular margins and course punctuation and mosaics vi) High grade CIN: lugols iodine remains mustard colour showing areas of dysplastic epithelium.

Table 3.2 Colposcopic findings for 1318 women at first visit in relation to referral smear

	Colposcopic Impression (n=1318)			
	Normal	**Low Grade CIN**	**High Grade CIN****	**Other***
	22.08% (n=291)	56.60% (n=746)	17.22% (n=227)	4.10% (n=54)
Referral smear				
LSIL (n=778)	20.2% (157)	58.4% (454)	17.4% (135)	4.1% (32)
ASCUS (n=540)	24.4% (132)	54.4% (294)	17.0% (92)	4.0% (22)

*includes viral, atrophic cervicitis, unsatisfactory colposcopy

**includes one cases with ?microinvasion, three cases with glandular abnormalities

3.4.5 Histological diagnosis at first visit to colposcopy (T0)

The general practice for women referred to colposcopy with LSIL or ASCUS is that they are assessed by colposcopy and a biopsy taken if an atypical area is seen. Therefore, a cohort of 162 women who had a normal colpscopy, did not have a biopsy taken, cervical cytology was performed in 41 of these of which 10 women had a LSIL smear and 1 had a HSIL smear, the remaining 30 were negative.

Biopsies were taken from 1186 women at first visit, the majority, 91.5%, (1085/1186) had a colposcopic directed punch biopsy the remaining 8.5% (101/1186) received a LLETZ treatment. This is within the limits set out by CervicalCheck, which recommends that no more than 10% of minor abnormal referrals should receive a treatment on their first visit (Guidelines Quality Assurance in for Cervical Screening. 2009). Table 3.3 shows the association between histological diagnoses of biopsy compared with the initial referral cytology.

Table 3.3 Association between referral cytology and histology of biopsy at first visit

	Normal Colp No Biopsy † % (n)	Inadequate % (n)	Normal % (n)	CIN 1 % (n)	CIN 2 % (n)	CIN 3 % (n)
All (n=1339)*	9.1% (162)	3.4% (46)	36.4% (488)	23.2% (311)	19.0% (249)	6.2% (83)
LSIL (n=795)	10.9% (87)	3.3% (26)	33.5% (266)	25.3% (201)	20.3% (161)	6.8% (54)
ASCUS (n=544)	13.5% (75)	3.7% (20)	40.8% (222)	20.2% (110)	16.2% (88)	5.3% (29)

*2 cases of AIS, 6 cases of CIN uncertain grade and one case of VAIN 1 not included on table

† 41 had a smear taken, this showed 10 low grade abnormalities, 1 HSIL and the remaining were normal

The prevalence of CIN at first visit estimated from those patients who underwent colposcopy with resultant histological diagnosis was 48.0% (643/1339). Cervical biopsy taken at this first visit (n= 1177* cases) revealed 36.4% (488/1339) had a normal biopsy. The distribution of CIN was as follows; CIN 1 23.2% (311/1339), CIN 2 19.0% (249/1339) and CIN 3 6.2% (83/1339). An inadequate biopsy sample was taken in 3.4% (46/1339) of cases. The remaining 12.2% (163/1340) did not have a smear or biopsy and appeared normal by colposcopy (table 3.3). There was one case of VAIN1, two AIS (adenocarcinoma in situ) and 6 biopsies reported as uncertain grade.

The proportion of CIN 2+ detected following referral with LSIL was significantly higher than those referred with ASCUS. Figure 3.5 graphically illustrates the histological grade of biopsies taken at first visit in relation to their corresponding referral smear. The prevalence CIN 2+ was significantly higher in women presenting with LSIL compared to ASCUS referral (Pearson's Chi Square p=<0.05 respectively).

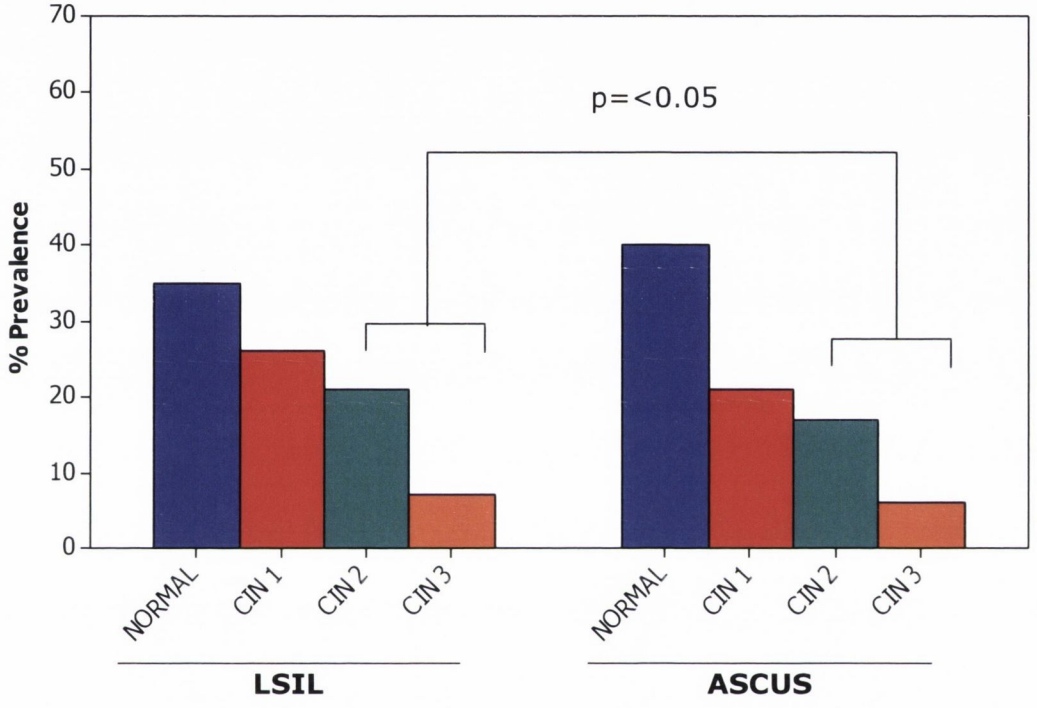

Figure 3-5 Association between referral cytology and histological findings at first visit to colposcopy

Referral based on LSIL can be seen on the left and referral based on ASCUS smear on the right. The % prevalence of histology findings are compared using Pearson's Chi Square. LSIL referral had a significantly higher prevalence CIN 2+ compared to ASCUS (p=<0.05).

3.4.6 Management at colposcopy–18 month follow up

Following enrolment into the study at first visit to colposcopy women were followed over a course of 18 months. Once entering colposcopy, women were assessed at their first visit, they were then followed up with six monthly smears at the colposcopy clinic. Cancellations and rescheduling of appointments modified follow up intervals with many patients being followed up at 6-9 months intervals. Two consecutive negative (normal) smears warranted discharge from follow up back to routine cervical screening with no treatment. If a high grade abnormality was identified the patient was returned in less than six months and a LLETZ treatment was performed. Figure 3.6 illustrates the visit at which i.) treatment was performed, ii.) a patient was discharged with two consecutive normal smears and no treatment and iii.) the cumulative rate at which patients exited the study. Each chart is representative of an 18 month follow up period. To date 951 women have completed their 18 month follow up from their initial presentation at colposcopy.

Exit 1: treatment by LLETZ

First Visit: 22.2% (79/356) of LLETZ treatments were performed at the initial colposcopy visit, 68.9% (51/74) of these were on the basis of an abnormal colposcopic impression (colposcopic impression available on 74 cases). The remaining treatments were women over the age of 40 or those with pervious persistent minor abnormal smears.

Second visit: 60.1% (214/356) of LLETZ treatments were performed at the second visit, between 2-6 months following initial assessment in colposcopy. 90.2% (193/214) were following a high grade cytology/histology result from the previous visit.

Third visit: 7.5% (27/356) of LLETZ treatments were performed at third visit, approximately 9 months following initial colposcopy, 85.2% (23/27) following a high grade cytology or histology result from previous visit.

Fourth visit: 10.1% (36/356) of LLETZ treatments were performed at fourth visit, up to 18 months after initial colposcopy visit. 38.9% (14/36) following high grade cytology or histology result the remaining 61.1% (22/36) were performed on the basis having persistent minor abnormalities (figure 3.5i).

Exit 2: Two consecutive normal smears (no treatment)

Women were discharged from the study as normal following two sequential normal smears and no treatment. The majority of women, 71.5% (244/341), were discharged on their third visit (12 months following initial presentation at colposcopy) following a colposcopy and biopsy at first visit (168 normal, 65 CIN 1) followed by two sequential normal smears. 10.3% (35/341) of women were discharged from colposcopy at their second visit and 18.2% (62/341) at their fourth visit (figure 3.5ii).

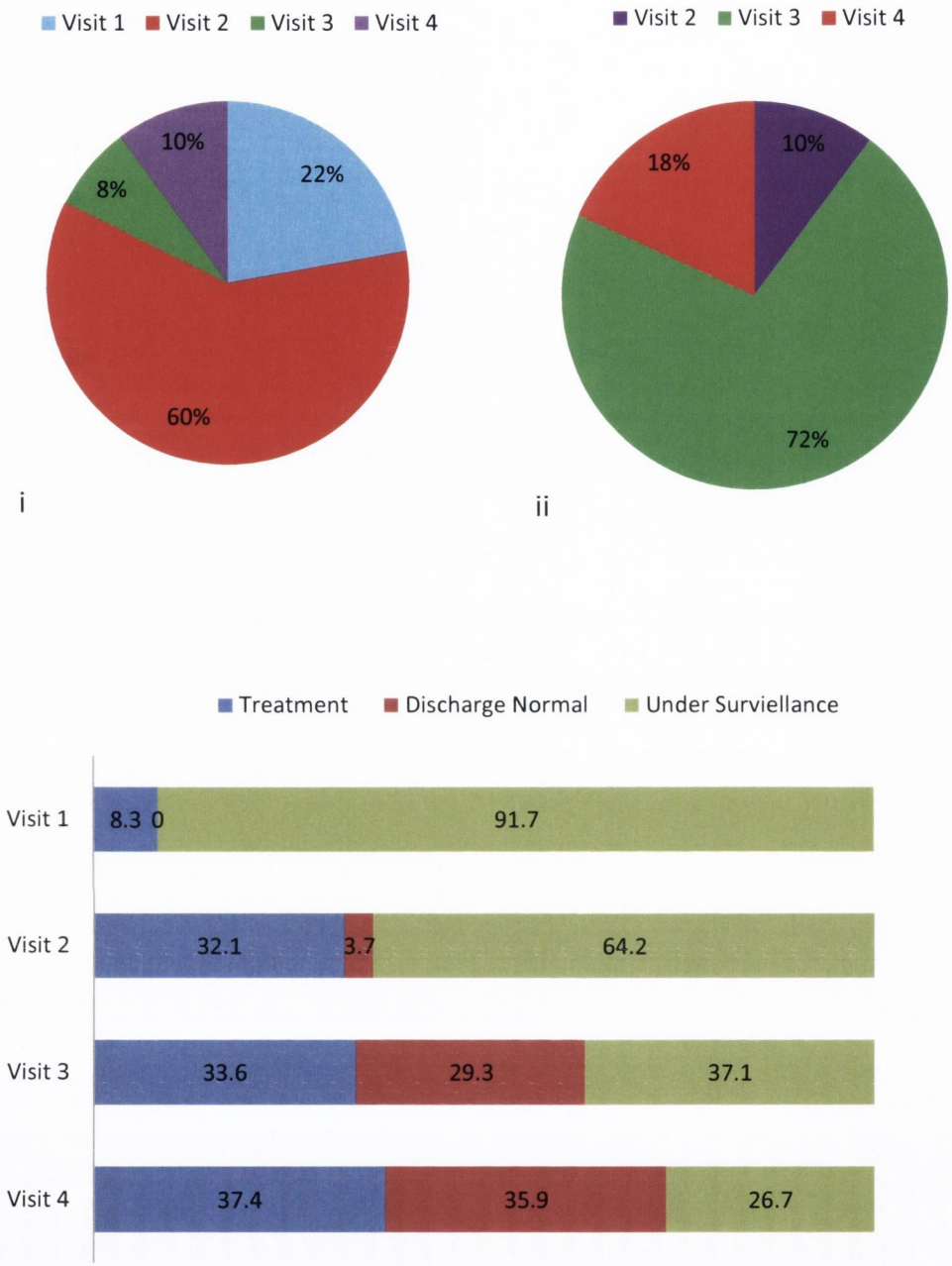

Figure 3-6 Treatment and discharge rate

i). Pie chart displaying the % distribution of treatment time over an 18 month follow up period. ii). Pie chart displaying the % of patients discharged at follow up over an 18 month period. iii). The % cumulative rate at which patients exit the study.

In total, 951 women have completed an 18 month follow up. Within an 18 month follow up period, a total of 37.4% (356/951) women received a LLETZ treatment and 35.8% (341/951) exited the study having had two sequential normal smears and no treatment. The stacked bar chart in figure 4.i shows that 26.7% (254/951) of women were still under surveillance at 18 months (visit 4), this represents women that have had persistent minor abnormalities since their visit to colposcopy and have not received any excisional treatment. It is important to note that not all of these 254 patients have attended all four visits. This is mainly due to postponing or missing follow up appointments. This accounts for 25% of women under surveillance that have only attend three visits, the last one within six months of the 18 month period. In addition, women still currently under surveillance were predominately younger women, 79.5% (202/254) of women still under surveillance were under the age of 40 years.

Figure 3.5.i illustrates the time at which treatment occurred, in most cases high grade pre-cancer was identified prior to performing treatment. Within an 18 month period, there were a total of 294 cases of CIN 2+, representing 30.9% (294/951) of the women. In instances where a range was given or multiple findings of high grade pre-cancer were reported, the highest grade was recorded. Of the 294 cases of high grade pre-cancer (CIN 2+) 65.3% (192/294) were classified as CIN 2 and 34.4% (101/294) were classified as CIN 3. The majority 81.6% (240/294) of high grade CIN was in fact identified at first visit. Most of these were identified by punch biopsy, 87.0% (209/240), following an abnormal colposcopic impression. The remaining 54 cases of CIN 2+ were identified at follow up visits, 17 at second visit, 17 at third visit and 20 at fourth visit.

3.4.7 Cumulative rate of CIN 2+ within an 18 month follow up

The overall cumulative rate over the 18 month follow up period, of histologically confirmed CIN 2+ was 30.9% (294/951), of which, 20.2% (192/951) were CIN 2, 10.6% (101/951) were CIN 3 and there was one case of microinvasion (Table 3.4). Within the 18 month period 69.1% (657/951) had no detectable high grade pre-cancer, of which, 40.0% (381/951) were discharged from colposcopy following two sequential normal smears and no treatment. A total of 8.0% (76/951) were treated by LLETZ and had a histological diagnosis of CIN 1 or Normal.

Table 3.4 Cumulative rate of CIN 2+ within an 18 month follow up

	<CIN 2 n=657	**CIN 2+ n=294**
All (n=951)	69.1% (657/951)	30.9% (294/951)
LSIL (n=596)	65.9% (393/596)	34.0% (137/596)
ASCUS (n=355)	74.3% (264/355)	25.6% (91/355)

The percentage of women with histologically confirmed CIN 2+ (either punch biopsy or LLETZ) at any time during follow up. The highest grade of CIN was taken as final result.

Excludes 61 cases who did not attend more than 2 follow up appointments after initial enrolment and those who most recent visit was more than 6 months prior to 18 month cut off point.

The cumulative rate of CIN 2+ was significantly higher in women referred with LSIL compared to ASCUS referral (Pearson's Chi-Square p=0.006). (figure 3.7).

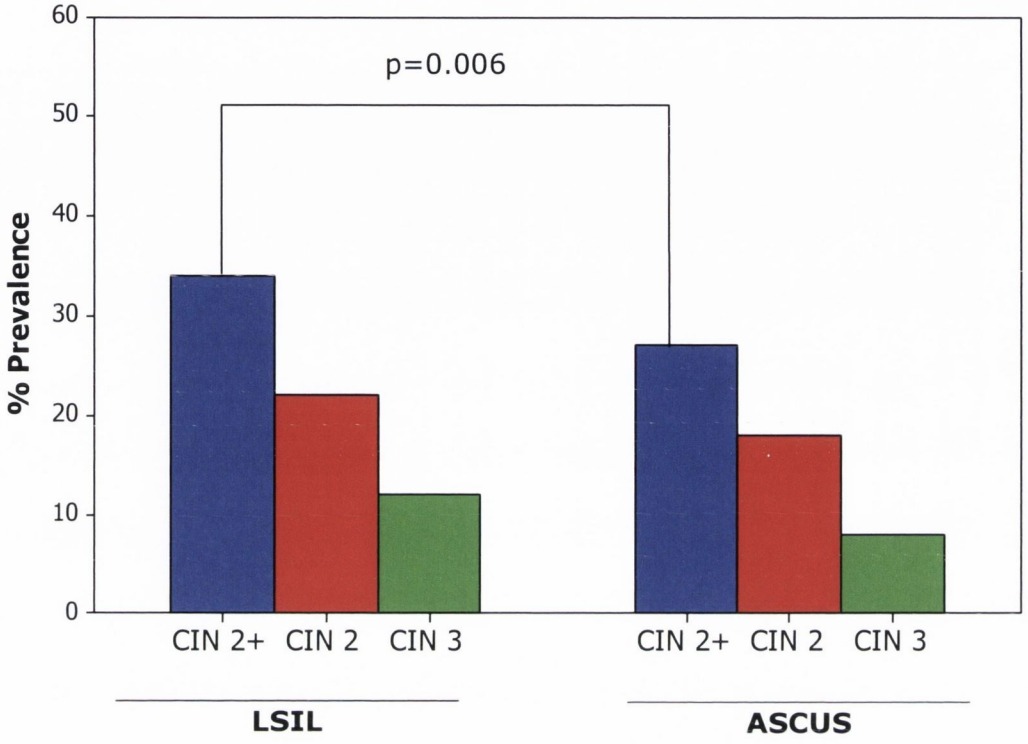

Figure 3-7 Association between referral smear LSIL or ASCUS and the cumulative incidence of CIN 2+, CIN 2 and CIN 3 over an 18 month follow up period

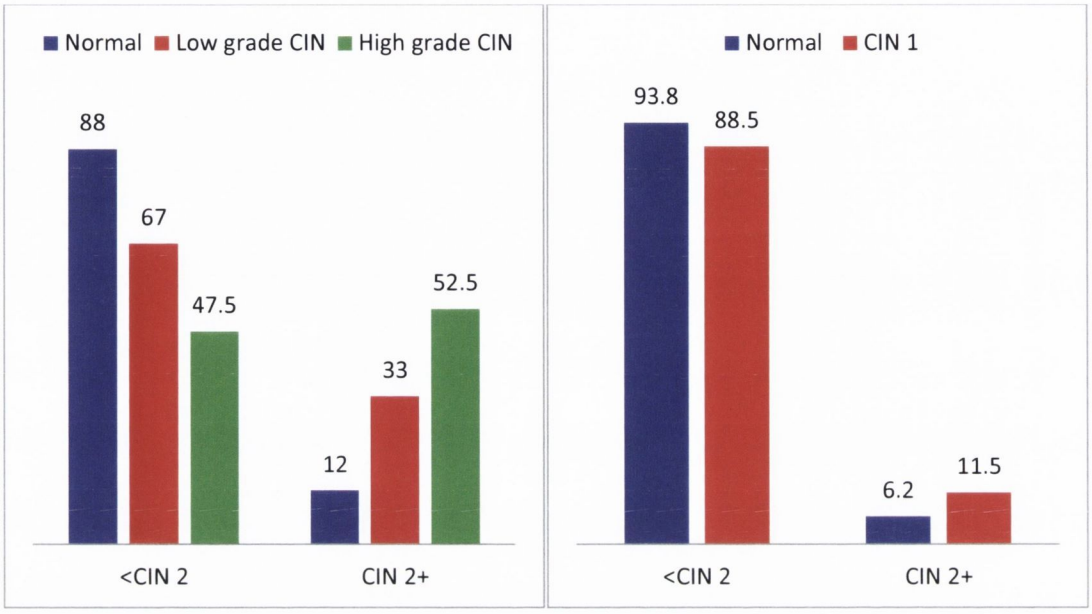

Figure 3-8 Risk of subsequent CIN 2+ diagnosed by histology according to first colposcopy and biopsy result

The risk of CIN 2+ according to colposcopic impression at first visit is shown in figure 3.7. CIN 2+ was identified in 18.0% (36/200) of patients with a reported normal colposcopy at first visit. The risk of CIN 2+ when colposcopy was abnormal at first visit was higher compared to a normal colposcopy at first visit. The detection rate of CIN 2+ increased with increasing severity of lesion identified on colposcopy at first visit. The prevalence of CIN 2+ was 33.0% (175/530) and 52.5% (83/158) in those women with colposcopy findings of low grade CIN and high grade CIN respectively. Detection of a high grade lesion following normal or CIN 1 histology at first visit was low 6.2% (23/373) and 11.5% (21/183) respectively.

3.4.8 Risk factors for developing CIN 2+

Cigarette smoking, hormonal contraception use, parity and age were examined for risk of developing CIN 2+. Table 3.8 shows the incidence of CIN 2+ over 18 months in relation to smoking, contraceptive use and parity. Heavy smokers (>10 per day) were found to be at an increased risk of CIN 2+ compared to non-smokers (OR 1.58 95% CI 1.00-2.51). Users of combined hormonal contraceptives (oral contraceptive pill, patch, vaginal ring) had a higher risk of CIN 2+ compared to non-users (OR 1.44 95% CI 1.08-1.93). There was no significant risk of women having one or more full term pregnancies and development of CIN 2+. Risk of high grade pre-cancer has been previously reported to be associated with high parity, there are insufficient numbers in this cohort to perform this analysis. Within this population only 13 women had 5+ full term pregnancies, 5/13 developed high grade pre-cancer. Risk of CIN 2+ decreased with age (39-40 v's 40+ OR 1.89 (95% CI 1.15-3.13) and 18-29 v's 40+ OR 2.45 (95% CI 1.4 -4.16).

Table 3.5 Risk of subsequent CIN 2+ according to smoking behaviour, contraception use and parity at time of visit first to colposcopy

	Cumulative CIN 2+	OR (95% CI)
Smoking behaviour		
Non Smoker	28.1% (155/551)	-
Light (<10/day)	32.9% (72/219)	1.12 (0.76-1.65)
Heavy (>10/day)	43.0% (52/121)	1.58 (1.00-2.51)
Recorded contraception use		
Combined oral contraception	33.8% (112/331)	1.33 (0.94-1.88)
Combined hormonal contraception	35.7% (123/345)	1.44 (1.08-1.93)
Hormonal contraception	35.2% (142/403)	1.43 (1.07-1.90)
Parity >1	11.6% (97/837)	1.44 (0.98-2.11)
Age		
40+	21.2 % (202/951)	-
30-39	35.2% (335/951)	1.89 (1.15-3.13)
18-29	43.5% (414/951)	2.45 (1.44-4.16)

3.5 Discussion

The primary objective of this chapter was to determine the prevalence of high grade CIN in a population of women referred to colposcopy with minor abnormalities on cytology. Based on these findings the objective was to further investigate the time to identification of CIN 2+ and the variation in prevalence in relation to referral smear LSIL or ASCUS. The rationale behind this work is to identify if LSIL and ASCUS provide a similar risk to CIN 2+ or in fact differ and could possibly be treated as two differed modalities in triage.

The data presented in this study established that a proportion of women with minor abnormalities will have a subsequent high grade lesion. In this chapter it has been identified that women presenting to colposcopy with LSIL are more likely to develop CIN 2+ (34%), than those referred with ASCUS (26%) ($p=0.006$). The findings from this study are in line with what has been previously reported for the prevalence of CIN 2+ in low grade and equivocal populations. A lower rate of CIN 2+ was reported in a meta-analysis by Melnikow et al. Which reported pooled progression rates over 24 months of 20.8% and 7.13% for LSIL and ASCUS referral smears, respectively (Melnikow et al 1998). An overall prevalence in the range of 2%-42% for CIN 2+ based on a population referred with LSIL has been reported (Bolger and Lewis, 1988, Melnikow et al., 1998, Giannopoulos et al., 2005, Ahmed et al., 2008, Cox et al., 2003, Rana et al., 2004, Cooper et al., 1992). For women with smears showing ASCUS the risk of CIN 2+ is between 2%-30% (Emerson et al., 2002, Rana et al., 2004, Shanbhag et al., 2003, Edwards et al., 2002, al-Nafussi et al., 2000).

The majority of CIN 2+ cases (81.6%) were identified at first visit suggesting that a significant amount of women referred with minor cytological lesions LSIL and ASCUS have in fact high grade CIN. This highlights the importance of colposcopy as a key factor in the management of minor cervical abnormalities. However, the management of minor abnormalities still offers considerable challenges. As seen, a high proportion, over 60% did not have high grade pre-cancer within 18 months of initial assessment in colposcopy, with 36% returning to normal without any treatment. Several studies have recommended immediate referral to colposcopy following first LSIL event. It is

evident that a number of LSIL cases have underlying CIN 2+. However immediate referral would undoubtedly impose an increased burden on already stretched colposcopy services. Ahmed et al found 67.5% of an LSIL population regressed to normal after one year follow up following immediate colposcopy, a high proportion remained normal following first normal follow up smear, 87% of which remained normal over 4 years follow up. A meta-analysis by Arbyn et al found risk of CIN 2+ following prior ASCUS was only 10% for CIN 2 and 6% CIN 3. As the evidence indicates there is a high chance of spontaneous regression of low grade lesions, thus a balance must be found to minimise the risk of cancer but also prevent over treatment of women without clinically significant disease. Achieving this will subsequently avoid unnecessary attendance at colposcopy and consequently avoid patient anxiety and possible over treatment in addition to benefiting health services by reducing pressure on resources. Randomised clinical trials have found that testing for high risk HPV DNA may offer a good approach for triage for women with ASCUS and help reduce referrals to colposcopy. This is discussed further in chapter 4. It is important to note that a number of women will benefit from surveillance. In this study, 18% of women with normal colposcopy at first visit had CIN 2+ detected over an 18 month period. Similarly 6% of women with a normal histological diagnoses and 12% of women with CIN 1 diagnoses at first visit went on to have a diagnosis of CIN 2+ over an 18 month period.

Considerations need to be taken when it comes to the management of minor abnormalities. Local excision of CIN is the preferred treatment method. Procedures such as LLETZ are successful at eradicating CIN, however many of these are associated with some level of morbidity. For instance there may be bleeding, discharge and infection, also, an increase frequency of low birth weight and premature delivery has been reported in a systematic review by Kyrgiou *et al.* In addition, it is well documented that there is an adverse psychological impact suffered by women receiving a minor abnormal smear result (Gray et al., 2006, Monsonego et al., 2012). Not only do minor cytological abnormalities produce anxiety for patients there are problems of high default rate associated with on-going follow up. Furthermore, expenditure on resources due to on-going cytological surveillance is an issue and of particular concern in a period of escalating limitations on health care expenditure and the allocation of resources.

The secondary objective of this chapter was to investigate various risk factors associated with the development of CIN 2+ in women presenting to colpsocopy with minor abnormalities LSIL and ASCUS. Two factors were considered, cigarette smoking and hormonal contraception use. It has been identified that heavy smoking and use of hormonal contraception's offer increased risk of developing high grade lesions.

The number of women reporting as smokers represented 38% of the population. This represents a high proportion compared to the overall Irish population where the prevalence of female smokers is only 22%, and 30% in individuals age 25-34 according to the office of tobacco control. An increased risk was seen in the incidence of CIN 2+ in smokers compared to non-smokers. Risk appeared significant only in those reporting a higher intensity of smoking (10 or more cigarettes per day). Self-reported smoking can be an inaccurate measurement of actual exposure to tobacco smoke. Due to under reporting and differences in smoking behaviour, biomarkers such as nicotine metabolites can be used to measure true exposure to tobacco smoke (Zielinska-Danch et al., 2007). In chapter 5 the association between smoking and high grade cervical disease will be examined using the nicotine metabolite cotinine to measure exposure to tobacco smoke. There was no association with oral contraception and high grade pre-cancer CIN 2+. The population of women currently using oral contraception was quite young 29 years. In addition it is long term use of oral contraception that has been associated with increased risk. We did not collect data on the duration or past contraception use. Use of all forms of hormonal contraception indicated an increased risk to high grade pre-cancer compared to non-use.

Cigarette smoking and oral contraception use have been recognised as risk factors in the development of cervical cancer however, most studies focus on risk associated with CIN 3 and invasive carcinoma (Plummer et al., 2003, Haverkos et al., 2003). It is not too surprising that light smoking and oral contraception use had no significant association with CIN 2+. CIN 2 lesions represented the majority of this group of women in this population with a high grade pre-cancer. While CIN 2 is classified as a high grade abnormality it is more heterogeneous in nature than CIN 3. In fact there is considerable controversy over a diagnosis of CIN 2 and whether it is truly a high grade lesion (Palma et al., 2009). Increased interobserver variation in diagnosing

CIN 2 has been reported. In particular, the differential diagnosis between immature squamous metaplasia and CIN1/2, or between low-grade (CIN1) and high-grade (CIN2/3) lesions, tends to be difficult (Robertson et al., 1989, Stoler and Schiffman, 2001). Lesions diagnosed as CIN 3 are more likely to represent a true precursor to cervical cancer. It is important to note that 52% of CIN 3 cases were current smokers compared to 36% of CIN 2+. Additionally 55% of CIN 3 cases were current users of oral contraception compared to 34% of CIN 2+.

The exact mechanism which cigarette smoking increases risk to cervical cancer is unknown. A number of methods have been postulated and are discussed in chapter 5. For example, nicotine derivatives, including cotinine have been found in cervical mucus (Simons et al., 1995, Szarewski et al., 2001) possibly inducing genomic damage by tobacco related toxins. It has also been demonstrated that cigarette smoking causes local immunosuppression of the cervix by reducing the number of Langerhan cells (Apseloff et al., 1994) this may contribute to prolonging HPV infection. Persistent HPV infection appears to be critical to development of cervical cancer. Hormonal contraception is thought to be associated with increased risk to cervical cancer (Moreno et al., 2002,Smith et al., 2003). However it unknown whether it is due to a direct effect or confounding factors such as number of sexual partners and age. The International collaboration of epidemiological studies of cervical cancer found that current use of oral contraceptives for 5 years or more is associated with an increased risk of invasive cervical cancer (Sasieni, 2007). The risk falls after use has ceased however it can take up to 10 years to returns to that of never users (Sasieni, 2007).

It was observed that at 18 months there remained a number of women still under surveillance. A large proportion of these was young nulliparous women and is likely that they were kept under observation rather than treated. The rate of default in this population was 4%, this is within the guidelines set out by CervialCheck Guidelines contained in the Quality Assurance in Cervical Screening document which states that the rate of defaulted appointments without any prior notice given should be maintained below 15% (CervicalCheck, 2009). There was no relationship between age or smoking habits in women who defaulted.

First this chapter seeks to address the current situation in the management of minor cytological abnormalities. The noted observations emphasize the need for improved efforts in triage of minimally abnormal cytology. This is of particular importance in Ireland due to the high prevalence of minor abnormalities, notably ASCUS compared to other countries. When exploring alternative options it is important to preserve minimising the risk of cancer but also to prevent overtreatment of women without clinically significant disease. Thus, maintaining safety and acceptance in screening and exceeding cost efficiently and effectiveness of current methods in identification of clinically significant disease. The knowledge that many minor abnormalities regress spontaneously has prompted the use of alternative tests for the detection of high grade pre-cancer. Evidence suggests that HPV triage may provide such an approach. Chapter 4 examines such approaches using HPV DNA based detection methods. The advent and adaptation of HPV molecular testing particularly in combination with cytology has led to the establishment of new algorithms in the management of minor cervical abnormalities. These strategies in addition to the use of novel biomarkers, such as HPV mRNA detection and over expression of the cellular protein $p16^{INK4A}$ will be discussed in chapters 5 and 6.

This chapter has also shown that smoking in women with LSIL or ASCUS is associated with an increased risk in the development of CIN 2+; confirming that smoking is an important co-factor in the risk of cervical cancer. The role of smoking based on urinary cotinine concentration, and the development of high grade pre-cancer and HPV status will be further investigated in chapter 5. It is event that smoking has an impact on the development of high grade pre-cancer emphasizing that smoking cessation campaigns targeting this population and young women could be very valuable.

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-Chapter 4-

Evaluation of the clinical performance of the cobas® 4800 HPV test in a colposcopy referred population

Overview

- Testing for high-risk HPV types has now been suggested as a tool to help identify women at risk of high grade CIN who will benefit most from colposcopy.
- HPV 16 and HPV 18 are the most important HPV genotypes identified in approximately 70% of all invasive cervical cancers. Therefore, additional risk stratification may be achieved by identifying these specific HPV types.
- The recently developed cobas® 4800 HPV test detects a pooled group of 12 high-risk HPV types in addition to individual genotypes HPV 16 and HPV 18.
- In this chapter the clinical performance of the cobas® 4800 HPV test is assessed in comparison with the gold standard HPV test, Hybrid Capture 2 for detection of carcinogenic HPV and CIN 2+.

4 Evaluation of the cobas® 4800 HPV test

4.1 Introduction

Based on the strong etiological link between HR HPV and cervical cancer, HPV has now become an important tool in developing strategies for cancer prevention. Papillomavirus cannot be cultured reliably *in vitro.* Initially Southern blot and Northern blot hybridizations were used for HPV detection. However, these methods were laborious and require a large volume of input material (Snijders et al., 2010). Today, detection relies on molecular technologies that detect HPV nucleic acids in cervical smears. The use of liquid based cytology has allowed for the application of molecular based tests on residual smear material. This not only broadens our testing capability but also allows for reflex testing, eliminating the need for repeat patient visits. There are currently a number of HPV DNA based detection technologies commercially available. These can be sub-divided into (1) signal-amplification and (2) target amplification. Hybrid Capture 2 is the best known signal amplification technique, it is based on an initial hybridisation step of nucleic acids with target specific probes which encompass the entire HPV genome. Hybrids are subsequently detected by an alkaline phosphatase conjugated antibody, and signal, resulting from a chemiluminescent reaction, is generated. Target amplification utilise PCR, assays have been developed to detect multiple HPV types in one assay using primers directed against highly conserved regions of the HPV genome, typically L1. The cobas® 4800 HPV test involves real-time PCR using a mixture of type specific fluorescently labelled probes allowing individual genotyping of HPV 16 and 18, a pool of 12 HR HPV types and β-globlin as a control.

Hybrid Capture 2 (hc2) was the first HPV DNA detection test to receive FDA approval in March 2003. There is a strong body of evidence demonstrating its good clinical sensitivity and high Negative Predictive Value (NPV) for detection of high grade abnormalities (Manos et al., 1999, Solomon et al., 2001). It is believed to improve patient management by providing a more accurate risk assessment for cervical cancer and its precursors (Petry et al., 2003, Arbyn et al., 2006, Cuzick et al., 2006). Based on the success of hc2, it was recognized that new HPV DNA detection tests would populate the market and it was recommended that new tests report performance relative to hc2 (Meijer et al.,

2009). In order to validate candidate HPV tests for primary screening, the inferiority test was developed by Meijer *et al* (Meijer et al., 2010). Relatively new to the market, the cobas® 4800 HPV test has been analytically and clinically validated (Heideman et al., 2011, Lapierre et al., 2012, Stoler et al., 2011) and in April 2011 received FDA approval for use in cervical screening.

Testing for carcinogenic HPV DNA has been proposed for triage of minor cytological abnormalities. Numerous clinical studies have been performed worldwide which have found testing for HPV DNA has improved accuracy for detection of CIN 2+ compared to repeat cytology (Manos et al., 1999, Cuzick et al., 2006, Arbyn et al., 2006). In the case of ASCUS, there is general agreement that HPV triage has improved accuracy for detection of CIN 2+, a meta-analysis by Arbyn *et al* reported a pooled estimated sensitivity and specificity of 94.8% (95% CI: 92.7%-96.9%) and 67.3% (95% CI: 58.2%–76.4%) respectively (Arbyn et al., 2004). However, in the case of LSIL, conflicting findings exist on the use of HPV triage. The ALTS (ASCUS-LSIL Triage Study) study was initiated by The National Cancer Institute of America in 1997. Findings indicated that HPV DNA has improved accuracy in the detection of CIN 2+ in women with ASCUS and women over the age of 30 years (ASCUS-LSIL Triage Study group 2003). However, due to the high HPV positivity rate in LSIL the test is limited (ASCUS-LSIL Triage Study group 2003). Findings from the English screening program differ slightly. In 2001, pilot studies began in England to evaluate HPV DNA testing in the triage of borderline smears and mild dyskaryosis (translate to ASCUS and LSIL), this proceeded to the Sentinel Sites program in 2007 covering 10% of the screening population (Kelly et al., 2011). Findings from the Sentinel Site studies indicated that HPV DNA triage of LSIL and ASCUS was well accepted, cost effective and resulted in a more rapid return to routine recall compared to repeat cytology. These findings led to the UK National Health Service Cervical Screening Programme (NHSCSP) in England introducing HPV triage after a primary LSIL/ASCUS cytology test in 2011. Women who test negative for HR HPV are returned to routine screening, while women who test positive are referred to colposcopy.

While HPV DNA testing has been demonstrated to be accurate for detection of high grade abnormalites (CIN 2+) there remains limitations. A proportion of HPV DNA positive women will not develop CIN 2+. Despite the fact a number

of HPV types have been characterised as high risk types in the development of cancer, not all high risk types have the same carcinogenic potential (Schiffman et al., 2005, Kjaer et al., 2010). Kjaer *et al* found absolute risk of CIN 3 or worse after an infection with HR HPV types other than 16, 18, 31 and 33 to be 6% (Kjaer et al., 2010). It is HPV 16 and HPV 18 that account for approximately 70% of all invasive cervical cancer cases (Smith et al., 2007b). HPV 16 appears to be exceptional in its ability to persist and cause neoplastic progression. Schiffman *et al* described it *"as a remarkably powerful carcinogen that merits separate clinical consideration"* (Schiffman et al., 2005). HPV 18 is more often associated with high grade lesions in addition to its association with difficult to detect lesions of the endocervical canal (de Sanjose et al., 2010). Taking this into consideration genotyping for HPV 16/18 might provide useful risk stratification for high grade abnormalities (Stoler et al., 2011). Many commercially available next-generation HPV tests include a genotyping capability. The cobas® 4800 HPV test, Cervista 16/18 HPV test and Abbott Real Time HPV tests, all detect a pool of 12 HPV types with separate detection of HPV 16 and HPV 18. However, while these types have a high carcinogenic potential not all HPV 16 and 18 infections will go on to develop cervical cancer. One study reported among HPV 16 and HPV 18 positive women the 10 year cumulative incidence of CIN 3+ was 17.2% and 13.6% respectively (Khan et al., 2005).

In this chapter the clinical performance of the recently FDA approved cobas® 4800 HPV test and hc2 are compared in a colposcopy referred population. The hc2 test is a nucleic acid hybridization method, which detects 13 HR HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) by a cocktail of type specific of full length RNA probes, detecting the entire genome, without distinction of individual genotypes. The cobas® 4800 HPV test is an automated real time PCR based system that specifically genotypes HPV 16 and 18 while concurrently detecting 12 other HR HPVs (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), in addition to a separate β-globin control for each sample. The test targets the highly conserved L1 region of the HPV genome which undergoes PCR amplification. The assays have been validated and compared in previous studies in women with ASCUS cytology and women ≥30 years (Heideman et al., 2011, Stoler et al., 2011, Lapierre et al., 2012, Gargano et al., 2012). This chapter seeks to add further knowledge to the performance of both tests in detecting CIN 2+ taking in to consideration age

and cytological classification. In order to resolve discordant results a subset were genotyped using Linear Array.

4.2 Aims

- To compare HR HPV detection rates using the cobas® 4800 HPV test compared with hc2, for detection of HPV in a population of women referred to colposcopy with LSIL/ASCUS.

- To evaluate the clinical performance of the cobas® 4800 HPV test and hybrid capture 2 for the detection of CIN 2+ and CIN 3 in a population of women attending colposcopy with abnormal cytology.

- To determine the risk of CIN 2+ and CIN 3 in women testing positive for HPV 16 and HPV 18 with the cobas® 4800 HPV test.

4.3 Materials and Methods

4.3.1 Study population

Participants were enrolled from colposcopy clinics at the Coombe Women and Infants University Hospital, Dublin and the National Maternity Hospital, Dublin. A subset of 465 patient samples from a larger population of n=1348 (minor cytology referrals) and 96 patient samples from a population of n=640 (high grade referrals) were included. Selection was based women enrolled from July 2010 - July 2011. A cervical smear was obtained and collected in PreservCyt medium prior to colposcopic examination. Colposcopy-guided biopsy specimens were obtained if an area of abnormality was identified.

4.3.2 Hybrid Capture 2

HR HPV DNA testing was performed using the Hybrid Capture 2 (hc2) (Qiagen, UK) as described in section 2.2. hc2 is a semi-quantitive nucleic acid hybridisation assay with signal amplification that utilizes chemiluminescent detection for the quantitative detection of 13 HR HPV types. HR HPV DNA is detected by a full length RNA probe cocktail for the detection of oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The RLU positive cut off value is 1.0pg, representing 5000 copies of HPV genome. Specimens below this detection limit are considered negative. A value between 1-2.5 RLU was considered to be borderline results and retested where possible.

4.3.3 cobas® 4800 HPV test

The cobas® 4800 HPV test is a fully automated system involving sample preparation combined with real-time PCR technology from 400µl of cervical smear sample. The test was carried out as described in section 2.3. The cobas® 4800 HPV test individually detects HPV 16 and HPV 18 and 12 pooled HR HPV genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Complementary primer pairs were used to amplify the L1 region of the HPV genome and fluorescent oligonucleotide probes specific for HPV16, HPV18, and the 12 other HR HPV types. The assay also detects the human β-globin gene as an internal control and to provide a measure of sample adequacy.

4.3.4 HPV genotyping using the Linear Array Genotyping Test

Specific HPV genotyping was performed on cases that were positive for HR-HPV by hc2 or Amplicor tests. This genotyping was performed using Roche Linear Array HPV Genotyping test, as described in Section 2.4. Hybridisation of amplicons to probes which are bound to test strips was performed to detect the various genotypes. The strips were then read visually by comparing the pattern of blue lines to the linear array HPV genotyping test reference guide and interpreted as described in Section 2.4.4.

4.3.5 Statistical Analysis

Agreement between hc2 and cobas® 4800 HPV was calculated for HR HPV detection based on concordant positive or negative results by both assays. Specimens in which HR HPV was detected by both the hc2 and cobas® 4800 HPV, regardless of genotype, were considered concordantly positive. The Cohen's kappa coefficient was used to ascertain the agreement between hc2 and the cobas® 4800 HPV test. The McNemar's test was used to compare differences in rates of detection between HPV tests. The clinical performance of the HPV tests was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the relative 95% confidence intervals (CI) for detecting CIN 2+ and CIN 3. HPV results for individual genotypes HPV 16, HPV 18 and 12 HR HPV types were assessed for risk of high grade CIN using binary logistic regression. A significance level of <0.05 was considered statistically significant.

4.4 Results

4.4.1 Study Population

Cervical smears from a total of 561 women between the ages of 18-65years were included in the study. The median age of the population was 32 years (interquartile range 28-39). In total, 465 of the population were attending their first visit to colposcopy on the basis of a minor cytological abnormality referral and 96 were attending for treatment following a high grade abnormality. Of the 561, 2 had missing or invalid (borderline) hc2 results and 1 had an invalid cobas® 4800 HPV resulting in 558 patient samples available for analysis. All women were assessed by colposcopy, 88.0% (491/558) had a punch biopsy or LLETZ result available.

4.4.2 Concordance between cobas® 4800 HPV and hc2

A population of n=558 samples were tested using the cobas® 4800 HPV test and compared with hc2. The overall prevalence of HPV detected by the cobas® 4800 HPV test was lower, 62.7% (350/558), compared to 65.8% (367/558) for hc2 (McNemar 0.015). A comparison of hc2 testing and cobas® 4800 HPV testing on the 558 cases demonstrated an overall agreement of 92.3% (95% CI 91.7%-92.9%) (kappa 0.834). Both tests were positive in 60.4% (337/558) and both tests were negative in 32.1% (178/558) of cases. There were 43 discordant results, among them 69.8% (30/43) were cobas® 4800 HPV negative, hc2 positive. The remaining 30.2% (13/43) were hc2 negative, cobas positive (table 4.1).

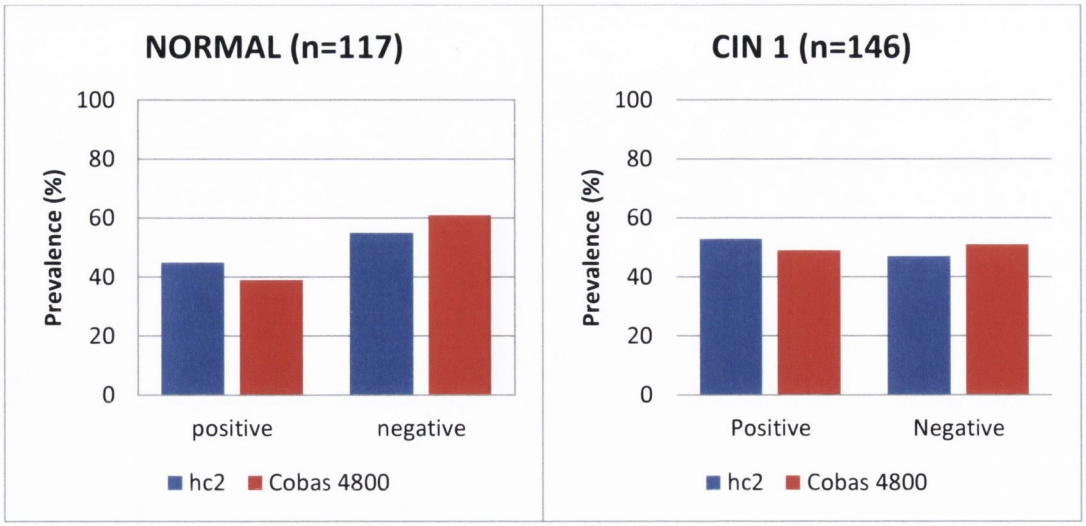
Table 4.1Concordance between hc2 and Cobas 4800

	cobas® 4800 HPV +	**cobas® 4800 HPV –**
Hybrid Capture 2 +	337	30
Hybrid Capture 2 –	13	178

Cohen's Kappa Coefficient: 0.834 (95% CI 0.784-0.881)

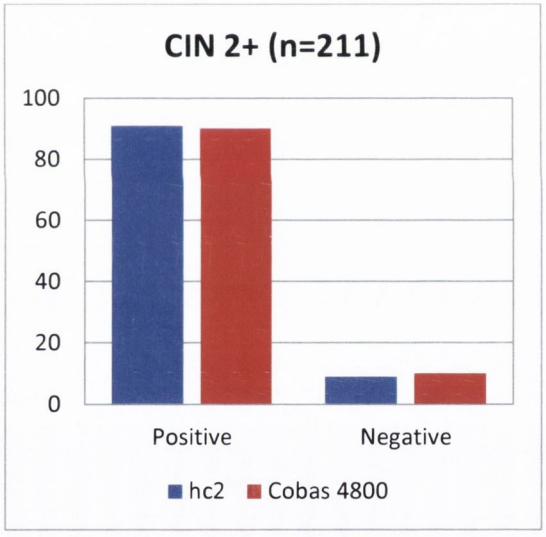
4.4.3 HPV detection rates across various grades of CIN.

Among 558 women referred to colposcopy histological confirmed diagnosis was available for 491. Biopsy confirmed CIN 1, CIN 2 and CIN 3 were identified in 29.7% (146/491), 22.8% (112/491) and 20.2% (99/491) respectively, 23.8% (117/491) were normal on histology. There was one case of adenocarcinoma in situ, 3 uncertain grade CIN and 13 inadequate biopsy samples. The positivity rate and test agreement for both tests increased with increasing grades of CIN (figure 4.1). Women with a histological diagnosis of normal had a HPV positivity rate of 45.3% and 39.3% for hc2 and cobas® 4800 HPV, respectively. HPV DNA was detected in 52.7% and 48.6% for CIN 1 and 90.5% and 90.0% in CIN 2+ by hc2 and the cobas® 4800 HPV test respectively.



Agreement: 87.2% (95% CI 85.2%-89.2%) Agreement: 92.5% (95% CI 91.35-93.6%)

Kappa: 0.594 (95% CI 0.454-0.733) Kappa: 0.850 (95% CI 0.764-0.935)



Agreement: 93.8% (95% CI 93.1%-94.6%)

Kappa: 0.649 (95% CI 0.464-0.837)*

Figure 4-1 HPV detection rates in relation to CIN grade

HR HPV detection by hc2 and cobas 4800 across the different histological confirmed grades of CIN in women referred to colposcopy with an abnormal smear.

*The low kappa value demonstrated for CIN 2+ is most likely due to the small number of negative cases.

4.4.4 Assessment of discordant HPV test results

Of the 367 cervical samples reported as HR HPV positive by the hc2 test, 30 were reported as HR HPV negative by the cobas® 4800 HPV. In 7 of the 30 hc2 positive cases, a positive result was generated from repeat borderline hc2 results (where RLU/CO was 1.0-2.5), and of the 350 cervical samples reported as HR HPV positive by the cobas® 4800 HPV test, 13 were reported as HR HPV negative by hc2. The Linear Array (LA) HPV Genotyping Test from Roche was employed to help elucidate the HPV status of a subset of 23 discordant HPV test results as part of an MSc project in Medical Diagnostics by Patrick Kerr.

In the case of HPV test results hc2 positive/cobas® 4800 HPV negative, 70.6% (12/17) were found to contain low-risk HPV types only. The most common genotype in this subset was HPV 53, which was detected in 41% (7/17) of cases, 23% (4/17) were negative for HPV of which 3 cases were histologically normal on biopsy (Table 4.2). There were 6 cases which were hc2 negative/cobas® 4800 HPV positive, 83% (5/6) of this sub-group were found to contain multiple HPV infections, at least one of which was a high-risk type (Table 4.3).

Table 4.2: Genotyping by LA of hc2 positive/the cobas® 4800 HPV negative cases

AGE	Referral	Histology	hc2	cobas 4800 HPV	LA HPV Genotypes
53	LSIL	Normal	**Positive**	Negative	53
27	LSIL	CIN 2	**Positive**	Negative	82
36	LSIL	CIN 1	**Positive**	Negative	70, 81
30	ASCUS	CIN 2	**Positive**	Negative	53,61
28	LSIL	CIN 1	**Positive**	Negative	84
28	LSIL	Normal	**Positive**	Negative	42, 53
52	LSIL	CIN 2	**Positive**	Negative	70
24	LSIL	CIN 2	**Positive**	Negative	53,73
52	LSIL	Not Available	**Positive**	Negative	53
34	LSIL	Normal	**Positive**	Negative	53
28	LSIL	CIN 1	**Positive**	Negative	53
34	ASCUS	Normal	**Positive**	Negative	Negative
26	LSIL	Normal	**Positive**	Negative	Negative
54	ASCUS	Normal	**Positive**	Negative	Negative
21	LSIL	CIN 1	**Positive**	Negative	42, 83
29	LSIL	CIN 1	**Positive**	Negative	Negative
58	LSIL	CIN 1	**Positive**	Negative	66

Table 4.3: HPV Genotyping by LA of hc2 negative/cobas® 4800 HPV positive cases

AGE	Referral	Histology	hc2	cobas 4800 HPV	LA HPV Genotypes
27	ASCUS	Normal	Negative	**HR HPV**	59, 83, 61, 54, cp6108
35	LSIL	CIN 2	Negative	**HPV 16**	16, 61
36	ASCUS	CIN 2	Negative	**HR HPV**	Negative
28	LSIL	CIN 3	Negative	**HR HPV**	42, 45
57	ASCUS	CIN 1	Negative	**HR HPV**	71, 58, 40, 52*
31	ASCUS	Normal	Negative	**HR HPV**	18, 31, 42, 54

4.4.5 Clinical test performance of the cobas® 4800 HPV test

To assess the performance of the cobas® 4800 HPV test for the detection of CIN 2+ the sensitivity, specificity, NPV and PPV were calculated and compared with the same figures calculated for hc2 (table 4.4 and 4.5). Histological diagnosis of CIN 2+ was used as gold standard. Table 4.3a shows the sensitivity of cobas® 4800 HPV for detection of CIN 2+ was comparable to hc2 (90.0% vs 90.5%). The specificity (55.5%) and PPV (61.9%) of the cobas® 4800 HPV to detect CIN 2+ was marginally higher than hc2 but this did not reach significance. Both tests demonstrated comparable NPVs. Sensitivity increased for detection of CIN 3 to 98.0% for hc2 and reaching 100% for cobas® 4800 HPV. There was a 10% reduction in specificity to 40.0% and 44.5% for hc2 and the cobas® 4800 test respectively, when CIN 3 was used as the endpoint. When the analysis was restricted to women over the age of 30 years, shown in table 4.3b, the sensitivity for detection of CIN 2+ decreased slightly but not significantly for detection of CIN 2+ and remained the same for CIN 3. Specificity increased significantly by approximately 10% for detection of CIN 2+ and over 6% for detection of CIN 3 in women over the age of 30 years.

Table 4.4 Clinical performance of hc2 and the cobas® 4800 HPV test for detection of CIN 2+ and CIN 3

	CIN 2+ (n=211)		CIN 3 (n=99)	
	cobas 4800	**hc2**	**cobas 4800**	**hc 2**
Sensitivity	90.0% (88.8-91.3)	90.5% (89.4-91.7)	100% (-)	98.0% (97.6-98.4)
Specificity	55.5% (52.5-58.5)	50.2% (47.2-53.2)	44.5% (42.0-47.0)	40.0% (37.6-42.4)
PPV	61.9% (59.3-64.5)	59.3% (56.7-62.0)	31.6% (29.2-34.0)	30.1% (27.8-32.4)
NPV	87.4% (85.8-89.1)	86.8% (85.0-88.7)	100% (-)	98.7% (98.5-98.9)

Table 4.5 Clinical performance of hc2 and the cobas® 4800 HPV test for detection of CIN 2+ and CIN 3 in women over 30 years

≥30 years	CIN 2+ (n=129)		CIN 3 (n=56)	
	cobas 4800	**hc2**	**cobas 4800**	**hc2**
Sensitivity	89.1% (87.5-90.8)	89.1% (87.5-90.8)	100 (-)	98.2% (97.8-98.7)
Specificity	64.8% (61.4-68.1)	60.2% (56.7-63.8)	51.4% (48.3-54.5)	47.8% (44.7-50.9)
PPV	65.0% (61.6-68.3)	62.2% (58.8-65.6)	31.6% (28.5-34.8)	29.7% (26.7-32.7)
NPV	89.1% (87.4-90.8)	88.3% (86.5-90.2)	100 (-)	99.2% (99.0-99.3)

4.4.6 Clinical performance of the cobas® 4800 HPV test for use in triage of LSIL and ASCUS

The clinical performance of the cobas® HPV test for use in triage of women referred with either LSIL or ASCUS is shown in table 4.6. This relates specifically to a subpopulation of n=465. The positivity rate for LSIL was 64.5% (158/245) and 71.8% (176/245) for cobas® 4800 HPV and hc2 HPV respectively (McNemars p=0.803). In contrast, the positivity for ASCUS was 47.7% (105/220) both for cobas® 4800 HPV and hc2 (this does not represent 100% agreement, it is merely coincidence that the number of positive cases for each test is n=105. Within the 105 positive cases for each test there were 16 discordant results). The sensitivity, specificity, PPV and NPV for detection of CIN 2+ in LSIL and ASCUS is shown in table 4.6. The cobas® 4800 HPV test had comparable sensitivity to hc2 for detection of CIN 2+ in both LSIL (86.8% v's 89.7%) and ASCUS (81.0% for both tests) referral groups. Specificity of the cobas® 4800 HPV was comparable to hc2 in ASCUS referral. However in women referred with LSIL cytology, the cobas® 4800 HPV test demonstrated a higher specificity at 55.2% compared to 35.1% for hc2.

Table 4.6 Clinical performance of the cobas® 4800 HPV test for detecting CIN 2+ in LSIL and ASCUS referral

	LSIL		ASCUS	
	hc2	**Cobas 4800**	**hc2**	**Cobas 4800**
Sensitivity	89.7% (87.5-91.9)	86.8% (84.0-89.5)	81.0% (77.1-85.0)	81.0% (77.1-85.0)
Specificity	35.1% (31.5-41.6)	55.2% (51.3-59.1)	62.7% (58.9-66.4)	63.3% (59.6-67.1)
PPV	37.9% (34.3-41.6)	46.1% (41.8-50.4)	45.6% (40.8-50.4)	46.1% (41.3-50.9)
NPV	88.5% (86.0-91.1)	90.4% (88.7-92.2)	89.5% (87.7-91.3)	89.6% (87.9-91.4)

4.4.7 Distribution of HPV genotypes detected by cobas 4800 HPV

The prevalence of HPV genotypes 16 and 18, in addition to the pooled group of 12 HR HPV types detected by the cobas® 4800 HPV test, stratified by CIN grades is shown in figure 4.2. Overall the prevalence rates of 12 pooled HR HPV, HPV 16 and HPV 18 detected by the cobas® 4800 HPV was 34.2% (191/558), 12.5% (70/558) and 3.2% (18/558). There were 70 cases that demonstrated a positive result for two or more of the HPV types detected by the cobas® 4800 HPV test, HPV 16, HPV 18 and HR HPV. The prevalence of HPV 16 and 18 was 9% in women who were normal on colposcopy, 6% with CIN 1, 15% with CIN 2 and 40% with CIN 3. The remaining 12 HR HPV types were detected in a higher proportion of cases, specifically normal, 21% and CIN 1 35%. HR HPV was detected in 46% of CIN 2 and 39% of CIN 3.

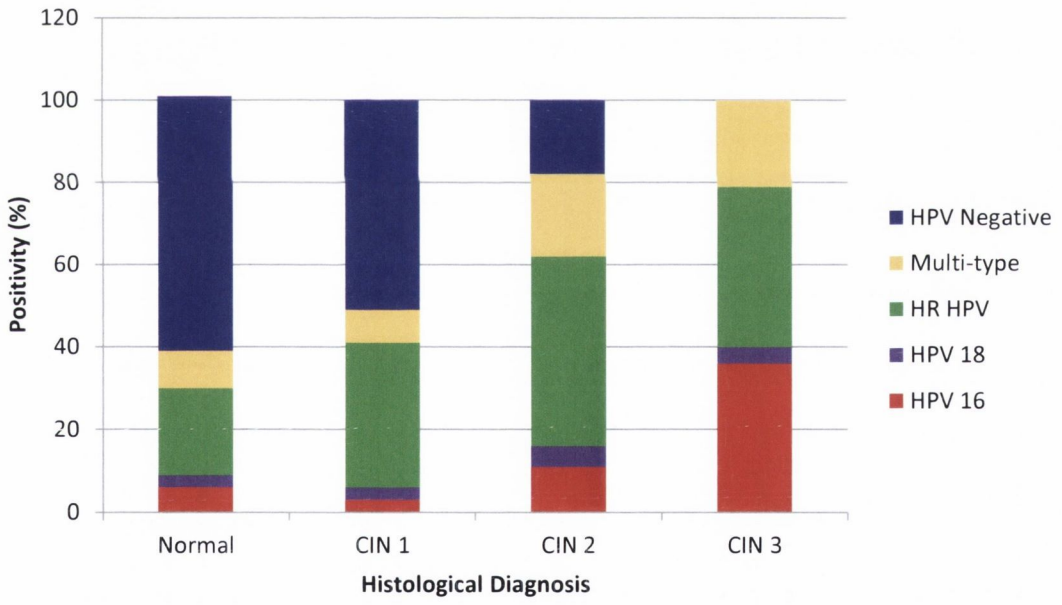


Figure 4-2 Distribution of HPV according to histological grade in n=491 women with biopsy result available.

Binary logistic regression was used determine crude odds ratios to determine the risk of CIN 2+ based on infection with HPV 16, HPV 18 or HR HPV. HPV 16 positive results were considered as HPV 16 positive alone and HPV 16 positive in the presence of HPV 18 and/or HR HPV (n=111). HPV 18 positive results included those positive for HPV 18 alone and positive for HPV 18 in the presence of HR HPV (n=29). HR HPV was classified as those positive for HR HPV alone (n=165). Analysis demonstrated that HPV 16 generated the highest risk to CIN 2+, OR 5.90 (95% CI 3.63 - 9.57) followed by HR HPV, OR 1.86 (95% CI 1.27 - 2.73). Those positive for HPV 18 were at an increased risk to CIN 2+ by comparison to HPV negative but not significantly OR 1.36 (95% CI 0.64 - 2.89).

4.5 Discussion

The aim of this chapter was to compare the performance of the cobas® 4800 HPV and hc2 tests for detection of HR HPV in women attending colposcopy with cervical abnormalities. Overall, the cobas® 4800 had a lower positivity rate of 62.7% compared to hc2 at 65.7%. However, agreement between the two tests remained high at 92.3% producing a kappa value of 0.832 (95% CI 0.784-0.881). The strength of agreement appeared to increase with severity of the lesion. A significantly higher level of agreement was identified in CIN 2+ cases of 93.8% (95% CI 93.1%-94.6%) compared to 87.2% (95% CI 85.2%-89.2%) in those that were normal on histology. Similar findings have been found in previous studies reporting a high concordance >87% which tended to increase with lesion severity (Wong et al., 2012, Stoler et al. (2011), Lapierre et al., 2012).

Recommended guidelines by Stoler *et al* state that a HPV test should include at least 13 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 but has also endorsed HPV 66 as desirable (Stoler et al., 2007). The cobas® 4800 HPV detects HPV 66 in addition to the 13 HR HPV types detected by hc2. However, HPV 66 alone does not explain the discordant samples observed here, in fact it is thought that hc2 does detect HPV 66 due to cross-hybridisation in addition to other LR HPV types (Lapierre et al., 2012). Genotyping by linear array was performed on 17 samples within this population that were found to be hc2 positive/cobas® 4800 HPV negative. LR HPV was identified in 12 of these samples, in particular HPV 53 representing 58% (7/12). It has been reported that the hc2 test detects up to 15 different HPV genotypes, not included in the high-risk probe cocktail, with low-risk HPV 53 observed to be the most common (Poljak et al., 2002, Lapierre et al., 2012). Interestingly, there was one hc2 positive case that LA detected as HPV 66. The remaining 23% (4/17) were found to be HPV negative using the Linear Array. This may be a result of differences in the amplification targets between hc2, which targets the full length probe, and the cobas® 4800 HPV and LA, which target the L1 region of the genome. Loss or partial loss of L1 has been reported following viral DNA integration into the host genome (Corden et al., 1999, Wagatsuma et al., 1990, Cone et al., 1992). Of the four cases three were normal and one was CIN 1 on histology suggesting viral integrations unlikely. 5/6 samples, which produced negative hc2 results but

positive cobas® 4800 HPV results, were found to contain multiple infections, at least one of which was HR HPV. The negative result produced by hc2 may be a result of analytical sensitivity of the hc2 which is slightly less than PCR-based target amplified techniques. The limit of detection of hc2 is reported to be between 0.2-1pg HPV-DNA/ml (approximately 5000 copies)(hybrid capture 2 product insert) compared to a limit of detection for the cobas® 4800 HPV test reported as 600 copies/ml for HPV 16 and 18 (cobas® 4800 HPV test product insert). For the Linear Array HPV Genotyping assay the limit of detection varies according to the genotype studied, ranging from 76 copies/ml for HPV 59 to 200 copies/ml for HPV 16 to 20,000 copies/ml for HPV 82 (Linear Array HPV Genotyping Test product insert). (Observed LOD concentration with > or equal to 95% positive hit rate for all assays).

Evaluation of the clinical performance of the cobas® 4800 HPV and hc2 HPV was achieved by determining their proficiency in detecting CIN 2+ and CIN 3. This was accomplished by calculating sensitivity, specificity, PPV and NPV for both tests. The sensitivity and specificity rates generated by the cobas® 4800 HPV for detection of CIN 2+ were 90.0% (95% CI 88.8-91.3) and 55.5% (95% CI 52.5-58.5). This was comparable with hc2 which demonstrated sensitivity and specificity of 90.5% (95% CI 89.4-91.7) and 50.2% (95% CI 47.2-53.2). When disease endpoint was confined to detection of CIN 3, sensitivity for both cobas® 4800 HPV and hc2 increased to 100% and 98.0% (95% CI 97.6-98.4) respectively. However, this resulted in a significant loss of specificity by approximately 10%.

Most previous studies evaluated the cobas® 4800 HPV in triage of ASCUS. Focusing on the ASCUS population in our study, the cobas® 4800 HPV had a sensitivity and specificity of 81.0% (77.1-85.0) and 63.3% (59.6-67.1). Lapierre *et al* showed equivalent results, reporting sensitivity of 89.7% (95% CI 72.8-97.2) in a population of women over 24 with at least one ASCUS (Lapierre et al., 2012). Stoler *et al* evaluated the clinical performance of the cobas® 4800 HPV in a population of women over 21 with ASCUS, reporting sensitivity for detection of CIN 2+ (90.0% 95% CI 81.5-94.8) and a higher specificity (70.5%: 95% CI 68.1-72.7) and lower PPV (14.0%: 95% CI 12.8-15.3) to what is reported for this study in our ASCUS population. This is likely owing to the higher prevalence of high grade disease in our population (26.0% v's 5.1%) (Stoler et al., 2011).

It is recognised that no screening test will achieve 100% clinical performance (Stoler et al., 2007). Any improvement in clinical sensitivity almost always results in a reduction in clinical specificity and vice versa. It is important to recognise that a balance between clinical sensitivity and specificity for detection of CIN 2+ is important in order to identify women at risk of high grade pre-cancer while minimising unnecessary follow up procedures in those who are not. The use of HPV detection is not without its limitations and due to the repeatedly reported low specificity, it has been found triaging with HPV testing can result in significant increased referrals to colposcopy. Thus appropriately management strategies are needed for HPV positive cases. In England, the NHS have incorporated HPV DNA triage for LSIL and ASCUS using hc2 however a higher RLU cut off value of 2.0 was adapted in their Sentinel Sites Studies (Kelly et al., 2011) rather than that recommended by the manufacture of a RLU/CO ratio of 1.0. This is based on findings from the ARTISTIC trial which found that increasing the threshold RLU/CO ratio to ≥2 generated a positive balance between sensitivity and detection of CIN 3 lesions, reducing unnecessary colposcopies without reducing the frequency of true precursor lesions detected as CIN 3 (Sargent et al., 2010). Other recommendations have suggested that the identification of the most carcinogenic HPV genotypes eg. HPV16 and HPV18 could be useful. This could aid in identifying those at true risk, Stoler *et al* suggested that HPV 16 only should be managed and followed up more closely while other HR HPV types followed up in one year (Stoler et al., 2011). Recently more specific tests have been developed such as detection of E6/E7 HPV mRNA and the cellular protein $p16^{INK4A}$ which will be discussed in chapters 5 and 6.

When the results were restricted to those referred with LSIL or ASCUS it was found that both tests had a similar sensitivity in both ASCUS and LSIL referral. The PPV for both tests is quite low, this is most likely due to the low prevalence of disease in this population. For LSIL, a higher sensitivity was demonstrated and a significantly lower specificity than was shown for ASCUS, which is consistent with what has previously been reported using hc2 (Arbyn et al., 2006). However, the cobas® 4800 HPV was more specific than hc2 for detection of CIN 2+ in women presenting to colposcopy with LSIL cytology 55.2% (95% CI 51.3-59.1) and displayed a specificity comparable with that shown by hc2 in ASCUS cytology 62.7% (95% CI 58.9-66.4). Overlapping confidence intervals were also identified with what was reported in a meta-

analysis by Arbyn et al for specificity of hc2 in detecting CIN 2+ with ASCUS cytology (62.5%, 95% CI 57.8-67.3) (Arbyn et al., 2009).

These findings suggest that the cobas® 4800 HPV may be a more specific test for the detection of CIN 2+ in women with LSIL than hc2. This is most likely due to the fact that hc2 is more likely to cross-react with LR-HPV types, risking identifying a small number of clinically irrelevant infections. The higher specificity of the cobas® 4800 HPV is an important finding as there remains controversy on the appropriate management of LSIL cytology. Whereas the use of HPV triage of women with ASCUS is now an accepted management option. ASCUS/HPV positive smears warrant immediate colposcopy (Wright et al., 2007, Jordan et al., 2008). Furthermore, the ability of the cobas® 4800 HPV to individually detect HPV 16 and HPV 18 could offer an improved triage method. Genotyping for HPV 16 and 18 has been previously demonstrated to have potential to identify women at higher risk of developing high grade disease (Stoler et al., 2011). In particular HPV 16 has been found to confer the highest risk of CIN 2+. It has been suggested that women positive for HPV 16 be followed up more closely (Stoler et al., 2011). This may be of benefit in triage of women with LSIL, given that one of the primary concerns with using HPV as a triage test in this population is the high prevalence of transient HPV infections in this group. Reported HPV positivity rates have been in the region of 76% (95% CI 71%–81%) compared to 43% (95% CI 40%–46%) in ASCUS (Arbyn et al., 2009) Within our study population, the risk of CIN 2+ was highest amongst those women positive for HPV 16 (OR 5.90) followed by HR HPV (OR 1.86). Infection with HPV 18 did not appear to significantly increase risk of CIN 2+, OR 1.36 (95% CI 0.64 – 2.89), however the wide confidence intervals demonstrated are reflective of the low sample number (n=29) compared with other HPV types. In addition, HPV 18 has been shown to be more often associated with adenocarcinoma lesions of the endocervical canal (de Sanjose et al., 2010). There were 70 cases where co-infections of the HPV genotypes detected by the cobas® 4800 HPV were identified. The significance of multiple HPV infections in unclear, previous studies have demonstrated conflicting evidence on the role of multiple infections and association with development of high grade CIN (Cuschieri et al., 2004a, Ferreux et al., 2003). The role of multiple infections requires further investigation and cannot be fully explored within this study as only HPV 16 and HPV 18 are individually identified.

In summary, the cobas® 4800 HPV demonstrated a high level of agreement with hc2 and comparable clinical performance. The cobas® 4800 HPV may offer some potential advantages to the hc2, as it identified more true HR HPV infections, as confirmed by Linear Array, in addition it permits individual identification of HPV 16 and 18, which together are responsible for up to 70% of cervical cancer (Smith et al., 2007b). HPV persistence is considered one of the most important predictors for high-risk of cervical disease and requires the exact HPV genotype to be identified. Therefore, tests which allow the specific HPV genotype to be classified appear to have great potential for improving future screening programs. It is also important to note that HPV 16 and HPV 18 are the two HR HPV types currently targeted by commercially available HPV vaccines. Furthermore, the cobas® 4800 HPV test requires a smaller specimen volume of 400µl compared to 4mls for hc2 and contains an internal control for each sample, which the hc2 lacks. While an internal control is an important feature to prevent false negatives it may be of marginal importance given the repeatedly confirmed high clinical sensitivity of hc2 (Solomon et al., 2001, Cuzick et al., 2003, Ferreccio et al., 2012).

This study formed part of a larger study aimed to investigate alternative triage strategies for women presenting to colposcopy with minor abnormal smears. Additional modalities including biomarkers such as E6/E7 mRNA and $p16^{INK4A}$ will be discussed in subsequent chapters 5 and 6.

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-Chapter 5-

HPV DNA and HPV E6/E7 mRNA as a triage tool in low grade cervical abnormalities LSIL and ASCUS

Overview

- HPV DNA testing is an effective tool, but it is transforming infection that is considered the true pre-cursor to neoplastic progression.
- Detection of HPV E6/E7 mRNA has potential to identify transforming HPV infections and may serve as a better triage test for minor cervical abnormalities.
- This chapter will examine the use of the PreTect HPV Proofer for the detection of E6/E7 mRNA compared to Hybrid Capture 2 for detection of HR HPV DNA for detecting high grade cervical lesions CIN2+.
- While HPV infection is the primary risk factor for developing cervical cancer, cigarette smoking is also one of the main co-factors.
- Women with high levels of urinary cotinines (by-products of nicotine), are more likely to harbour integrated oncogenic HPV virus and are therefore at increased risk of developing high grade cervical pre-cancer
- This chapter will for the first time correlate urinary cotinines with HPV DNA, HPV mRNA and disease status in women referred to colposcopy with low grade disease to better understand the role of smoking in progression of cervical pre-cancer.

5 HPV DNA and HPV E6/E7 mRNA as a triage tool

5.1 Introduction

The ASCUS and LSIL Triage Study (ALTS) firmly established the clinical utility of HPV testing in women with ASCUS. However, for LSIL, due to the high HPV DNA positivity rate, in excess of 80%, the utility of HPV DNA testing is limited (ASCUS-LSIL Triage Study group 2003). Similar findings were found in a meta-analysis by Arbyn *et al*, which also reported reduced accuracy of HPV testing in LSIL due to the high HPV positivity rate compared to ASCUS, concluding that a more specific method for triaging LSIL is needed (Arbyn et al., 2006). Most screening programs, including in Ireland, begin screening women at the age of 25 years, with some countries screening women as young as 20 years. In Ireland, from 2009-2010, 16.9% (representing 47,387 women) of women receiving a cervical smear were under the age of 30 years (CervicalCheck Report 2009-2010). In addition, 23.87% (10,289 women) of abnormal cervical smears in the same period were reported as LSIL (CervicalCheck Report 2009-2010). This represents a considerable proportion of women within the screening program, in which the utility of HPV DNA testing in the context of triage may be suboptimal.

It is recognised that women with minor cytological abnormalities require careful management. However, there continues to be diversity in how they are managed worldwide. The European guidelines for quality assurance in cervical cancer screening recommend several management options for LSIL and ASCUS. Triage algorithms include repeat cytology and immediate referral to colposcopy, in the case of ASCUS, reflex HR HPV testing is preferred only when LBC is used (Jordan et al., 2008). In some countries, including Ireland, repeat cytology for both LSIL and ASCUS remains standard. In England, HPV triage after a primary LSIL/ASCUS cytology test was introduced in 2011. While previous suggestions state that HPV DNA triage is limited in LSIL (Cotton et al., 2010)ASCUS-LSIL Triage Study group 2003) findings from the Sentinel Site studies indicated that HPV DNA testing was well accepted, cost effective, and resulted in a more rapid return to routine recall compared to repeat cytology for LSIL and ASCUS. However, there remained a high proportion of women who were negative at colposcopy due to either a negative biopsy or colposcopic assessment. A negative outcome at colposcopy was seen in 59.9%

of women with initial ASCUS and 48.3% of women with initial LSIL (Kelly et al., 2011).

Despite the role HPV plays in cervical cancer, many HPV infections are transient, especially those in younger women, with estimates that up to 80% of sexually active women will become infected at some stage in their lifetime (Tjalma et al., 2005). The fate of these infections can take various directions, most HPV infections clear within a year (Franco 1999, Ho 1995, Plummer et al 2008). Clearance of HPV can be used to predict pre-malignant lesions which will regress spontaneously. It has been found that 50% of women with LSIL can clear HR HPV infection within one year with HPV clearance preceding cytological regression by an average of 3 months (Nobbenhuis et al., 2001). In fact, neoplastic transformation is a rare complication of a HPV infection (Helmerhorst and Meijer, 2002, Nobbenhuis et al., 2001). It is persistent infection that is a major risk factor for initiating malignant transformation.

HPV contributes to malignancy through the activity of oncoproteins E6 and E7. Their persistent expression is considered fundamental for transformation and maintainence of a neoplastic state (Watanabe et al., 1989, zur Hausen and de Villiers, 1994). Malignancy is manifested by changes in host cell cycle regulatory proteins. Altered trancriptional regulation of viral oncogenes E6/E7 can occur once the virus becomes intregrated into the host genome. E7 targets and inactivates hypophosphorylated pRB disrupting its association with transcription factor E2F (Munger et al., 1989). The resulting activation of E2F leads to transactivation of genes such as cyclin A and E involved in DNA synthesis and progression through S phase (Zerfass et al., 1995). Such deregulated cell growth would ordinarily result in apoptosis; however, the activity of E6 counteracts such a response. The E6 oncogene interacts with cellular factors, primarily p53, targeting it for ubiquitin ligase degradation. This results in resistance to apoptosis and induces cellular immortalisation (Huibregtse et al., 1993, Scheffner et al., 1990). Both E6 and E7 oncoproteins can function independently however, as their activity complements one another co-expression results in pronounced transformation potential.

Active transforming infections can be monitored directly through detection of increased expression of E6/E7 oncogenes. This is achieved by exclusively detecting mRNA from E6 and E7. Identification of these transcripts provides the capacity to identify HPV infections with transforming potential that are at

higher risk of causing disease progression. Thus, detection of E6/E7 mRNA could serve as a more specific indicator of high grade pre-cancer (Cuschieri et al., 2004b).

The PreTect™ HPV Proofer (Norchip, AS) assay has been developed for the detection of HPV E6/E7 mRNA. The assay makes use of nucleic acid sequence based amplification (NSABA) technology. E6/E7 mRNA transcripts from 5 HR HPV types (HPV 16, 18, 31, 33 and 45) are amplified under isothermal conditions and detected in real-time using molecular beacon probes. The PreTect™ HPV Proofer assay has been evaluated in a number of studies (Molden et al., 2005, Rijkaart et al., 2012a, Benevolo et al., 2011, Keegan et al., 2009). The clinical performance of the test has shown that it has enhanced specificity over HPV DNA testing and has been proposed for use as a "test and treat" approach in women with HSIL/mRNA positive results (Sorbye et al., 2010). In a study by Molden et al, a comparison of HPV DNA and mRNA was performed on 77 women with an initial diagnosis of ASCUS or LSIL on cervical cytology, with a 2-year follow-up period (Molden et al., 2005). In this study, women with a positive HPV-Proofer assay were approximately 70 times more likely to be diagnosed with CIN 2 or greater than women who tested negative. In addition, the PreTect™ HPV Proofer assay has the ability to specifically detect E6/E7 mRNA from 5 specific high risk genotypes, which allows for indication of persistent infection with the 5 main carcinogenic HPV types.

Minor cytological abnormalities represent a large proportion of referrals to colposcopy. Many of these minor abnormal smears will not develop into a clinically significant lesion. Currently in Ireland, all women with minor cytological abnormalities are followed up in the same manner. Only a proportion of these women are at true risk of developing high grade pre-cancer. In chapter 3 it was demonstrated that 34.0% of women referred to colposcopy with LSIL and 25.6% referred ASCUS had detectable CIN 2+ within 18 months. It is therefore important that these women are managed carefully. It would be of enormous benefit to be able to identify women with true precancerous lesions requiring immediate colposcopy and follow up, from those with little or no risk of progression. However, challenges currently exist in identifying those women at risk of underlying high grade lesions and those likely to regress to normal. Many ASCUS and LSIL cases are related to transient HPV infections. However, a significant number of infected women will

successfully clear HPV without any adverse pathological effects, with only a proportion developing CIN 3. Due to the nature of HPV infection and the fact that in the majority of cases infections represent a transient event, the false positive rate is high and clinical specificity is low for HPV DNA testing. Taking this in to consideration, it is logical to investigate a strategy that will provide a more articulate diagnosis in identifying women at true risk. HPV testing is an effective tool but it is transforming infection that is considered the true precursor to neoplastic progression. The hypothesis of this chapter is that women who test positive for HPV mRNA are at an increased risk of high grade pre-cancer than those testing positive for HPV DNA. This chapter compares the performance of the PreTect™ HPV Proofer test for detection of E6/E7 HPV mRNA to Hybrid Capture 2 for detection of HR HPV DNA for detecting high grade cervical lesions CIN 2+ in a population of women referred to colposcopy with LSIL and/or ASCUS.

While HPV infection is the primary risk factor for developing cervical cancer, cigarette smoking is also one of the main co-factors (Plummer et al., 2003, Winkelshein, 1977, Szarewski et al., 1998). Nicotine derivatives have been found in cervical mucus as well as DNA adducts (Apseloff et al., 1994) (Benowitz, 1999). It has been demonstrated that cigarette smoking causes local immunosuppression of the cervix by reducing the number of Langerhan cells (Nadais Rda et al., 2006). Self-reported smoking status is the cheapest and easiest way to collect information in relation to an individual's smoking habits, however it is well recognised that patients under report smoking habits (Apseloff et al., 1994). Not only this, but smoking behaviours vary from person to person, depending on depth of inhalation and exposure to passive smoke. Urinary cotinine is a commonly used biomarker specific to tobacco smoke that can be measured easily in a urine sample. We hypothesise that persistent infection by HPV in the cervix is better facilitated in women who smoke, as a consequence of the reduced local immune response in the cervix.

5.2 Aims

- To determine the prevalence of HR HPV DNA in a population of women attending colposcopy for LSIL/ASCUS.

- To determine the prevalence of HR HPV mRNA in a population of women attending colposcopy for LSIL/ASCUS.

- To investigate the utility of HPV DNA and HPV mRNA testing in detecting high grade cervical lesions in a population of women with LSIL/ASCUS.

- To examine the effect of cigarette smoking through urinary cotinine analysis on HPV oncogene expression and the subsequent development of CIN in this defined patient population.

5.3 Materials and Methods

5.3.1 Study Population

Women were recruited through the colposcopy clinics at the National Maternity Hospital, Holles Street from October 2008 to July 2011. Enrolment was on the basis of being referred to colposcopy with two or more minor abnormal smears including LSIL or ASCUS. A cervical smear for HPV testing was collected during colposcopy examination. Patient's clinical and demographic details including referral smear, age at enrolment, contraception use, previous treatments, smoking and parity results were collected for each patient. A biopsy was taken during the colposcopy procedure for histological confirmation of the diagnosis, as described in chapter 2 section 2.1.1.

Women were followed over the period of time they spent under surveillance at the clinic or until they reached defined study endpoints which included; having two consecutive normal smears and discharge from the clinic without treatment alternatively having a LLETZ (Large Loop Excision of the Transformation Zone) treatment for presence of high grade pre-cancer.

5.3.2 HPV DNA testing

HR HPV DNA testing was performed using the Hybrid Capture 2 (Qiagen, UK) as described in section 2.2. hc2 is a semi-quantitative nucleic acid hybridisation assay with signal amplification that utilizes chemiluminescent detection for the quantitative detection of 13 HR types. HR HPV DNA is detected by a full length RNA probe cocktail for the detection of oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The results were given as the ratio of relative light units (RLU). The intensity of the light emitted indicates the presence or absence of HR HPV DNA in the specimen. The RLU positive cut off value is 1.0, representing 5000 copies of HPV genome. Specimens below this detection limit were considered negative. A value between 1-2.5 RLU was considered to be borderline and retested where possible.

5.3.3 E6/E7 HPV mRNA detection

Cell lysis was performed on an aliquot of 5mls of cervical PreservCyt specimen using Qiagen Lysis buffer. Total mRNA was extracted from lysed specimen using the RNeasy Minikit (Qiagen, UK) on the Qiagen M48 BioRobot. Detection of E6/E7 HPV mRNA was performed using the PreTect™ HPV Proofer (NorChip AS, Norway) as described in section 2.6. The assay detects E6/E7 mRNA from 5 HR HPV types (16, 18, 31, 33 and 45) as well as an internal control gene U1A. Detection was measured by the relative fluorescence signal, according to the manufacturer's protocol. A specimen was considered positive when the relative fluorescence signal was >1.7. Results in the range 1.4-1.7 were considered intermediate and were retested where possible.

5.3.4 Urinary Cotinine Analysis

Self-reported smoking habits were collected from all women enrolled in the study. In addition a urine sample obtained for cotinine analysis to confirm smoking status. Women were divided into three smoking status categories, non-smokers, light smokers and heavy smokers. The nicotine metabolite cotinine, the principle metabolite of nicotine was measured using Nicotine Metabolite Immunoassay (Seimens, UK) on Siemens Immulite 2000 analyser as described in section 2.6.

5.3.5 Statistical Analysis

The clinical performance of the HPV tests was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the relative 95% confidence intervals (CI) for detecting CIN 2+ within 18 month follow up. McNemar's test was used to compare HPV tests in the cytological groups LSIL and ASCUS and age group <30 years and >30 years. The Pearson's Chi-square test was applied for all comparisons between proportions. Binary logistic regression was used to calculate odd ratios to determine associated risk factors. A significance level of <0.05 was used.

5.4 Results

5.4.1 Subject population

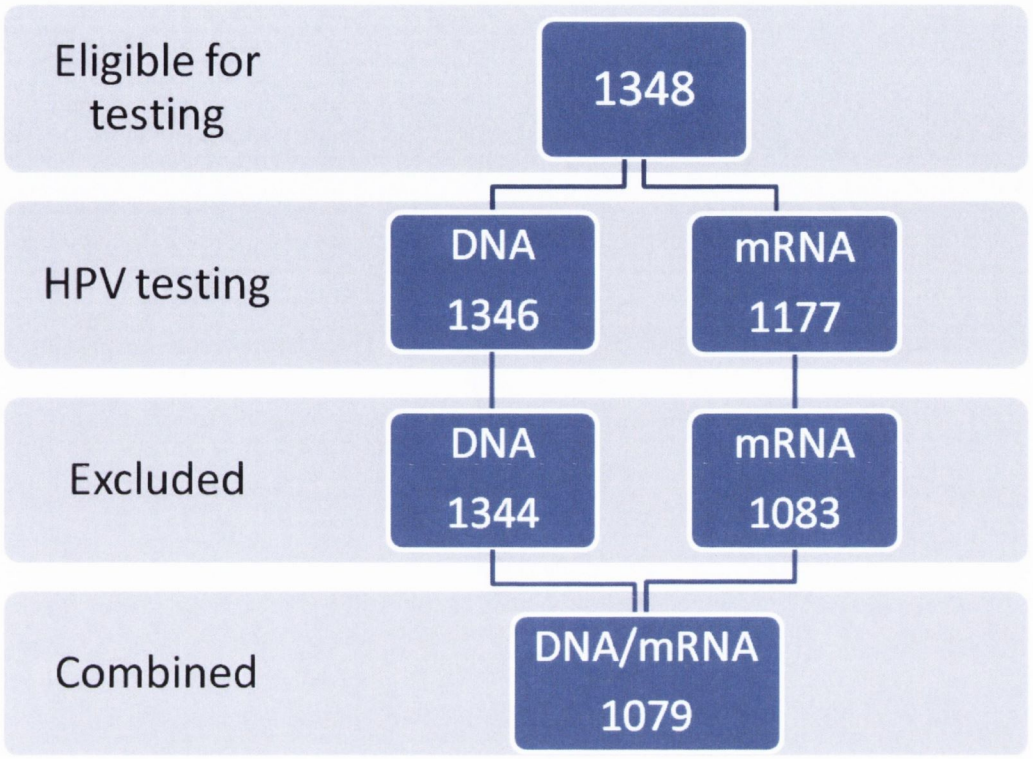


Figure 5-1 Breakdown of study population

1348 women enrolled in the study were eligible for analysis based on meeting the correct criteria. Of those eligible for analysis 1346 had a HPV DNA test and 1177 had a HPV mRNA test. There were two exclusions based on a HPV DNA test result, this was due to incomplete results due to borderline results and insufficient sample to retest. A total of 94 HPV mRNA tests were omitted. Exclusions were as follows, 33 failed the internal control of the assay and were considered invalid as per the manufacturer's protocol. 45 results were below the test cut off and deemed intermediate as per the test protocol. In such instances the manufacturer recommends re-extracting 5ml of specimen and repeating the test. This however could not be carried out on all samples due to lack of specimen. A further 17 were excluded due to inconsistent results on repeated samples. In total, there were 1079 women with both complete HPV DNA and HPV mRNA test results available for analysis.

5.4.2 Study population: baseline characteristics

Demographics were collected from women at their first visit to colposcopy (Time 0) including referral smear, age, smoking behaviour, contraceptive use and parity. Table 5.1 outlines the socio-demographic characteristics of the population. The majority of women, approximately 80%, were under the age of 40 years, with 42.9% (463/1079) under the age of 30 years. LSIL referral was more common representing 60.3% (651/1079) of referrals compared to ASCUS at 39.6% (428/1079). Smoking, contraceptive use and parity data was available on a subset of patients. Smoking behaviour, available from 1028 patients found that 37.3% (383/1028) reported as active smokers. Oral contraception was the most common form of contraception used, 38.6% (381/986) of women were currently taking oral contraception at time of enrolment.

Table 5.1 Socio-demographic characteristics for 1079 women referred to colposcopy with LSIL/ASCUS at baseline

Characteristic	Subjects n=1079 (%)
Age	18-65
Mean	33.1 ±8.6
Median	30 (27-38)
18-29	42.9% (463/1079)
30-39	36.1% (389/1079)
>40	21.0% (227/1079)
Referral	
LSIL	60.3% (651/1079)
ASCUS	39.6% (428/1079)
Non-smoker	62.5% (642/1025)
Active smoker	37.3% (383/1025)
<10 per day	24.1% (248/1025)
>10 day	13.1% (135/1028)
Oral Contraceptive Pill	38.6% (381/986)
Number of pregnancies	
0	61.7% (595/965)
1	14.3% (138/965)
2	11.7% (113/965)
3	7.6% (73/965)
4	3.1% (30/965)
>5	1.7% (16/965)

5.4.3 Prevalence of CIN at first visit to colposcopy

In Ireland, under the standard recommendations of CervicalCheck, the national screening programme, women are referred for colposcopy following two LSIL or three ASCUS cervical smears. A cervical biopsy was taken at this first visit when an abnormal area was seen by colposcopy (n= 962 cases). This revealed 42.6% (409/961) were normal on histology. The distribution of CIN was as follows: CIN 1 25.1% (241/961), CIN 2 20.7% (199/961) and CIN 3 7.3% (70/961), an inadequate biopsy sample was taken in 3.7% (36/961) of cases (figure 5.2). There were two cases of AIS, 4 uncertain grade and one VAIN 1. The remaining 118 did not have a biopsy and appeared normal by colposcopy. Among these 118 women, 29 had a smear taken which revealed 51.7% (15/29) normal, 0.3% (1/29) LSIL and 27.6% (8/29) ASCUS. There were 5 unsatisfactory smears. When examining the outcome at first visit, cases that appeared normal on colposcopy or smear only were classified as normal. Where a smear demonstrated an abnormality, it was classified as the corresponding histology result. 9 smears showing LSIL and ASCUS were placed in CIN 1. The 5 unsatisfactory smears were grouped with the 37 inadequate biopsy results.

5.4.4 HPV detection in relation to outcome at baseline

A total of 61.7% (666/1079) of the population were positive for HPV DNA and 36.1% (390/1079) were positive for HPV mRNA. Overall, detection of HPV DNA was significantly higher than detection of HPV mRNA (McNemar p=<0.001). The prevalence of HPV DNA and mRNA in relation to outcome result at first visit is shown in figure 5.2. Overall 49.4% (251/508) of those with a normal outcome at first visit were positive for HPV DNA compared to 26.1% (133/508) who were positive for HPV mRNA. Both HPV DNA and mRNA increased with severity of lesion, HPV DNA was detected in 60.0% (153/255) CIN 1, 85.0% (170/200) CIN 2 and 94.3% (66/70) CIN 3 cases while HPV mRNA was detected in 30.6% (78/255) CIN 1, 55.0% (110/200) CIN 2 and 75.7% (53/70) CIN 3 cases. Furthermore, women having an inadequate biopsy or smear result at first visit had a higher HPV DNA (53.7%) positivity compared to HPV mRNA (31.7%).

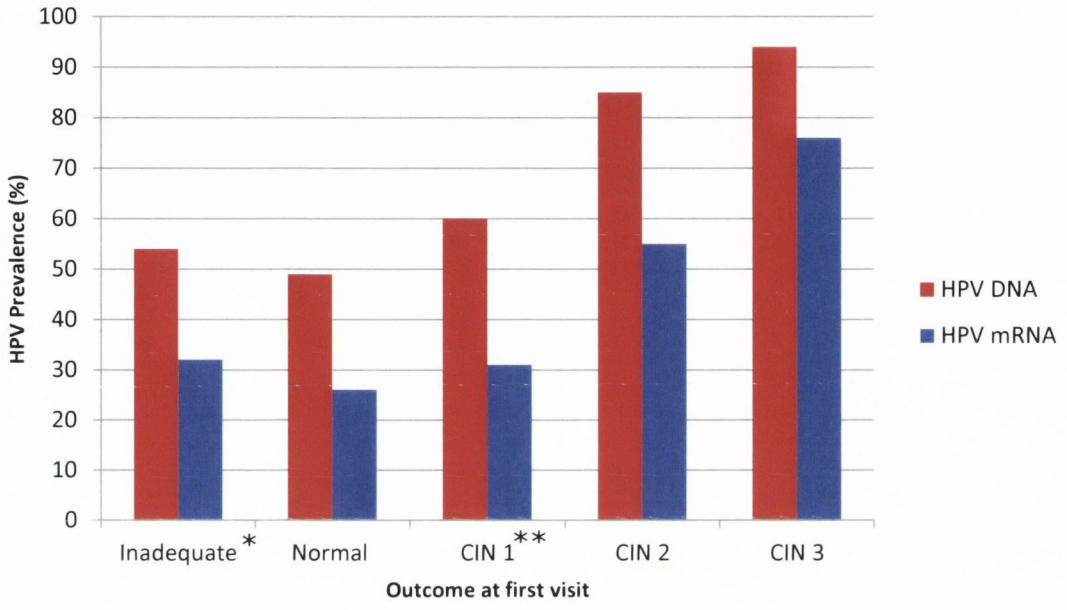


Figure 5-2 Overall prevalence of HPV DNA and HPV mRNA in relation to histology outcome at baseline

Both HPV DNA and mRNA positivity increased with severity of lesion. There is an overall higher prevalence of HPV DNA compared to HPV mRNA across all outcome categories (McNemars p=<0.05).

*Inadequate included 37 inadequate biopsy and 5 unsatisfactory smears.

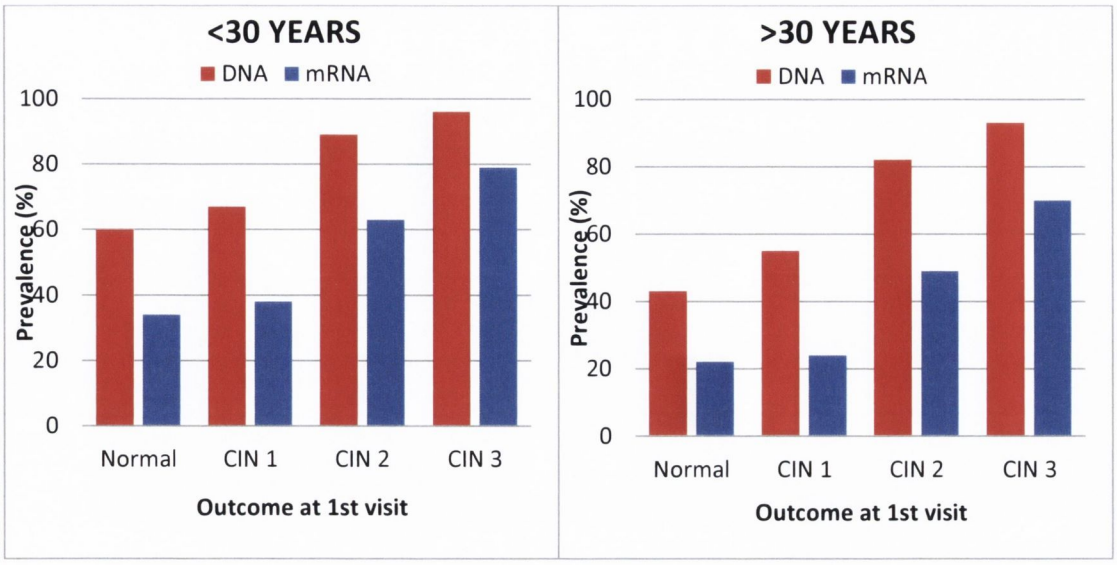
**CIN 1 included 241 showing CIN1, 8 smears showing LSIL and 1 smear showing ASCUS.

5.4.5 Age stratification and HPV status

The overall prevalence of HPV DNA was 61.7% (666/1079); decreasing positivity rates were associated with increasing age as follows, 71.3% (330/463) for 18-29 years, 61.4% (239/389) for 30-39 years and 42.7% (97/227) for women over the age of 40 years. Similarly, of the 36.1% (390/1079) of the population positive for HPV mRNA, positivity rates decreased with increasing age. A total of 45.6% (211/463) were positive in the 18-29 age group, 33.9% (132/389) of 30-39 years and 20.7% (47/227) for women over the age of 40 years.

The relationship between age, HPV status and disease diagnosis at first visit is shown in figure 5.3. The prevalence of HPV was highest among women under the age of 30 years. Based on this and the published literature, a cut off point of 30 years of age was chosen. HPV status was compared between each age groups <30 years and >30 years. Women under the age of 30 years who were normal or CIN 1 had a significantly higher prevalence of HPV DNA (p=<0.05) than those >30 years. There was no significant difference in the prevalence of HPV DNA between each age group with a diagnosis of CIN 2 (p=0.147) or CIN 3 (p=0.629). In contrast, HPV mRNA rates in women under the age of 30 years with a diagnosis of normal, CIN 1 and CIN 2 had a significantly higher prevalence of HPV mRNA (p=<0.05) than those >30 years. There was no significant difference in prevalence of HPV mRNA (p=0.409) in women with CIN 3, in either age group.

The prevalence of HPV DNA was then compared to the prevalence of HPV mRNA within each age group. In women under the age of 30 years with a diagnosis of normal, CIN 1 and CIN 2 but not CIN 3, the frequency of HPV DNA positive women was significantly higher (McNemar's p=<0.001) than the frequency of HPV mRNA positive women. While the prevalence of HPV DNA was higher in CIN 3 cases, 95.9%, compared to 79.1% for HPV mRNA, this was not significant. Whereas in women over the age of 30 years the prevalence of HPV DNA was significantly higher than HPV mRNA in all outcomes (McNemars p=<0.05) (figure 5.3).



Age	Test	Normal	CIN 1	CIN 2	CIN 3	Total
<30	DNA+	**61.4%**	**66.7%**	**89.0%**	**95.9%**	**71.0%**
		(121/197)	(76/114)	(81/91)	(41/43)	(316/445)
	mRNA+	**34.0%**	**38.6%**	**62.6%**	**79.1%**	**45.4%**
		(67/197)	(44/114)	(57/91)	(34/43)	(202/445)
	McNemar	**<0.001**	**<0.001**	**<0.001**	**0.07**	**<0.05**
>30	DNA+	**36.3%**	**54.6%**	**81.7%**	**92.6%**	**55.1%**
		(133/311)	(77/141)	(89/109)	(25/27)	(324/588)
	mRNA+	**21.2%**	**24.1%**	**48.6%**	**70.4%**	**29.4%**
		(66/311)	(34/141)	(53/109)	(19/27)	(173/588)
	McNemar	**<0.001**	**<0.001**	**<0.001**	**0.04**	**<0.05**

Figure 5-3 HPV results stratified by age and outcome at baseline

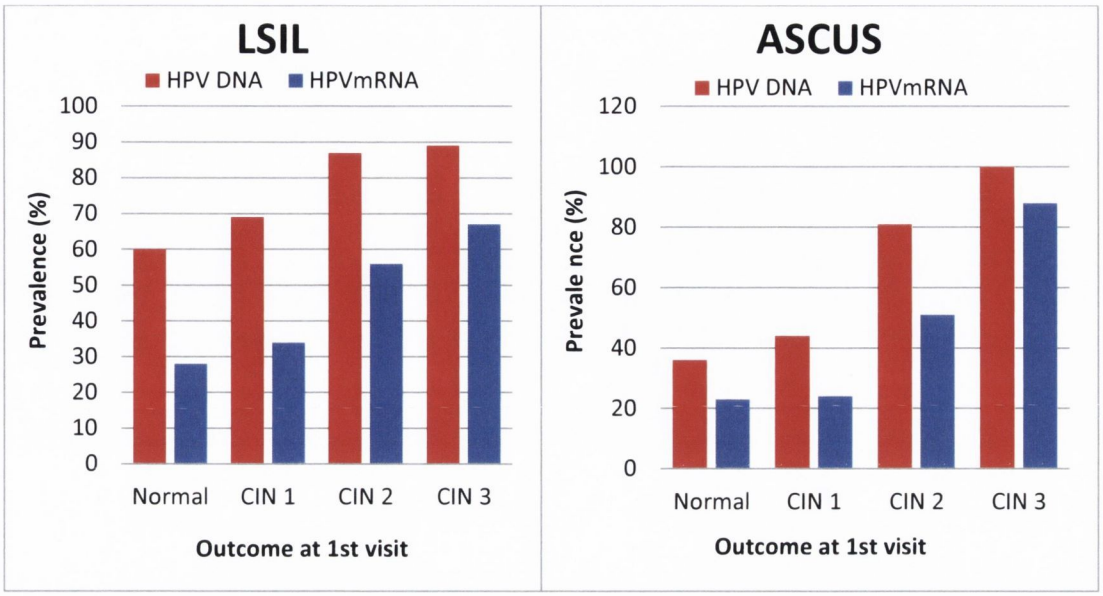
The prevalence of HPV DNA and HPV mRNA for each category of disease based on age (<30 years or >30 years). The results reported are based on n=1033 patients, 42 inadequate biopsy and smear results and 4 biopsies with uncertain grade were not included. McNemars p=<0.05 for prevalence of HPV DNA and HPV mRNA in all outcomes with the exception of CIN 3 in women <30 years.

5.4.6 HPV status in relation to referral smear

HPV status was assessed based on patients referral smear. A higher proportion of women referred with LSIL were positive for both HPV DNA and HPV mRNA (70.0% and 38.7%) compared to ASCUS referral (45.9% and 32.0%). Chi square analysis was used to test the difference between prevalence of HPV DNA in independent groups LSIL and ASCUS. The prevalence of HPV DNA was significantly higher in women referred with LSIL compared to women referred with ASCUS ($p=<0.001$). Additionally, the prevalence of HPV mRNA was significantly higher, in women referred with LSIL compared to those referred on the basis of an ASCUS smear ($p=0.03$).

The relationship between referral smear and HPV status, in relation to diagnosis at first visit, is shown in figure 5.4. The prevalence of HPV between the two referral groups, LSIL and ASCUS, was compared for each outcome at first visit. Detection of HPV DNA in <CIN 2 (normal and CIN 1) was higher in those referred with LSIL compared to ASCUS ($p=<0.001$). There was no significant difference between referral smear and the prevalence of HPV DNA in women with CIN 2 ($p=0.299$) and CIN 3 ($p=0.125$) diagnosed at first visit. In relation to mRNA, there was no significant difference between women referred with LSIL and those referred with ASCUS for normal, CIN 1, CIN 2 and CIN 3 ($p=0.136$).

The prevalence of HPV DNA and mRNA was then compared within each referral group. HPV DNA was significantly higher for all outcomes Normal, CIN 1, CIN 2 and CIN 3 in those referred with LSIL ($p=<0.05$)(figure 5.4). Whereas HPV DNA was significantly higher in normal, CIN1 and CIN 2 ($p=<0.001$) but not CIN 3 ($p=0.248$) in ASCUS referrals (figure 5.4).



Referral	TEST	Normal	CIN 1	CIN 2	CIN 3	Total
LSIL	DNA +	**59.6%**	**68.5%**	**86.9%**	**91.1%**	**69.9%**
		(170/285)	(113/165)	(113/130)	(41/45)	(437/625)
	mRNA+	**28.4%**	**33.9%**	**56.9%**	**68.9%**	**38.7%**
		(81/285)	(56/165)	(74/130)	(31/45)	(242/625)
	McNemar	**<0.001**	**<0.001**	**<0.001**	**0.016**	**<0.001**
ASCUS	DNA +	**36.3%**	**44.4%**	**81.4%**	**100%**	**49.8%**
		(81/223)	(40/90)	(57/70)	(25/25)	(203/408)
	mRNA+	**23.8%**	**24.4%**	**51.4%**	**88%**	**32.6%**
		(53/223)	(22/90)	(36/70)	(22/25)	(133/408)
	McNemar	**<0.001**	**<0.001**	**<0.001**	**0.248**	**<0.001**

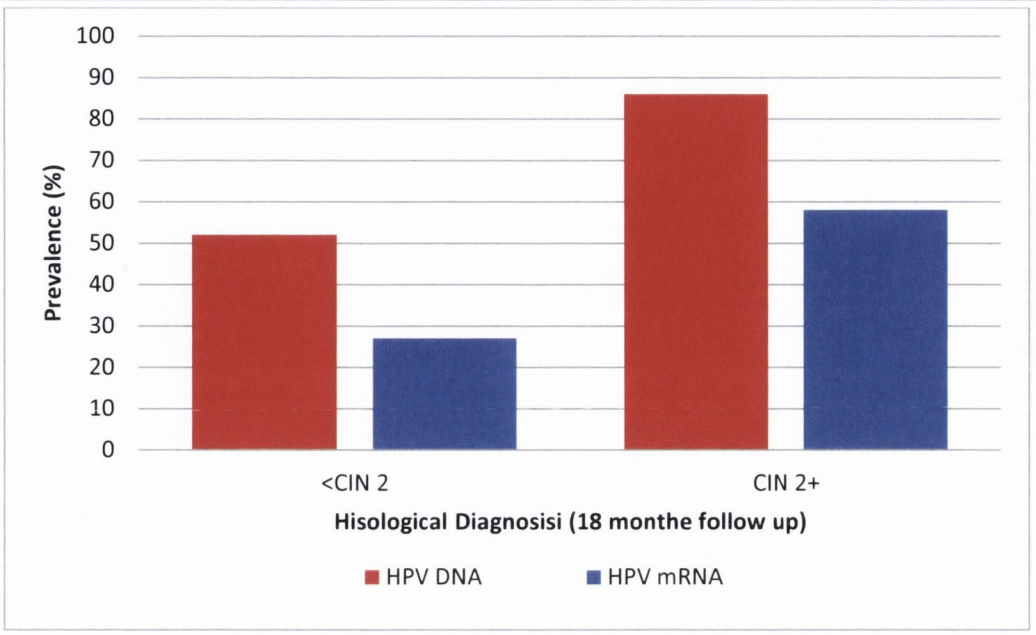
Figure 5-4 HPV results stratified by referral smear and outcome at baseline

The results reported are based on n=1033 patients, 42 inadequate biopsy and smear results and 4 biopsies with uncertain grade were not included. McNemars p=<0.05 for prevalence of HPV DNA and HPV mRNA in all outcomes with the exception of CIN 3 in women referred with ASCUS.

5.4.7 HPV DNA and HPV E6/E7 mRNA status in relation to histological outcome: an 18 month follow up

Following enrolment into the study women were followed over their time at colposcopy. Once entering the study women were assessed by colposcopy and had a biopsy taken where necessary, they were then followed by six monthly smears. Cancellations and rescheduling of appointments modified follow up intervals with many patients being followed up at 6-9 month intervals. Two consecutive negative smears warranted discharge with no treatment. If a high grade abnormality was identified the patient was returned in less than six months and a LLETZ treatment was performed. Within this population of n=1079 a sub group of n=800 patients have completed an 18 month follow up following the same criteria outlined in chapter 3 section 3.4.6. Histology results obtained over this period identified a total of 247/800 cases with a diagnosis of CIN 2+, comprising of 161 cases of CIN 2 and 86 cases of CIN 3 and one case of query microinvasion. This resulted in a cumulative rate of 31% for CIN 2+.

HPV prevalence was examined in relation to presence of high grade lesion (CIN 2 and CIN 3) compared to no high grade lesion (Normal and CIN 1). Women with no histological diagnosis that were normal on colposcopy at first visit and followed up with double normal cytology (n=79) were considered to have no CIN 2+ and have been included with outcome <CIN 2. Both HPV DNA and HPV mRNA positivity increased with severity of histological abnormality. This ranges from 52.0% and 27.0% in Normal/CIN 1 to 86.0% and 57.5% for CIN 2+ for HPV DNA and mRNA respectively (figure 5.5). This can be further broken down to 83.2% (HPV DNA) and 53.0% (HPV mRNA) for CIN 2 and 91.0% (HPV DNA) and 66.3% (HPV mRNA) for CIN 3. The one case of query microinvasion was negative for both HPV DNA and mRNA.



	HR HPV DNA	HR HPV mRNA
<CIN 2	52.0% (287/553)	27.0% (147/553)
≥CIN 2	86.0% (212/247)	57.5% (142/247)
CIN 2	83.2% (134/161)	53.0% (85/161)
CIN 3	91.0% (78/86)	66.3% (57/86)

Figure 5-5 Comparison of HPV DNA and mRNA for detection of CIN 2 and CIN 3 within an 18 month follow up period

HPV DNA detected a higher proportion of CIN 2+ lesions compared to HPV mRNA. However there were also a higher proportion of women with no detectable high grade disease (CIN 2+) positive for HPV DNA compared to HPV mRNA.

5.4.8 Clinical performance of HPV DNA and HPV mRNA

The performance of HPV DNA and mRNA testing was assessed by calculating the sensitivity and specificity for detection of CIN 2+ using histological diagnosis as gold standard in the cohort of 721 patients that had completed an 18 month follow up. The patients with no histological diagnosis that were normal on colposcopy at first visit and followed up with double normal cytology (n=79) were not included. Table 5.2 shows the sensitivity and specificity for detection of CIN 2+ for HPV DNA test was 85.8% and 48.1% respectively, compared to 57.5% and 73.4% for the HPV mRNA test. Overall the sensitivity for detection of CIN 2+ by HPV DNA was significantly higher than that of HPV mRNA (86% v's 58%) (McNemar <p=0.001). Conversely, mRNA demonstrated a significantly higher specificity than DNA (McNemar p=0.001) and consequently a higher PPV than HPV DNA. The low PPV demonstrated by both tests is owing to the low prevalence of disease in this cohort.

Table 5.2 Sensitivity, Specificity, PPV and NPV of HPV DNA and HPV mRNA testing for detection of CIN 2+

	HR HPV DNA	HR HPV mRNA
Sensitivity	**85.8%**	**57.5%**
95% CI	(84.3-87.4)	(54.4-60.5)
Specificity	**48.1%**	**72.4%**
95% CI	(45.9-50.4)	(70.6-74.2)
PPV	**46.3%**	**52.0%**
95% CI	(44.0-48.6)	(49.1-55.0)
NPV	**86.7%**	**76.6%**
95% CI	(85.3-88.0)	(74.9-78.2)

Analysis of sensitivity and specificity for detection of CIN 2 and CIN 3 indicated improved sensitivity with increase in lesion severity but at the risk of a loss in specificity as shown in table 5.3. The sensitivity and specificity of HPV DNA testing for detection of CIN 3 was 90.7% and 41.0% respectively while sensitivity of HPV mRNA testing increased to 66.3% with a specificity of 67.5%.

Table 5.3 Sensitivity and specificity of HPV DNA and HPV mRNA for detection of CIN 2 and CIN 3

	HR HPV DNA		HR HPV mRNA	
	Sensitivity	**Specificity**	**Sensitivity**	**Specificity**
CIN 2 (n=161)	**83.2%** 0.812-0.854	**48.1%** (45.9-50.4)	**52.8%** 0.490-0.566	**72.4%** (70.6-74.2)
CIN 3 (n=86)	**90.7%** 0.889-0.925	**39.6%** 37.6-41.4	**66.3%** 0.616-0.710	**66.0%** 64.2-67.7

5.4.9 Test performance in relation to age and referral smear

In order to examine the utility of HPV DNA and mRNA testing stratified by referral smear (LSIL and ASCUS) and age group (<30 years and >30 years) the sensitivity, specificity, PPV and NPV was calculated for detection of CIN 2+, in relation to each category. Results for all categories are shown in table 5.4 and graphically in figure 5.6a. The sensitivity for detection of CIN 2+ was significantly higher in women under the age of 30 years for both HPV DNA and mRNA testing. However the specificity was substantially lower in women under 30 years for HPV DNA (37.8% vs 55.2%) and HPV mRNA (65.3% vs 79.0%). The sensitivity of HPV DNA for detection of CIN 2+ did not differ significantly between LSIL and ASCUS, however the specificity increased from 38.2% in LSIL to 63.7% in ASCUS, the NPV was also significantly higher. Similarly the sensitivity for HPV mRNA detection of CIN 2+ did not differ between LSIL and ASCUS however specificity was significantly higher.

Table 5.4 Performance of HPV DNA and HPV mRNA testing for detection of CIN 2+ within 18 months according to age and referral smear

Referral	TEST	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
<30 years	DNA	88.7% (87.0-90.5)	37.8% (34.7-40.9)	44.0% (41.0-47.1)	85.9% (83.5-88.3)
	mRNA	66.1% (62.2-70.1)	65.3% (62.4-68.3)	51.3% (47.4-55.1)	77.8% (75.3-80.2)
>30 years	DNA	82.9% (80.4-85.4)	55.2% (52.5-57.9)	41.0% (38.0-44.0)	89.6% (88.3-91.0)
	mRNA	48.9% (44.4-53.2)	79.0% (77.2-80.8)	46.5% (42.2-50.8)	80.4% (78.7-82.2)
LSIL	DNA	86.4% (84.6-88.2)	38.2% (35.7-40.7)	41.% (38.6-43.7)	84.9% (82.8-86.9)
	mRNA	57.4% (53.7-61.1)	70.1% (67.9-72.4)	50.0% (45.5-52.5)	76.7% (74.7-78.7)
ASCUS	DNA	84.6% (81.7-87.5)	63.7% (60.6-66.8)	45.8% (41.8-49.9)	91.9% (90.8-93.1)
	mRNA	58.0% (52.3-63.1)	78.6% (76.4-80.9)	49.5% (44.3-54.6)	83.7% (81.8-85.6)

Figure 5.6a and 5.6b graphically illustrates the sensitivity and specificity according to each category for detection of CIN 2+ and CIN 3. It is evident that overall HPV DNA testing has a substantially higher sensitivity than HPV mRNA testing for both disease endpoints. Sensitivity increased for detection of CIN 3 in women referred with ASCUS from 85% and 58% to 100% and 73% for HPV DNA and mRNA testing respectively. The sensitivity of mRNA testing for the detection of CIN 3 also increased in women over the age of 30 from 49% to 58%. The sensitivity in all other categories did not differ significantly. Overall there was a decrease in specificity in all categories for both HPV DNA and mRNA testing, with the exception of DNA testing in ASCUS and LSIL for detection of CIN 3. The most notable change in specificity was seen in HPV mRNA testing in women referred with LSIL which demonstrated a decrease of 37% in specificity for detection of CIN 3 (figure 5.6a/b).

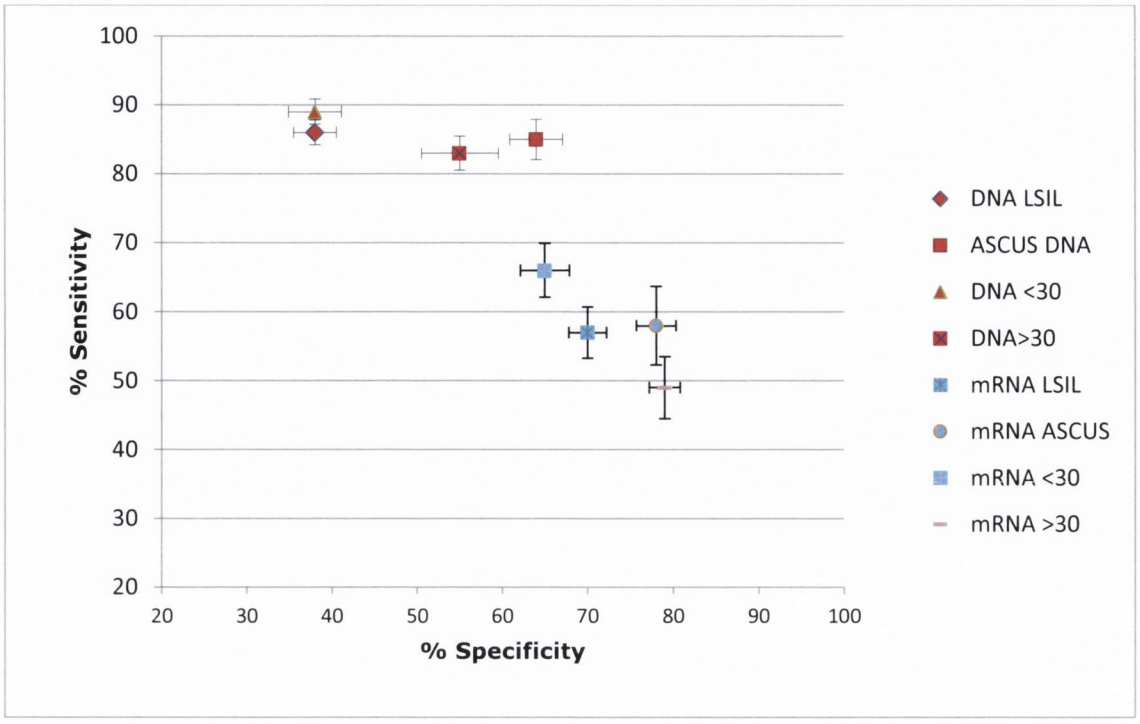


Figure 5-6 a: Comparison of sensitivity and specificity with 95% CI for detection of CIN 2+ according to age and referral smear.

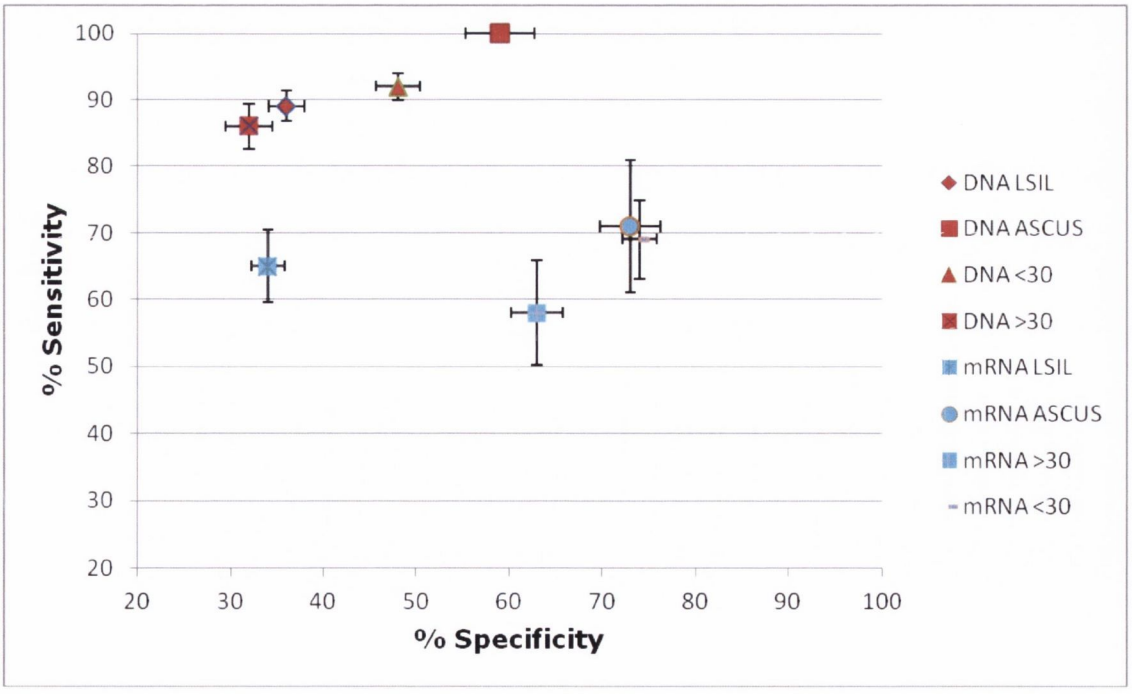


Figure 5-6b: Comparison of sensitivity and specificity with 95% CI for detection of CIN 3+ according to age and referral smear.

5.4.10 The distribution of HPV genotypes 16, 18, 31, 33 and 45 among mRNA positive cases

The PreTect™ HPV Proofer detects mRNA transcripts from 5 HR HPV types, 16, 18, 31, 33 and 45. Overall, 36.1% (289/800) of women tested positive for HPV mRNA. E6/E7 mRNA transcripts for HPV 16 were the most common type detected, representing 38.4% (111/289) of HPV mRNA positive cases. This was followed by HPV 45 at 17.3% (50/289), HPV 33 10.4% (30/289), HPV 18 9.7% (28/289) and HPV 31 8.3% (24/289). Multiple HPV types were detected in 17% (46/800) of the mRNA positive cases, the majority of which 76.0% (35/46) included HPV type 16. The distribution of HPV genotypes detected in CIN 2 and CIN 3 is shown in table 5.5. Based on these findings it is not surprising that HPV 16 was the dominant type detected in high grade lesions representing 44.6% (63/124) of CIN 2+ cases. The distribution of the remaining HPV types in CIN 2+ was as follows, HPV 18 11.3% (16/142), HPV 33 9.9% (14/142), HPV 31 8.5% (12/142) and HPV 45 7.0% (10/142). E6/E7 mRNA from HPV 45 appeared to be over represented in cases where no CIN 2+ was detected. This accounted for 80.0% (40/50) of HPV 45 mRNA positive results. Interestingly, out of the 40 cases that were mRNA positive for HPV type 45, thirteen were negative for HPV DNA, representing 41.9% (13/31) of HPV mRNA positive HPV DNA negative cases. The risk of CIN 2+ depending on genotype was examined. Multiple infections were not considered due to the complex distributions of types among the 46 women with multiple HPV mRNA types. Risk of CIN 2+ was highest for HPV 16 (OR 5.05 95% CI 3.29-7.82) and HPV 18 (OR 5.16 95% CI 2.37-11.23) followed by HPV 31 (OR 3.87 95% CI 1.60-7.15) and HPV 33 (OR 3.38 95% CI 1.60-7.15) mRNA's. mRNA from HPV 45 did not appear to have any significant risk in development of CIN 2+ (OR 0.97 95% CI 0.47-2.00).

Table 5.5 Prevalence of HPV mRNA genotypes according to outcome at 18 month follow up

	<CIN 2 (n=147)	**CIN 2** (n=85)	**CIN 3** (n=57)
HPV 16 (n=111)	32.7% (48/147)	41.2% (35/85)	49.1% (28/57)
HPV 18 (n=28)	8.2% (12/147)	11.8% (10/147)	10.5% (6/57)
HPV 31 (n=24)	8.2% (12/147)	11.8% (10/147)	3.5% (2/57)
HPV 33 (n=30)	10.9% (16/147)	4.8% (7/147)	12.2% (7/57)
HPV 45 (n=50)	27.2% (40/147)	4.1% (6/147)	7.0% (4/57)
Multiple types* (n=46)	12.9% (19/147)	11.6% (17/147)	17.5% (10/57)

5.4.11 Risk factors

In chapter 3 it is was highlighted that cigarette smoking and age were risk factors in the detection of CIN 2+ lesions. The association of cigarette smoking and age with HPV DNA and mRNA was examined. Table 5.6 shows the prevalence of HPV DNA and HPV mRNA in relation to self-reported smoking status and age.

Current smokers were found to have a higher rate of HPV DNA and HPV mRNA compared to non-smokers but this did not reach significance (OR 1.19 95% CI 0.91-1.56). The risk of being HPV DNA positive and HPV mRNA positive was highest in younger women, 18-29 vs 40 + (OR 3.13 95% CI 2.22-4.41) and (OR 3.14 95% CI 2.15–4.58) for DNA and mRNA respectively. Risk appeared to decrease with age, 30-39 vs 40+ (OR 1.99 95% CI 1.41-2.80 and OR 1.97 95% CI 1.33-2.91). Crude estimates of risk were calculated for current oral contraceptive pill (OCP) use and parity. There was no significant association of HPV DNA and OCP use (OR 1.11 95%CI 0.83-1.49) or mRNA (OR 1.05 95% CI 0.79-1.40). Parity was not associated with risk of HPV DNA (OR 0.73 95% CI 0.54-1.00) or mRNA (OR 1.04 95% CI 0.75-1.42).

Table 5.6 Associated risk of cigarette smoking and age with HPV DNA and HPV mRNA positivity

	HR HPV DNA	OR (95% CI)	HR HPV mRNA	OR (95%CI)
Smoking (n=1025)				
Non-Smoker	59.8% (384/642)	-	35.4% (227/642)	-
Smoker				
Light (<10/day)	65.3% (162/248)	1.14 (0.84-1.56)	39.1% (97/248)	1.02 (0.75-1.39)
Heavy (>10/day)	64.4% (87/135)	1.22 (0.82-1.81)	40.0% (54/135)	1.24 (0.84-1. 83)
Age				
40+	42.7% (97/227)	-	20.7% (47/277)	-
30-39	61.4% (239/389)	1.99 (1.41-2.80)	33.9% (132/389)	1.97 (1.33-2.91)
18-29	71.3% (330/463)	3.13 (2.22-4.41)	46.0% (211/463)	3.14 (2.15-4.58)

5.4.11.1 Urinary cotinine analysis

Cotinine levels were measured from a urine sample obtained at time of recruitment. When urinary cotinine concentration was used to examine exposure to tobacco smoke it was found that a proportion of women reporting as non-smokers had in fact detectable levels of cotinines. Figure 5.7 shows the distribution of cotinine concentration based on self-reported smoking habits. A number of patients had no smoking behaviour recorded. Cotinine levels in these women appear identical to non-smokers and were most likely not recorded as they reported as non-smokers. As shown in figure 5.7 there is a wide range of cotinine concentration across each category. Of those who reported as non-smokers, cotinine concentrations ranged between 10.0ng/ml-6111ng/ml, those reporting smoking 10 or less per day had a range of 10.0ng/ml-1425ng/ml, and those smoking greater than 10 cigarettes per day 10.0-19028ng/ml. The median concentration increased significantly with increasing intensity of smoking from 10ng/ml in non-smokers to 278ng/ml in light smokers and 2470ng/ml in heavy smokers (Kruskal-Wallis $p=<0.001$). As with self-reported smoking behaviour, younger women aged 18-29 years appeared to have higher levels of cotinine compared to older women aged 30-39 years and 40+years (Kruskal-Wallis $p=0.03$).

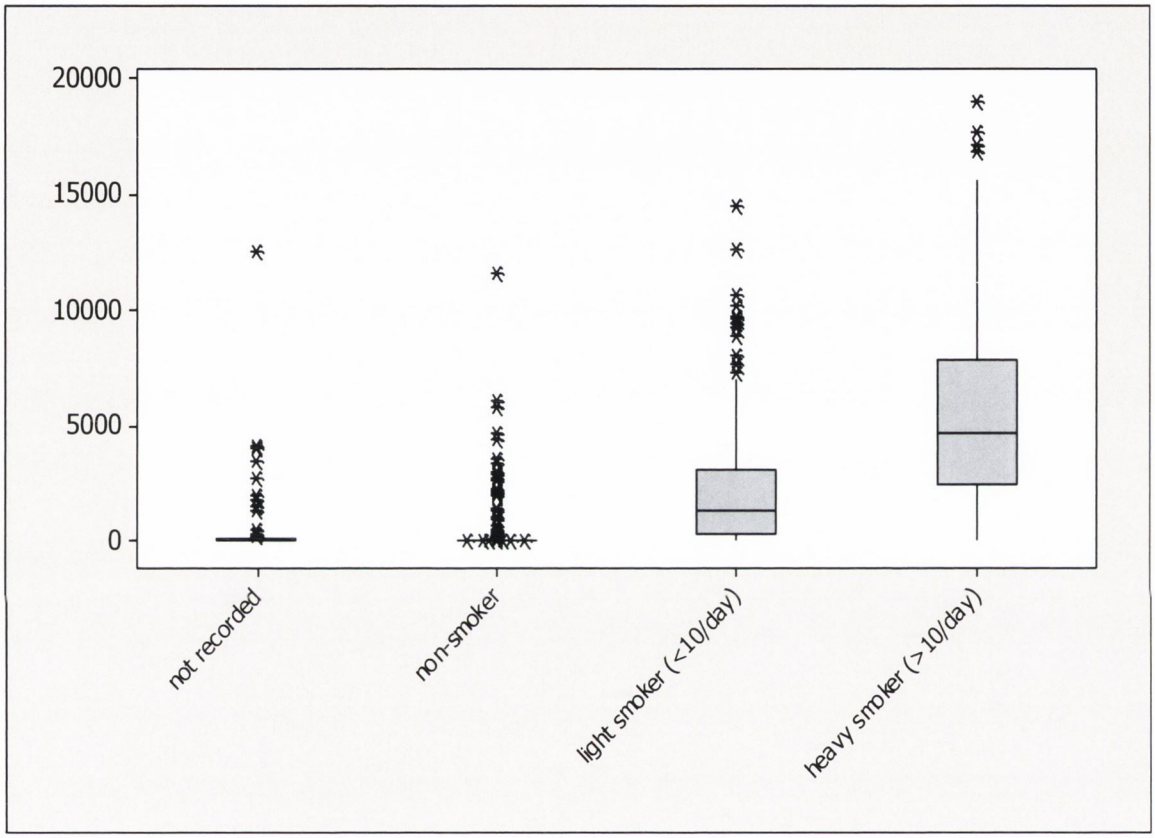


Figure 5-7 Cotinine concentration according to self-reported smoking status

Box and Whisker plot illustrating the distribution of cotinine concentrations within each reported smoking categories. The median cotinine concentration significantly increased from non-smoker to light smoker to heavy (Kruskal-Wallis p=<0.001)

A cut off value of 50ng/ml is suggested to distinguish non-smokers from those exposed to tobacco smoke (SRNT Subcommittee on Biochemical Verification, 2002). Of those who reported as non-smokers 9.4% (72/770) had cotinine levels above 50ng/ml. Under reporting smoking habits did not appear to be influenced by age. Using this cut off value it was found that women with detectable urinary cotinines above 50ng/ml were in fact at an increased risk of HPV DNA infection (OR 2.78 95% CI 1.83-4.23) and to a lesser extent at risk of mRNA positivity (OR 1.53 95% CI 1.03-2.28). To assess whether the risk of HPV infection and exposure to tobacco smoke was dose dependent, cotinine ranges were broken down into >50ng (non-smoker), 50-1000ng/ml, 1001-2500ng/ml, 2500-5000ng/ml and 5000+ng/ml. There was no significant increase in risk of HPV DNA positivity with increasing concentration of urinary cotinine. While, the risk of HPV mRNA positivity was only evident in lower cotinine concentrations 50ng/ml-1000ng/ml, no significant risk was identified above 1000ng/ml (table 5.7). There was no increased risk of CIN 2+ when cotinine concentrations were below 2500ng/ml. However, women with urinary cotinine concentrations above 2501ng/ml had an overall increased risk of CIN 2+.

Table 5.7 Association between urinary cotinine concentration and age with HPV DNA, HPV mRNA and CIN 2+

	HPV DNA	OR 95% CI	HPV mRNA	OR 95% CI	CIN 2 +	OR 95% CI
<50ng/ml	58.6% (373/636)	-	32.7% (208/636)	-	24.2% (145/559)	-
50-1000	69.4% (86/124)	2.78 (1.83-4.23)	46.0% (57/124)	1.53 (1.03 2.28)	31.5% (35/111)	1.18 (0.76-1.86)
1001-2500	67.0% (61/91)	2.69 (1.68-4.31)	41.8% (38/91)	1.48 (0.93 2.33)	35.7% (30/84)	1.58 (0.97-2.58)
2500-5000	71.4% (65/92)	3.31 (2.04-5.36)	40.2% (37/92)	1.41 (0.89 2.23)	44.4% (36/81)	2.29 (1.41-3.71)
5000+	61.9% (52/84)	2.10 (1.31-3.36)	38.1% (32/84)	1.21 (0.75 1.96)	38.6% (32/83)	1.76 (1.08-2.85)
40+	43.3% (94/217)	-	22.6% (46/217)	-	21.0% (41/195)	-
18-29	71.4% (315/441)	2.30 (1.63-3.24)	45.6% (201/441)	3.03 (2.07 4.42)	35.3% (141/399)	2.05 (1.36-3.08)
30-49	61.8% (228/369)	2.11 (1.48-3.01)	33.9% (125/369)	1.91 (1.29 2.83)	29.6% (96/324)	1.59 (1.04-2.42)

5.5 Discussion

5.5.1 Utility of HPV Testing

In this chapter the primary aim was to examine the use of two different HPV detection methods as triage tools for detecting CIN 2+ in women referred to colposcopy with LSIL and ASCUS. Assessment was based on the use of Hybrid Capture 2 for detection of HPV DNA and the PreTect™ HPV Proofer for detection of HPV mRNA.

Within this population of women presenting to colposcopy with minor cytological abnormalities, the overall prevalence of HPV DNA and mRNA was 61% and 38% respectively. It is expected that HPV DNA prevalence rates will be high among a population of women presenting at colposcopy with minor cytological abnormalities. This is particularly the case for younger women under 30 years. A majority of these women will clear HPV infection and CIN lesions will regress naturally. For this reason, it is believed testing for HPV mRNA may be a better test to predict women at risk of a subsequent diagnosis of a high grade lesion.

The data presented showed that HPV DNA and mRNA positivity rate increased with severity of lesion, these findings are in agreement with previous studies (Rijkaart et al., 2012a, Molden et al., 2005, Varnai et al., 2008). Findings indicated that 53% of women with a CIN 2 lesion and over 66% of women with a CIN 3 lesion over an 18 month period were positive for HPV mRNA at the time of their first visit to colposcopy. Importantly, of the women with no high grade pre-cancer (<CIN2) detected over 18 months, 27% were positive for HPV mRNA in comparison with nearly double, 52%, for HPV DNA. Many <CIN2 cases represent regressive lesions characteristic of transient HPV infections, it is expected that these non-transforming infections would not express E6 and E7 due to E2-induced down regulation of E6 and E7 (Romanczuk et al., 1990, Doorbar, 2006). The lower prevalence of HPV mRNA in <CIN2 lesions confirms that HPV mRNA testing may have a role as a more specific marker to stratify those women presenting at colposcopy with minor cytological abnormalities who are at risk of high grade pre-cancer.

As described in previous studies (Ratnam et al., 2010, Szarewski et al., 2012, Keegan et al., 2009) mRNA testing appears to be more specific (72% v's 48%), but less sensitive than DNA testing (86% v's 58%) for detection of CIN 2+. In our study, if the analysis is focused on CIN 3, the sensitivity of HPV mRNA increases to 66%. High sensitivity is an important attribute when considering any screening test. However, it is known that many CIN 2 and a smaller number of CIN 3 lesions will regress. It is possible that CIN 2+ cases that were missed by the PreTect™ HPV Proofer, were not destined to progress on to cervical cancer. Previous studies have also reported a number CIN 2+ cases that test negative for HPV E6/E7 mRNA by the PreTect™ HPV Proofer (Rijkaart et al., 2012b, Benevolo et al., 2011). Findings from a study by Rijkaart *et al* indicated that the PreTect™ HPV proofer missed slightly more HPV 18, 31, 33 and 45 associated CIN 2+ than HPV 16 associated CIN 2+. This may be due to the sensitivity of the assay, the HPV 16 primers and probes have been found to be the most sensitive of the assay (Molden et al., 2007). Interestingly, in our study those positive for HPV 45 were not significantly associated with risk of CIN 2+, the HPV 45 primers have been found to be the least sensitive with a limit of detection using oligo templates of 10nM compared to 0.1nM for HPV 16. Perhaps CIN 2+ cases of this type are more likely to be missed. Furthermore, HPV 45 has been found to have both a higher frequency and earlier rate of integration into the human genome compared to HPV 16 (de Sanjose et al., 2010, Tjalma et al., 2012) and together with HPV 18 it is significantly more commonly associated with harder to detect lesions of the endocervical canal (de Sanjose et al., 2010).

The lower sensitivity of the PreTect™ HPV Proofer compared to hc2 may also be due to the fact that it detects the presence of mRNA from 5 HPV types rather than 13 types detected by hc2. HPV 16, 18, 31, 33 and 45 have been reported as the most common HPV types associated with cervical cancer (Clifford et al., 2003, Munoz et al., 2003, Guan et al., 2012). However, recent studies have included HPV 58 as one of the top five most common types associated with cervical cancer (Mariani et al., 2010, Li et al., 2011b). The more recently developed APTIMA assay detects a larger pool of E6/E7 mRNA from 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) compared to only 5 detected by the PreTect™ HPV Proofer (16, 18, 31, 33 and 45). APTIMA is the first mRNA based HPV detection method to receive FDA approval. The assay makes use of Transcription Mediated Amplification

(TMA) technology to amplify E6/E7 mRNA without genotyping capability and has been found to demonstrate sensitivity comparable to hc2 with an enhanced specificity (Dockter et al., 2009, Szarewski et al., 2012). HPV mRNA detection technologies are now moving towards detecting a broader range of HPV types, the HPV OncoTect™ E6, E7 mRNA kit can detect any HPV type in conjunction with 3D cell morphology. It has been found to have high sensitivity and specificity for detection of CIN 2+. The PreTect™ HPV Proofer was chosen for this study as it was the only commercially available HPV E6/E7 mRNA detection kit when the study commenced. Increasing the number of genotypes detected may help overcome the low sensitivity previously demonstrated by the PreTect™ HPV proofer.

The appropriate management of LSIL and ASCUS remains unclear, recommendations for these women range from repeat cytology, immediate colposcopy referral or HPV testing (Jordan et al., 2008). A number of studies propose that HPV DNA triage represents the best approach (Arbyn et al., 2006, Cuzick et al., 2006). There are currently a number of technologies with FDA approval for detection of HPV DNA including Hybrid Capture 2, Cobas® 4800 HPV and Cervista® HPV. Nevertheless, conflicting evidence on the use of HPV triage in minor cytological abnormalities exists.

The sensitivity of hc2 for detection of CIN 2+ shown in the data presented is slightly lower (86% for LSIL, 85% for ASCUS) than previously described. A commentary by Arbyn *et al* recounted results from a number of large studies investigating HPV DNA triage reporting sensitivity for detection of CIN 2+ in the region of 95% with the exception of TOMBOLA (Arbyn et al., 2010). The Trial of Management of Borderline and other Low grade Abnormal smears (TOMBOLA) examined the role of HPV triage on 4439 women. Contrary to other studies TOMBOLA concluded that HPV testing was not effective in determining management of women under the age of 40 years with minor abnormal cytology. Studies evaluating HPV DNA testing report limitations with respect to specificity, the drawback with this is that it can lead to unnecessary referrals to colposcopy. Despite advantages offered by HPV DNA testing in diagnostic accuracy it is not capable of distinguishing from transient infections which are very common in sexually active women, from transforming infections. Hence, uncertainty still remains on the best management of minor cytological abnormalities that are HPV DNA positive.

The utility of HPV DNA based detection methods to stratify women at risk of CIN 2+ is an important topic in cervical cancer screening given that a number of screening programs are now adapting this strategy as a triage tool. The impact of referring all HPV DNA positive women to colposcopy, is the possibility of over-diagnosis leading to over treatment in addition to the high costs associated with colposcopy. Consequently, the need for more specific tests is pivotal now more than ever as we enter an era of HPV testing in primary screening and in the vaccinated population. The rationale is that detection of E6/E7 may provide higher specificity for CIN 2+ and be more indicative of a clinically significant lesion. This is due to the fact that the oncogenic potential of a HPV infection depends on expression of these two oncoproteins. Rijkaart *et al*, examined the use of HPV mRNA testing in HPV DNA positive cases from a range of cytology categories from a primary screening population, and reported that the risk of CIN 2+ associated with a single mRNA positive test in cytology normal cases was similar to two consecutive positive HPV DNA tests at 6 months intervals. However, they also found there remained a risk of CIN 2+ in mRNA negative women and suggested a repeat test in one year time. Benevolo *et al* also found that the sensitivity of HPV mRNA was significantly lower than that of HPV DNA however the specificity was significantly higher (Benevolo et al., 2011). The limited sensitivity of the HPV mRNA using the PreTect™ HPV Proofer for detection of CIN 2+ is in agreement with the findings of this work. There is a clear need to identify new strategies to improve the accuracy of cervical cancer screening. The identification of novel biomarkers will play a role in increasing the overall efficiently cervical screening. Specificity is an important characteristic for a triage test, it is important that new biomarkers also have a high sensitivity to match that of HPV DNA testing. $p16^{INK4A}$ has emerged as a strong contender and will be discussed further in chapter 6.

5.5.2 Urinary cotinine

The second element of this chapter was to investigate the risk of smoking through urinary cotinine analysis with HPV status and CIN. Persistent HPV infection is considered the strongest epidemiological risk factor for cervical cancer, while it is necessary, it is not a sufficient cause given the high

prevalence of transient infections. Cigarette smoking has been found to be significantly associated cervical disease (Winkelstein, 1977, Plummer et al., 2003, Haverkos et al., 2003) and has been associated with a longer duration of HPV infections (Giuliano et al., 2002). However, findings are mixed, many studies may be hampered by the fact that the disclosure of smoking habits is very unreliable. Furthermore, actual smoking behaviours differ from individuals, form depth of inhalation, total amount of cigarette smoked and exposure to second hand smoke (Benowitz, 1996). Therefore the use of a biomarker such as cotinine, offers a valuable approach to objectively identify true exposure to tobacco smoke. This study demonstrates for the first time a direct association between urinary cotinine levels, HPV infection and risk of developing CIN in a cohort of women with abnormal smears. The results show that a number of women reporting as non-smokers had cotinine levels seen among those reporting as active smokers. It is not unexpected that non-smokers should have some degree of urinary cotinine due to exposure to environmental tobacco smoke through passive smoking. However, cotinine levels would not be expected to be above 500ng/ml (Jarvis 1984, Bramer et al., 2003). 4% of those reporting as non-smokers had cotinine levels above 500ng/ml. In addition, there was a high degree of inter-subject variability and overlap of cotinine levels seen in women reporting smoking 10 or less cigarettes per day and those reported greater than 10 per day. Overall the results suggest two things, women were not revealing true smoking habits and there exists a high degree of variability in smoking behaviour between individuals. It has been previously reported that many people under report true smoking habits (Lindqvist et al., 2002, Apseloff et al., 1994). The findings of this study show that based on self-reported smoking habit, there was no significant increase in risk of being HPV DNA or mRNA positive compared to non-smokers. When urinary cotinine levels were used to assess smoking status a cut off of 50ng/ml was chosen. Cotinine levels slightly above this may indicate passive smoking (or very light smoking). It is well known that exposure to environment tobacco smoke is a health risk. Women with detectable urinary cotinine levels above 50ng/ml were at an increased risk of HPV infection and those with lower levels of cotinine appeared to be at risk of active transforming HPV infection as indicated by HPV mRNA positivity. There appeared to be a dose dependent relationship between risk of CIN 2+ and concentration of urinary cotinine. This is in agreement with findings from

chapter 3, that heavy smokers (greater than 10 per day) were at increased risk to CIN 2+. The increased risk of HPV DNA in active smokers suggests that current smoking may be associated with acquisition of a HPV infection, however only cotinine concentration above 2501ng/ml was associated with CIN 2+ suggesting that virus persistence is only associated with increased smoking intensity. However, in contrast to this risk of mRNA positivity was only associated with lower cotinine concentrations. The data presented has shown that a number of high grade lesions did not test positive for HPV E6/E7 mRNA, as discussed in the previous section this may be attributed to the fact that the PreTect™ HPV Proofer only detects mRNA from 5 HPV types. HPV mRNA may be under represented in this population and hence the risk of transforming infection and exposure to cigarette smoke through urinary cotinine is possibly underestimated.

The exact mechanism by which cigarette smoking increases risk of cervical cancer is unknown. A number of proposals have been put forward, cigarette smoking may promote DNA alterations in cervical epithelium due to the carcinogens present in cigarettes (Hellberg et al., 1986, Poppe et al., 1996). Tobacco smoke delivers over 4500 compounds containing a number of known carcinogens including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or NNK (Hoffmann et al., 1995). NNK as well as cotinine has been found at increased levels in cervical mucus of smokers compared to non-smokers (Prokopczyk et al., 1997, Kjellberg et al., 2000). These compounds may directly interact with cellular DNA (Simons et al., 1995) or impair local immunity prolonging cervical HPV infections. A number of studies have demonstrated a reduced population of langerhans cells (LC) in smokers compared to non-smokers (Nadais Rda et al., 2006). LC cells are antigen presenting cells that play critical role immunity. A reduction in the number of LC will affect generation of an adequate immune response, facilitating persistent infection and hence promoting malignant transformation.

5.5.3 Conclusion

In conclusion a HPV mRNA positive result confers an increased risk of CIN 2+ in women referred to colposcopy with LSIL and ASCUS. The PreTect™ HPV Proofer may be used to select those who require colposcopy and help prevent

women from extra follow up visits, ultimately reducing the number of unnecessary treatments. However, those who test negative for HPV mRNA should not be returned to routine recall but require follow up assessment as there remains a risk of CIN 2+. Thus, as a stand-alone test, to stratify women at risk of CIN 2+, HPV mRNA testing would not be accurate enough. In the context of screening, an important consideration will be the number of different HPV types required for efficient screening, increasing the number of HR HPV types detected by an assay will no doubt increase sensitivity, however this may be at the risk of reducing specificity. It will be at the discretion of health care providers to seek a balance between sensitivity and specificity, to achieve the most cost effect approach without putting the health of women undergoing screening at risk. The data presented here will be applied to triage models suggested by the NHS cervical screening program for the use of HPV DNA testing in the triage of LSIL and ASCUS. The results will be presented in chapter 7.

Furthermore it is evident that smoking has an impact on the development of high grade cervical pre-cancer emphasizing that smoking cessation campaigns targeting this population and young women might be valuable.

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-Chapter 6-

The performance of p16INK4A/Ki-67 protein co-expression in minor cervical abnormalities LSIL and ASCUS

Overview

- The cyclin dependent kinase inhibitor p16^{INK4A} is a well-established biomarker in cervical cancer and pre-cancer.
- However, p16^{INK4A} can be identified in non-neoplastic entities and consequentially it is morphology dependent.
- The CINtec® PLUS kit has been developed to combine p16^{INK4A} detection with the proliferation marker Ki-67 as a morphology independent marker.
- Simultaneous detection of p16 ^{INK4A} and Ki-67 may be a potential tool in the triage of minor cytological abnormalities.
- The use of p16^{INK4A}/Ki-67 dual staining was assessed for detecting CIN 2+ in a population of women referred to colposcopy with minor cytological abnormalities and compared to HPV DNA and HPV mRNA testing.

6 p16^{INK4A}/Ki-67 co-expression in LSIL and ASCUS

6.1 Introduction

Currently research is focused on discovering objective markers, both viral and cellular that can identify transforming HPV infections and predict disease severity. Our knowledge of HPV pathophysiology has allowed for a number of biomarkers to be identified with potential to detect those at risk of disease progression. It is well established that transforming infection is the true risk factor and that it is characterised by an increased expression of viral oncogenes E6 and E7 (Chakrabarti and Krishna, 2003). The use of E6/E7 mRNA detection has been suggested as a management option and was discussed in chapter 5. This chapter will focus on cellular proteins that are increased in the presence of transforming HPV infection.

Several host cell biomarkers have been evaluated for their potential to improve diagnostic specificity of cervical screening. These markers include p16^{INK4A} which plays a major role in cell cycle regulation, Ki-67 a proliferation marker, topoismerase 11-α (TOP2A) a regulator of DNA topology and minichromosome maintenance protein 2 (MCM2) a DNA licensing factor. It has been recently proposed that the combined detection of these novel biomarkers would have utility as surrogate markers of transforming HPV infections. This has led to the development of commercial assays, ProExC (BD) which simultaneous detects TOP2A/MCM2 and CINtec® PLUS (Roche mtm laboratories) which detects over expression of p16^{INK4A}/Ki-67. Initial studies showed ProEx C had an improved PPV for detection of CIN 2+ compared to pap (Kelly et al., 2006). A study on tissue samples, reported strong staining for ProEx C, it found 92% positive staining in CIN 2+, with staining seen in lower and upper layers of cervical epithelium (Badr et al., 2008). Siddiqui *et al* evaluated the stain on cervical cytology slides of ASC-H reporting a high sensitivity, 98.8% and specificity of 82.4%, for detection of CIN 2+ as well as improved prediction of CIN 2+ compared to HPV DNA testing (Siddiqui et al., 2008). However, ProEx C staining requires careful interpretation as expression of MCM can be found in some benign cycling squamous and glandular cells, morphological features need to be taken in to consideration for clarification of positive results (Shroyer et al., 2006, Siddiqui et al., 2008, Oberg et al., 2010). Thus, further studies are needed to explore standardised scoring

interpretation of ProEx C staining. CINtec PLUS has showed promising results and will be discussed further on.

One of the most promising cellular protein markers to be identified is the cyclin dependent kinase inhibitor p16^{INK4A}. It is known to be directly involved with the transforming activity of HPV E7 oncoprotein (Wentzensen and von Knebel Doeberitz, 2007). p16^{INK4A} is involved in the regulation of the pRB/E2F pathway and is associated with a negative feedback of functional pRB (Al-Khalaf et al., 2011, Johnson, 1995). However, in a transforming HPV infection, HPV E7 oncoprotein disrupts the association of pRB with E2F without having any effect on p16^{INK4A} expression (Figure 6.1). As a result, p16^{INK4A} accumulates in the cells and hence acts as a surrogate marker of transforming HPV infections. Over expression of p16^{INK4A} has demonstrated promising results in its capability to detect high grade pre-cancer (Klaes et al., 2001, Murphy et al., 2005, Cuschieri and Wentzensen, 2008). It has been identified in histological preparations and has been found to improve inter-observer reproducibility in diagnosing CIN compared to conventional H&E staining (Klaes et al., 2002, Horn et al., 2008). Expression directly correlates with grade of CIN, strong diffuse staining has been demonstrated in many CIN 2 and CIN 3 lesions (Klaes et al., 2001, Agoff et al., 2003, Wang and Lu, 2004) (Galgano et al., 2010). Furthermore, studies have shown that CIN 1 lesions which are p16^{INK4A} positive are far more likely to progress to more severe dysplasia compared to those that are p16^{INK4A} negative(Wang and Lu, 2004, Negri et al., 2004). Thus, p16^{INK4A} can also be used as a marker of progression.

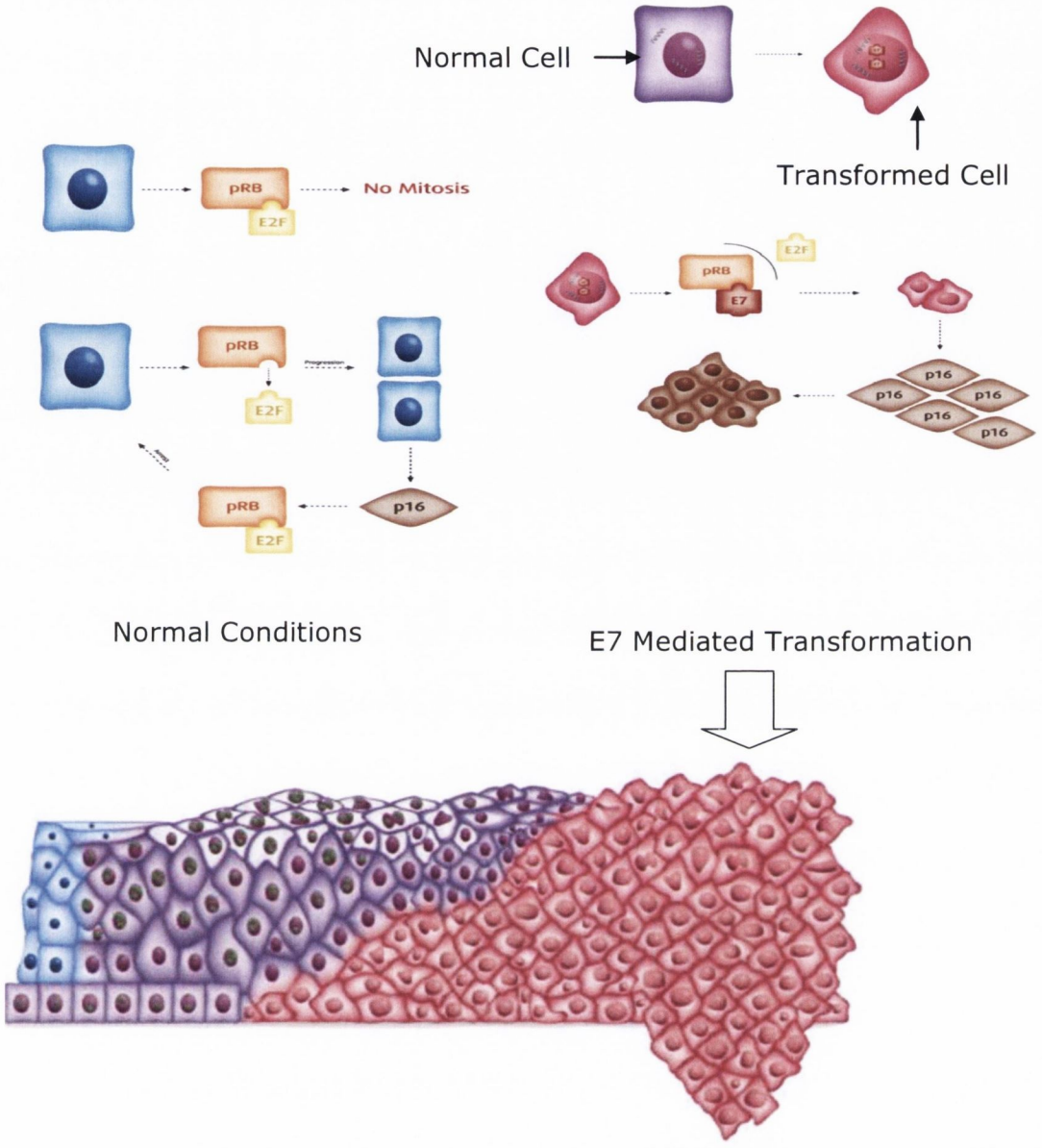


Figure 6-1 HPV E7 mediated over expression of p16[INK4A]

Under normal conditions pRB is bound to the transcription factor E2F preventing proliferation. p16[INK4A] serves as a negative regulator of cell cycle progression that blocks proliferation by promoting the interaction between E2F and pRb. In transforming HPV infections, due to the inactivation of pRB mediated by E7, the anti-proliferation function of p16[INK4A] is abolished and cells can proliferate despite the presence of high levels of p16[INK4A]. As E7 mediates oncogenic transformation, it causes cell cycle progression, resulting in disruption in maturation of cervical epithelial. If left untreated can become invasive if the lesion breeches the basement membrane in to underlying cervical stroma.(www.mtmlabs.com)

A number of studies have also examined the use of p16^{INK4A} in cytology specimens (Klaes et al., 2001, Murphy et al., 2005, Szarewski et al., 2012) and have investigated it as a triage tool for LSIL and ASCUS (Denton et al., 2010, Wentzensen and Klug, 2009, Monsonego et al., 2007). Their findings indicate it provided a similar sensitivity to HPV testing with an increased specificity. However, p16^{INK4A} can be over expressed in some non-dyskaryotic cells, it has been identified in reactive metaplastic cells, endocervicals and atrophic specimens (Trunk et al., 2004). Consequently there exist some limitations with respect to p16^{INK4A} in cytology specimens. Morphological criteria need to be applied when examining cytology slides stained by p16^{INK4A} in order to discriminate between normal and abnormal cells. This results in a level of subjectiveness due to the nature of interpretation of morphological criteria in classifying positive cells. Despite a number of proposed approaches there is a lack of standardised methods for the interpretation of p16^{INK4A} positivity. Disparity of p16^{INK4A} staining was reviewed in a meta-analysis by Tsoumpou *et al*. Out of 61 studies the range of positivity, especially in LSIL and ASCUS lesions, was extremely wide (Tsoumpou et al., 2009). Some studies used a method of counting positive cells and employing a cut off level, while others used morphology based criteria. An evaluation system has been suggested by Wentzensen *et al*, this is based on a nuclear scoring. Applying this scoring system increased the specificity of p16^{INK4A} staining (Wentzensen et al., 2005). However, morphologic assessment of p16^{INK4A} stained cells can be difficult and even, in cases, more challenging than interpretation of pap staining (Edgerton et al., 2011).

A meta-analysis by Tsoumpou *et al* concluded that p16^{INK4A} expression correlates with severity of cervical lesions (Tsoumpou et al., 2009). More recently a systematic review Roelens *et al* explored the clinical application and performance of p16^{INK4A} in the triage of minor cytological abnormalities in 17 studies. A subset of 8 studies compared p16^{INK4A} staining and hc2. The reported relative sensitivity for detecting CIN 2+ was 0.95 (95% CI 0.89-1.01) and 0.87 (95% CI 0.81-0.94) for ASCUS and LSIL respectively and relative specificity of 1.82 (95% CI 1.57-2.12) and 2.74 (95% CI 1.99-3.76) for ASCUS and LSIL respectively were reported compared with hc2 (Roelens et al., 2012). The authors concluded that p16^{INK4A} staining is more accurate than HPV testing by hc2 in the triage of ASCUS, however the lower sensitivity in women with LSIL indicates p16^{INK4A} negative women cannot be referred back

to routine screening. Hence, in order to achieve good reproducibility a more objective test is desirable.

A recently developed kit for dual staining of p16^{INK4A} and Ki-67 offers potential to eliminate morphologic interpretation and focus solely on immune-reactive cells. Ki-67 is a proliferation marker which has undergone extensive investigation as a marker for cervical pre-cancer (Dunton et al., 1997, Mimica et al., 2010, Agoff et al., 2003, Keating et al., 2001). Ki-67 is expressed during the interphase of the cell cycle and can be exclusively detected in the nucleus of actively cycling cells (Gerdes et al., 1984). Under normal conditions, p16^{INK4A} expression leads to cell cycle arrest and thus has an anti-proliferative effect, while Ki-67 is strictly associated with cellular proliferation. Under normal circumstances expression of both proteins should not co-exist. It is known that increased expression of p16^{INK4A} signals functional activation of E2F mediated by E7 and therefore co-expression of p16^{INK4A} and Ki-67 signals HPV transformed cells undergoing proliferation. Consequently, the presence of double immune-stained cells in cervical cytology could be used as a method of identifying progressive cervical lesions. Retrospective studies evaluating CINtec® PLUS have reported it as a potential triage tool that can accurately detect CIN 2+. Petry *et al* investigated HPV positive ASCUS smears in women aged over 30 years. Sensitivity and specificity of 91.9% and 81.2% was reported for detection of CIN 2+ (Petry et al., 2011). This is similar to what Schmidt *et al* presented for triage of LSIL and ASCUS in the same age group, sensitivity 89.1% and 92.7% and specificity 85.5% and 70.7% (Schmidt et al., 2011). Other studies have confirmed a high sensitivity and significantly higher specificity than compared to HPV testing for detection of high grade CIN using dual staining (Wentzensen et al., 2012, Loghavi et al., 2012).

In this chapter the performance of the CINtec PLUS kit for the simultaneous detection of p16^{INK4A} and Ki-67 is assessed for detecting CIN 2+ in a large study of women referred to colposcopy with LSIL/ASCUS, and compared with HPV DNA and HPV mRNA testing.

6.2 Aims

- Examine the co-expression of p16^{INK4A}/Ki-67 in population of women referred to colposcopy with LSIL and ASCUS.

- Assess the clinical performance of CINtec® PLUS for the dual staining of p16^{INK4A}/Ki-67 for detecting CIN 2+ in a population of women referred to colposcopy with LSIL and ASCUS.

- Compare the performance of p16^{INK4A}/Ki-67 dual expression with HPV DNA and HPV mRNA testing for the detection of CIN 2+.

6.3 Materials and methods

6.3.1 Study population

Women were recruited through the colposcopy clinics at the National Maternity Hospital, Holles Street from October 2008 to July 2011. Enrolment was on the basis of being referred to colposcopy with two or more minor cytological abnormalities including LSIL or ASCUS. A cervical smear for HPV testing was collected during colposcopy examination. Patient's clinical and demographic details including referral smear, age, contraception use, previous treatments, smoking, and parity results were collected for each patient. A biopsy was taken during colposcopy for histological confirmation of the diagnosis.

Women were followed over the period of time they spend under surveillance at the clinic or until they reached defined study endpoints which included having two consecutive normal smears and discharge from the clinic without treatment alternatively having a LLETZ (Large Loop Excision of the Transformation Zone) treatment for presence of high grade disease.

6.3.2 p16^{INK4A}/Ki-67 protein expression

ThinPrep cytology slides from residual PreservCyt material from the same smear used for HPV testing were prepared using a T2000 slide processor. The CINtec® PLUS kit (Roche mtm laboratories) was used for the immunocytochemical staining for p16^{INK4A}/Ki-67 in accordance with the manufacturer's instructions. Immunocytochemical staining was performed using a ready to use primary antibody cocktail containing a mouse monoclonal antibody (E6H4) directed against p16^{INK4A} and a rabbit monoclonal antibody (274-11 AC3) directed against Ki-67. Visualization was based on two visualisation reagents (HRP and AP) and two different substrate-chromogens (DAB and FastRed). Specimens were counterstained with Harris Hematoxylin followed by a two-step mounting procedure with an aqueous mounting medium followed by pertex mount. A positive result was interpreted as brown cytoplasmic staining for p16^{INK4A} expression and red nuclear staining for Ki-67 expression. The presence of one or more stained epithelial cells showing simultaneous expression signified a positive result. All cases were subjected to an additional pathologist review.

6.3.3 Statistical analysis

The clinical performance was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and 95% confidence intervals (CI) for detecting CIN 2+ within 6 month follow up. McNemars test was used to evaluate the difference between p16^{INK4A}/Ki-67 positivity and HPV DNA and HPV mRNA The Pearson's Chi-square test was applied for all comparisons between proportions.

6.4 Results

6.4.1 Optimisation

Expression of p16^{INK4A} and Ki-67 in cervical cancer and CIN has been well documented. The CINtec® PLUS kit is a fully validated assay with ready to use antibody. Initially in order to optimise staining protocols, expression of p16^{INK4A}/Ki-67 was assessed in HPV 18 positive HeLa cells. Cytospin preparations were made from cell pellets of 1x10^{6} cells/ml. Figure 6.2 shows HeLa cells demonstrating strong immunoreactivity. No primary antibody was added to negative control specimens, no immune reactivity was identified in these samples.

In order to develop a bank of positive and negative clinical control samples, cytology specimens with confirmed pap results of Negative (n=7), LSIL (n=6) and HSIL (n=7) were stained using the CINtec® PLUS kit. All the specimens reported as HSIL had strong immunoreactivity for p16^{INK4A} and Ki-67 (figure 6.3a), none of the specimens reported as LSIL demonstrated any immunoreactivity (figure 6.3b) and one normal case had a single positive cell. Numerous positive cells were identified in HSIL cases, positive cells were found both singly and in clusters (figure 6.3).

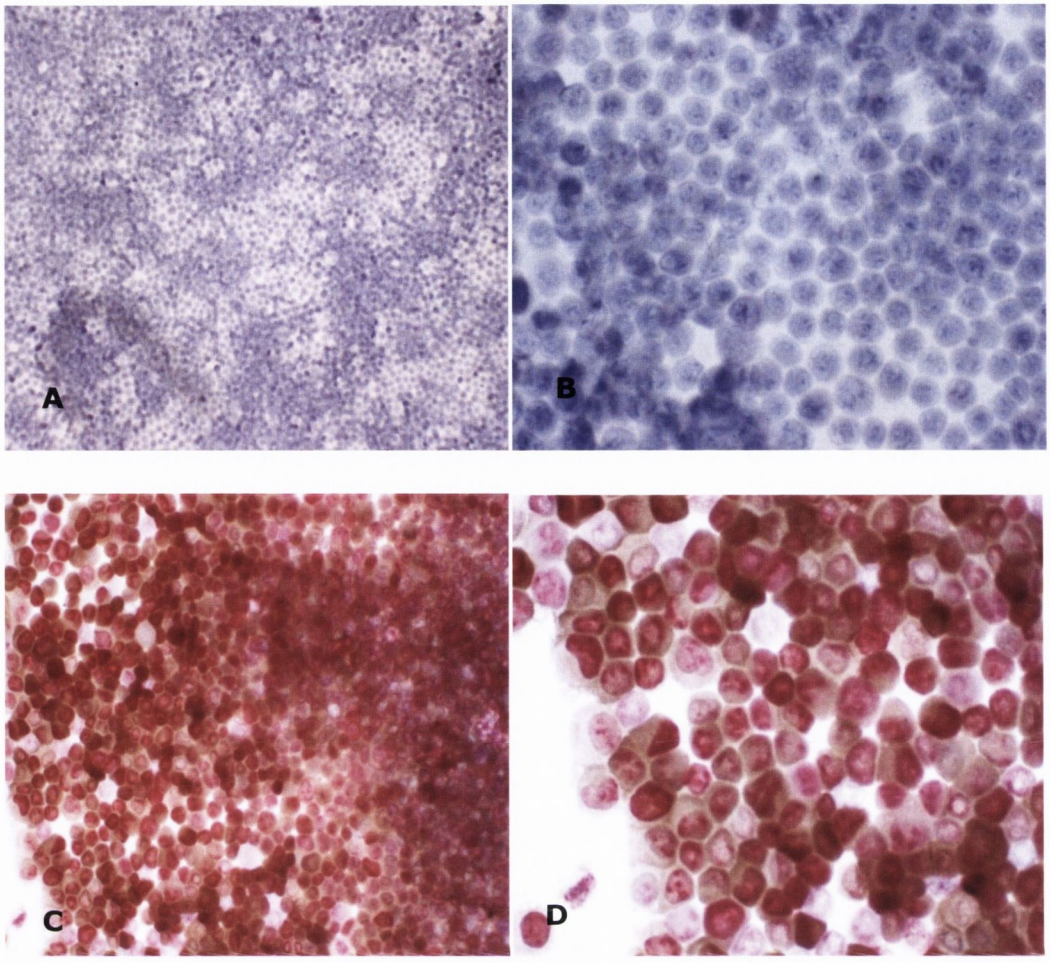


Figure 6-2 p16^{INK4A}/Ki-67 stained HeLa Cells

HeLa cells stained with the CINtec® PLUS p16^{INK4A}/Ki-67, positive cells show a red nuclear stain indicating Ki-67 expression and brown staining of the cytoplasm indicating p16^{INK4A} expression. A) HeLa cells at 20x magnification with no primary antibody. B) HeLa cells at 40x magnification with no primary antibody. C) p16^{INK4A}/Ki-67 positive stained HeLa cells at 10x and D) p16^{INK4A}/Ki-67 positive HeLa cells at 40x magnification.

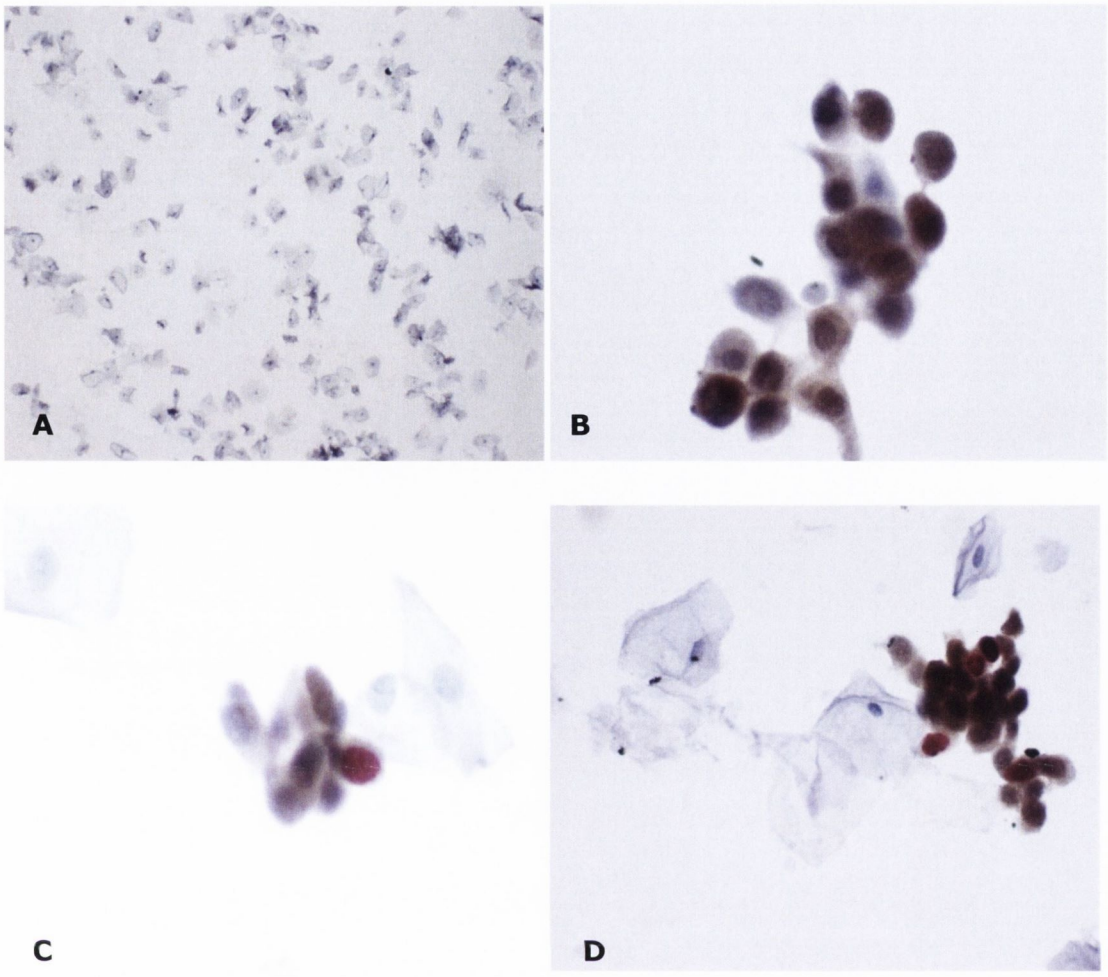


Figure 6-3 p16^{INK4A}/Ki-67 stained cervical cytology cells

Positive results characterised by brown cytoplasmic staining for p16^{INK4A} and red nuclear staining for Ki-67 within the same cell. The presence of at least one dual positive cell is a considered a positive result. A) NAD (no abnormality detected) demonstrating haematoxylin staining only, negative for p16^{INK4A} and Ki-67 staining (magnification 10x). B) positive cytoplasmic and nuclear staining for p16^{INK4A} only, negative for dual staining (magnification 40x). C) HSIL single dual stained positive cell (magnification 40x). D) HSIL cluster of positivity stained cells, brown cytoplasmic staining indicating over expression of p16^{INK4A} and red nuclear staining indicating over expression of Ki-67 with adjacent normal superficial cells (magnification 40x).

6.4.2 Study population

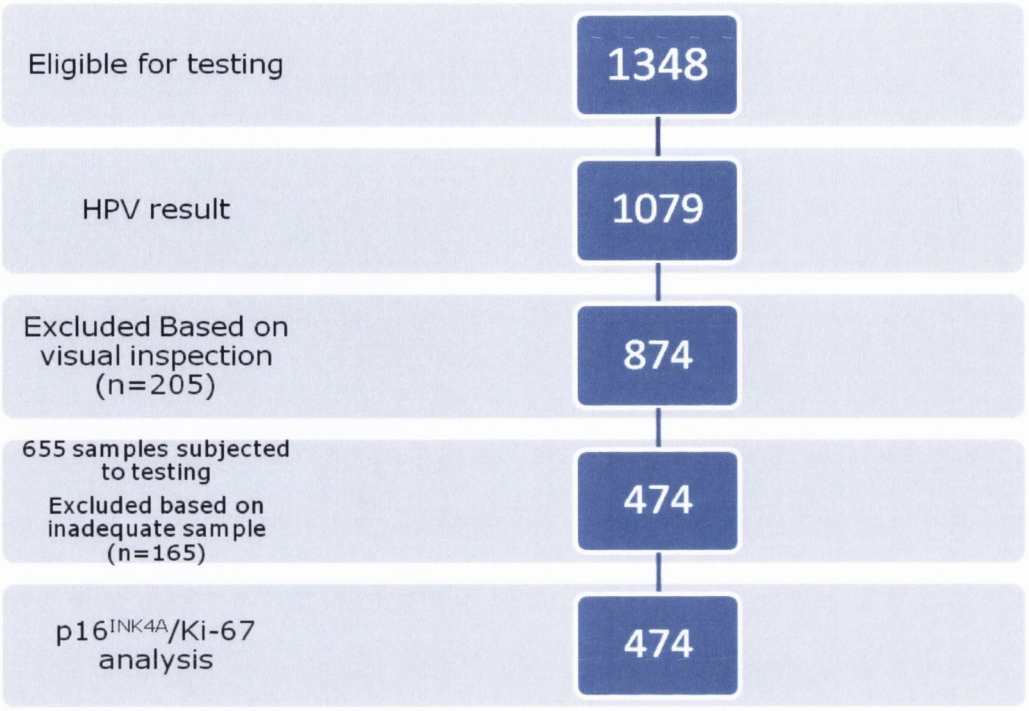


Figure 6-4 Breakdown of subject population

Testing was performed on residual ThinPrep material, remaining after at least 9ml of specimen was removed for HPV DNA and mRNA testing. For this reason a substantial number of samples (n=205) did not have sufficient cellular material remaining to prepare a slide for immunocytochemistry and were excluded. This resulted in 874 available for analysis, over 655 patient samples were subjected to immunocytochemistry using the CINtec® PLUS kit. As recommended by the manufacture a further 168 cases were excluded following staining as they did not meet the minimum cellularity criteria as specified by the Bethesda System 2001 (Solomon et al., 2002). Again, this was related to insufficient material remaining following all HPV DNA and mRNA testing. However, if a positive result was seen in such an inadequate sample it was considered valid (it is noted that this may have enriched the population for high grade lesions). Thus in total 474 patient samples were available for analysis.

6.4.3 Study population: baseline characteristics

Demographics were collected from women at their first visit to colposcopy (Time 0) including referral smear, age, smoking behaviour, contraceptive use and parity. Table 6.1 outlines the socio-demographic characteristics of the population. The median age was 31 years (interquartile range 27-38). The majority of women, approximately 80%, were under the age of 40 years, with 39.9% (189/474) under the age of 30 years. LSIL referral was more common representing 56.5% (268/474) of referrals compared to ASCUS at 43.5% (206/474). Smoking, contraceptive use and parity data was available on a sub-set of patients. Smoking behaviour, available from 459 patients found that 37.3% (171/459) reported as active smokers. Oral contraception was the most common form of contraception used, 42.7% (182/426) of women were currently taking oral contraception at time of enrolment. 44.1% (137/311) of women had at least one full term pregnancy.

Table 6.1 Socio-demographic characteristics for 474 women referred to colposcopy with LSIL/ASCUS at baseline

Characteristic	Subjects n=474 (%)
Age	18-65
Median	30 (27-38)
18-29	39.9% (189/474)
30-39	38.8% (184/474)
>40	21.3% (101/474)
Referral	
LSIL	56.5% (268/474)
ASCUS	43.5% (206/474)
Non-smoker	62.7% (288/459)
Active smoker	37.3% (171/459)
<10 per day	23.7% (109/459)
>10 day	13.5% (62/459)
Oral Contraceptive Pill	42.7% (182/426)
Number of pregnancies	
0	56.0 (174/311)
1	18.0 (56/311)
2	12.9 (40/311)
3	8.7 (27/311)
4	1.9 (6/311)
>5	2.6 (8/311)

6.4.4 p16^{INK4A}/Ki-67 dual expression in LSIL/ASCUS

Valid test results were available from 474 patient samples that had been referred to colposcopy with LSIL or ASCUS. As described in section 6.3.2 the test was considered positive by the presence of a minimum of one cell showing simultaneous dual staining irrespective of morphological interpretation (figure 6.4). Samples demonstrating no dual immunoreactivity were reported as negative for p16^{INK4A}/Ki-67 co-expression. Figure 6.5 shows test samples demonstrating double immune reactivity with corresponding pap stained samples. In figure 6.5 examples of p16^{INK4A}/Ki-67 positive staining in LSIL and HSIL.

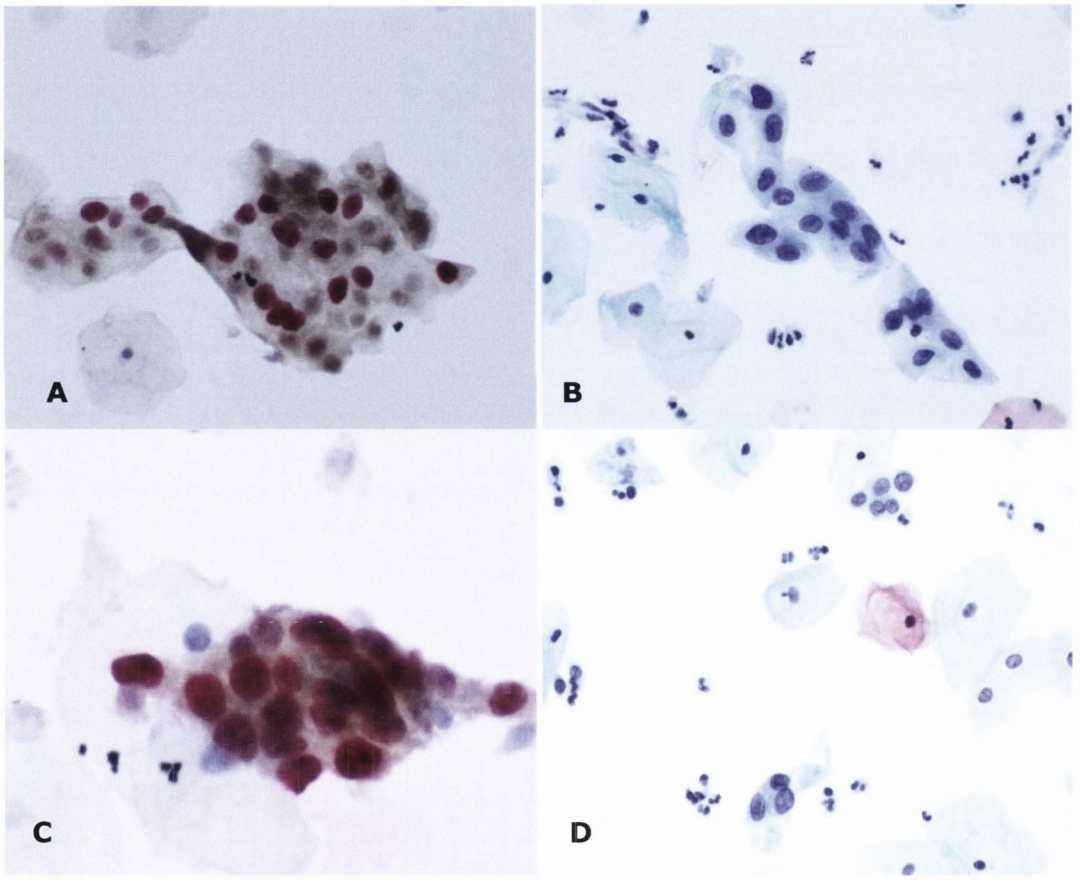


Figure 6-5 p16^{INK4A}/Ki-67 with corresponding pap stain

Patient sample demonstrating A) dual positive expression for p16^{INK4A}/Ki-67 and corresponding B) LSIL on pap cytology. Patient sample demonstrating C) dual positive expression for p16^{INK4A}/Ki-67 and D) corresponding pap cytology showing HSIL.

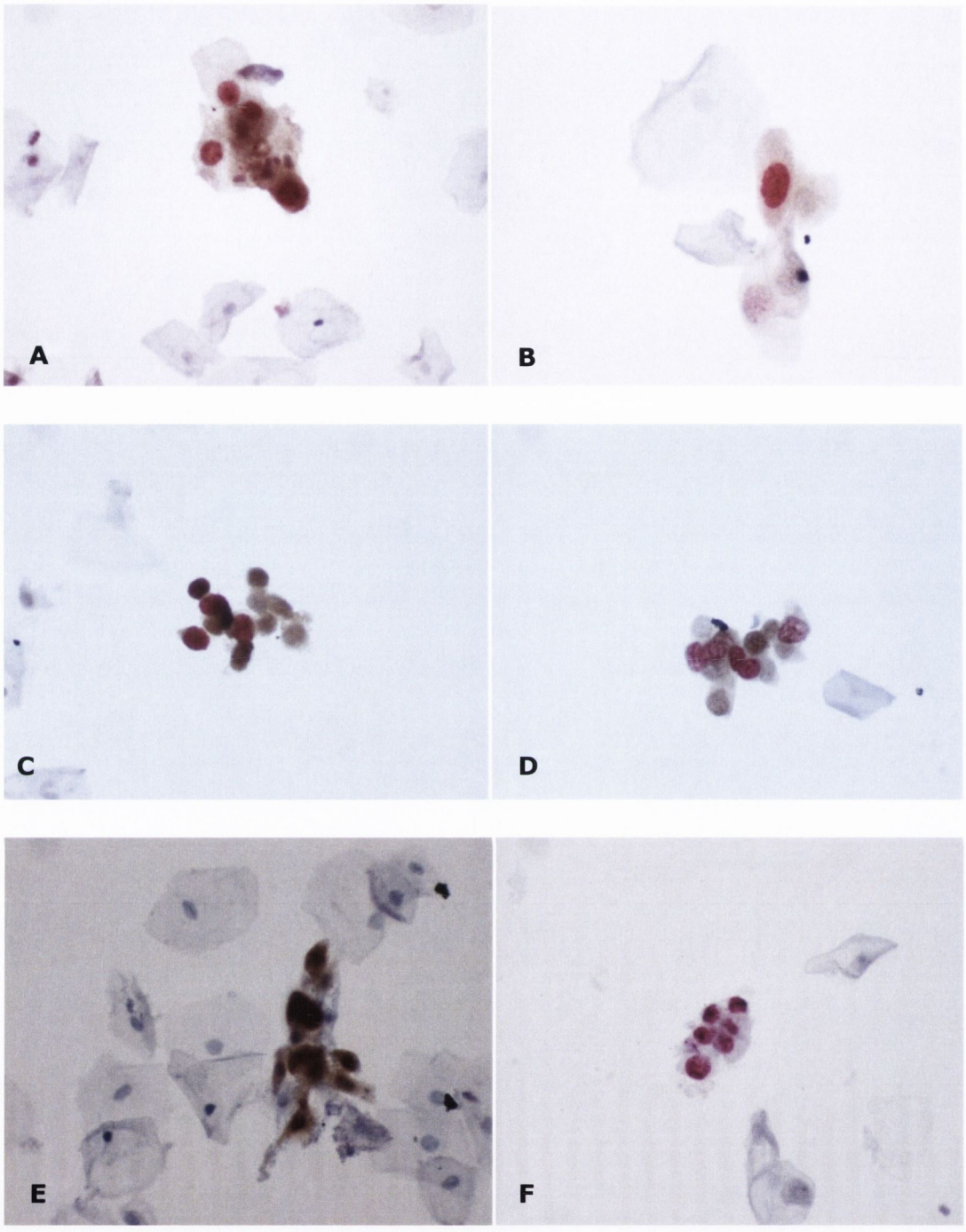


Figure 6-6 Positive p16[INK4A]/Ki-67 – LSIL and HSIL (40x)

Positive dual staining was seen in groups of cells A) and single cells B) as well as different levels of dyskaryosis LSIL A) and HSIL (C and D). E) Shows p16[INK4A] positive normal metaplastic cells. F) Ki-67 positive endocervical cells.

6.4.5 Comparison of p16^{INK4A}/Ki-67 expression with HPV status

Analysis of dual positively stained cervical specimens revealed that 34.6% (164/474) of the population were positive for co-expression of p16^{INK4A}/Ki-67. The histological diagnosis at baseline showed that overall 46.2% were normal on histology, 23.6% CIN 1, 19.4% CIN 2 and 6.6% CIN 3. 43 women had normal colposcopy without biopsy and were classified as Normal. There were 14 inadequate biopsy samples, 3 with uncertain grade of CIN and two showing AIS. The proportion of cases with dual positive staining increased with severity of histological diagnosis from 19.9% (35/176) Normal, 19.6% (22/112) CIN 1, 77.2% (71/92) CIN 2 and 87.5% (28/32) CIN 3 (figure 6.6). 9.3% (4/43) of women had normal colposcopy without biopsy showed positive staining. The percentage of p16^{INK4A}/Ki-67 positive subjects was significantly higher in women with CIN 2+ on histology compared to <CIN 2 (CIN 1 and Normal) (Pearson Chi-Square p=<0.001).

Of the 474 with valid p16^{INK4A}/Ki-67 results 447 had HPV DNA and mRNA results available. The results of p16^{INK4A}/Ki-67 dual stained cytology were compared to corresponding HPV DNA (hc2) and HPV mRNA (PreTect™ HPV Proofer) test results. p16^{INK4A}/Ki-67 was identified in 34.7% (155/447), HPV DNA was present in 64.0% (286/447) and HPV mRNA was present in 40.7% (182/447) of cases. Figure 6.7 compares dual positive specimens, HPV DNA and HPV mRNA results in relation to histological diagnosis within 6 months. For further analysis 11 samples with an inadequate biopsy result, 2 AIS and 3 uncertain grade were excluded.

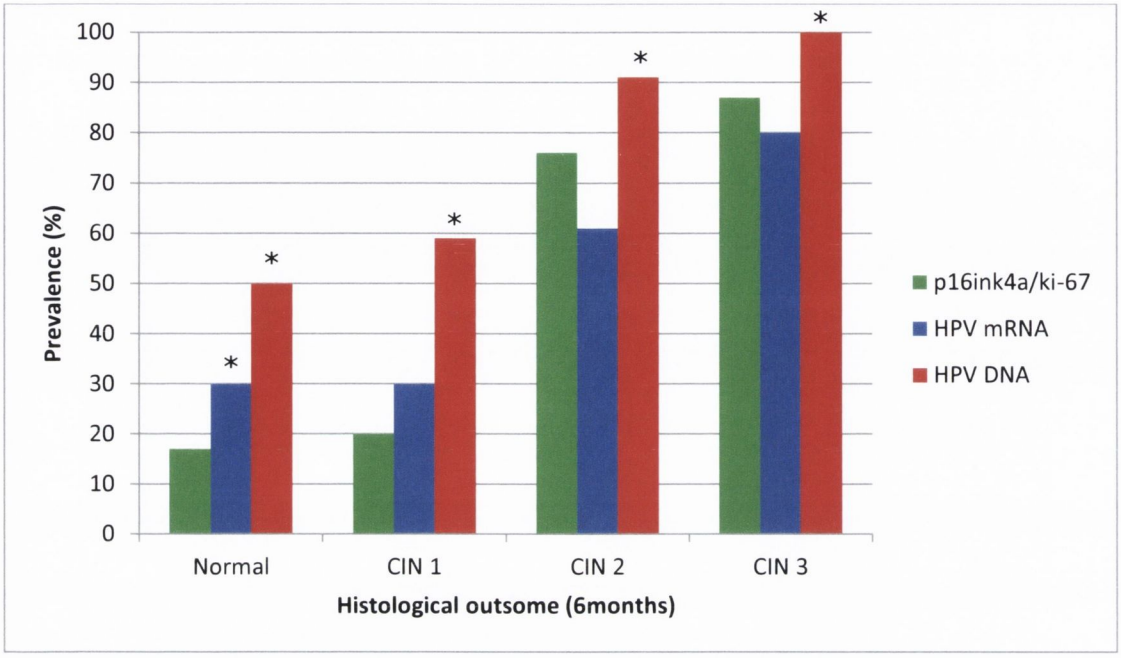


Figure 6-7 Frequency of positive test results from p16 [INK4A]/Ki-67, HPV DNA and HPV mRNA in relation to disease outcome at baseline (n=431).**

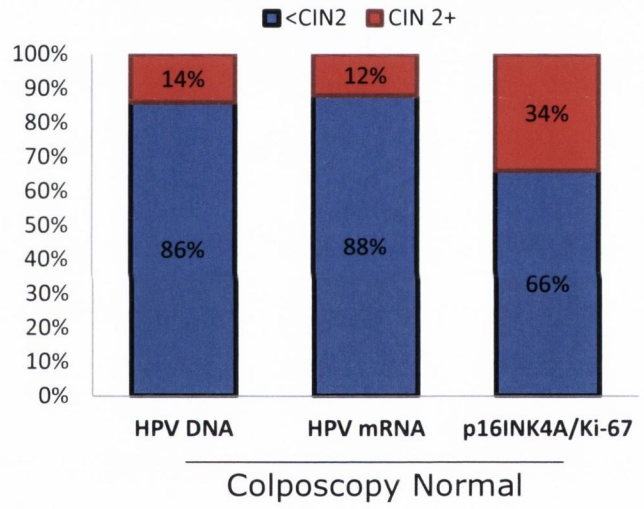
*Pearson Chi Square <0.05

**Not included: 11 samples with an inadequate biopsy result, 2 AIS and 3 uncertain grade.

Each of the three tests demonstrated increased positivity with increasing severity of lesion. p16[INK4A]/Ki-67 positive cases were less frequently positive in normal (17.3%) and CIN 1 (19.8%) cases compared to HPV mRNA which was positive in 29.8% and 30.2% and HPV DNA positive 51.9% and 58.5% for Normal and CIN 1 respectively. As shown in table 6.2, these differences were statistically significant for the prevalence of HPV mRNA in Normal cases and DNA in Normal and CIN 1 compared to p16[INK4A]/Ki-67 rates. Overall, HPV DNA positivity was higher across each grade of disease. A dramatic increase in p16[INK4A]/Ki-67 positivity was seen in CIN 2+ lesions compared to <CIN2 lesions. As shown in table 6.2 overall p16[INK4A]/Ki-67 dual staining was less frequently positive in <CIN 3 compared to both HPV DNA and HPV mRNA (McNemars p=<0.05).

6.4.5.1 Prevalence of HPV DNA, mRNA and p16INK4A/Ki-67 in colposcopy Normal cases

A total of 107 women had a normal colposcopic impression at first visit (time of recruitment). In this cohort the prevalence of HPV DNA was 46.7% (50/107), HPV mRNA 24.3% (26/107) and p16INK4A/Ki-67 14.0% (15/107). At 6 months the overall rate of CIN 2+ was 8.4% (9/107). Figure 6.8 shows the rate of CIN 2+ in test positive women, HPV DNA and mRNA had a similar rate of CIN 2+ at 14.0% (95% CI 10.7-17.3) and 11.5% (95% CI 7.6-15.5) respectively. The rate of CIN 2+ was significantly higher in who women tested positive for p16INK4A/Ki-67 at 33.3% (95% CI 22.1-44.6) (figure 6.8).



Test	% Overall
HPV DNA	46.7% (50/107)
HPV mRNA	24.3% (26/107)
p16INK4A/Ki-67	14.0% (15/107)

Figure 6.8 Rate of CIN 2+ in colposcopy normal cases with HPV DNA, HPV mRNA and p16INK4A/Ki-67 positive test results.

Table 6.2 p16^{INK4A}/Ki-67, HPV DNA, and HPV mRNA positivity in relation to histological grade (n=431)*

	Normal	CIN 1	CIN 2	CIN 3
p16^{INK4A}/Ki-67	17.3% (36/208)	19.8% (21/106)	75.6% (65/86)	87.1% (27/31)
HPV DNA	51.9% (108/208)	58.5% (62/106)	88.4 (76/86)	100% (31/31)
McNemar (P)	**<0.001**	**<0.001**	**0.006**	**0.134**
HPV mRNA	29.8% (62/208)	30.2% (32/106)	64.0% (55/86)	77.4% (24/31)
McNemar (P)	**<0.001**	**0.073**	**0.055**	**0.497**

*inadequate/uncertain/AIS not shown

The percentage of p16^{INK4A}/Ki-67 positivity in Normal cases was significantly lower than both HPV DNA and mRNA. p16^{INK4A}/Ki-67 positive rate did not differ significantly in all grades of CIN lesions compared with mRNA positivity. p16^{INK4A}/Ki-67 was significantly lower in CIN 1 and CIN 2 compared to HPV DNA. p16^{INK4A}/Ki-67 expression did not differ significantly from HPV DNA and mRNA in CIN 3 indicating the higher specificity of p16^{INK4A}/Ki-67 than HPV testing. HPV mRNA testing with the PreTect™ HPV Proofer detects and genotypes 5 HPV types 16, 18, 31, 33 and 45. Among those positive for HPV 16, 31, and 33 mRNA, 68%-72% demonstrated p16^{INK4A}/Ki-67 dual expression. Among those positive for HPV 18 mRNA, p16^{INK4A}/Ki-67 dual expression was identified in 48%. Samples positive for HPV 45 had a lower incidence of p16^{INK4A}/Ki-67 positivity at 32%.

6.4.6 Comparison of p16^{INK4A}/Ki-67 expression and HPV tests in relation to age

In chapter 5 it was shown that the overall prevalence of HPV was significantly higher in women under the age of 30 years. Positivity of p16^{INK4A}/Ki-67 was assessed in relation to age and referral smear. p16^{INK4A}/Ki-67 was co-expressed in a higher proportion, 41.6% (72/173), of women under the age of 30 compared to 29.8% (77/258) over the age of 30 (Chi Square p=0.012). This difference relates to the higher proportion of positive cases in <CIN 2 lesions in women under the age of 30 years (chi square p=<0.001). Table 6.3 illustrates p16^{INK4A}/Ki-67 positivity in relation to histological diagnosis stratified by age. Women under the age of 30 had a dual stain positivity rate of 41.6% (72/173) compared to 71.1% (123/173) positive for DNA and 47.4% (82/173) positive for mRNA.

Table 6.3 Dual expression of p16^{INK4A}/Ki-67, HPV DNA and HPV mRNA in relation to age (n=431)*

	Normal	CIN 1	CIN 2	CIN 3	Total
18-29 years					
p16^{INK4A}/Ki67	24.7%(20/81)	27.9%(12/43)	77.4%(24/31)	88.9%(16/18)	41.6%(72/173)
DNA	64.3%(52/81)	60.5%(26/43)	90.3%(28/31)	100%(18/18)	71.1%(123/17)
Mrna	37.0%(30/81)	32.6%(14/43)	77.4%(24/31)	77.8%(14/18)	48.6%(82/173)
30+					
p16^{INK4A}/Ki67	13.4%(17/127)	14.3%(9/63)	76.4%(42/55)	84.6%(11/13)	29.8%(77/258)
DNA	44.9%(57/127)	57.1%(36/63)	87.3%(48/55)	100% (13/13)	59.3%(153/258)
mRNA	25.2%(32/127)	28.6%(18/63)	56.4%(31/55)	76.9%(10/13)	35.3%(91/258)

*inadequate/uncertain/AIS not shown

6.4.7 Comparison of p16^{INK4A}/Ki-67 expression and HPV DNA/mRNA status stratified according to referral smear

Expression of p16^{INK4A}/Ki-67 was identified in a higher proportion of women referred with LSIL, 35.6% (88/247), compared to ASCUS, 33.2% (61/184) but not significantly (chi square p=0.593). Table 6.4 illustrates p16^{INK4A}/Ki-67 expression, HPV DNA and HPV mRNA positivity in relation to histological diagnosis stratified by referral smear. Dual staining demonstrated similar positivity rates for both LSIL and ASCUS across each disease outcome. HPV DNA and mRNA appeared to have a higher positivity rate in LSIL compared to ASCUS as shown in table 6.4.

Table 6.4 Expression p16^{INK4A}/Ki-67 stratified according to referral smear diagnosis, and overall disease outcome, defined in colposcopy (n=431)*

	Normal	CIN 1	CIN 2	CIN 3	total
LSIL					
p16^{INK4A}/Ki67	17.1%(20/117)	20.6% (13/63)	78.4% (40/51)	93.8% (15/16)	35.6% (88/247)
DNA	59.8%(70/117)	69.8% (44/63)	94.1% (48/51)	100.0% (16/16)	72.5%(179/247)
mRNA	31.6%(37/117)	33.3% (21/63)	66.7% (34/51)	62.5% (10/16)	41.3%(102/247)
ASCUS					
p16^{INK4A}/Ki67	17.6%(16/91)	18.6% (8/43)	74.2% (26/35)	80.0% (12/15)	33.2% (61/184)
DNA	42.9%(39/91)	41.9% (18/43)	80.0% (28/35)	100.0% (15/15)	55.4%(102/184)
mRNA	27.5%(25/91)	25.6% (11/43)	60.0% (21/35)	93.3% (14/15)	40.2% (74/184)

*inadequate/uncertain/AIS not included

6.4.8 Clinical Performance of p16^{INK4A}/Ki-67 for detection of CIN2+ compared to HPV DNA and HPV mRNA testing

As outlined previously, a follow up period of 18 months was chosen to assess clinical performance of all tests. However, given that the number of patients with p16^{INK4A}/Ki-67 and HPV testing represents a smaller population of 447 patients, an intermediate follow up period of 6 months was chosen for this part of the analysis, in order to avoid reducing numbers further. There were 43 cases with normal colposcopy and no biopsy within the 6 months, these were not included resulting in 404 available for analysis. Clinical performance characteristics were assessed by calculating the sensitivity, specificity, NPV and PPV for detection of CIN 2+ and CIN 3. The prevalence of CIN 2+ within a 6 month period was 30.2% (122/404), there was a small number of CIN 3 cases n=42 representing 8.9% of the population. The overall sensitivity and specificity of p16^{INK4A}/Ki-67 for detection of CIN 2+ was 79.8% and 82.3% respectively (Figure 6a). Sensitivity increased to 88.1% in detection of CIN 3 however, this was matched with a slight decrease in specificity to 70.6% (Figure 6b). The sensitivity and specificity for detection of CIN 2+ and CIN 3 was compared to that of HPV DNA and HPV mRNA testing on the same samples (figure 6.8a and b).

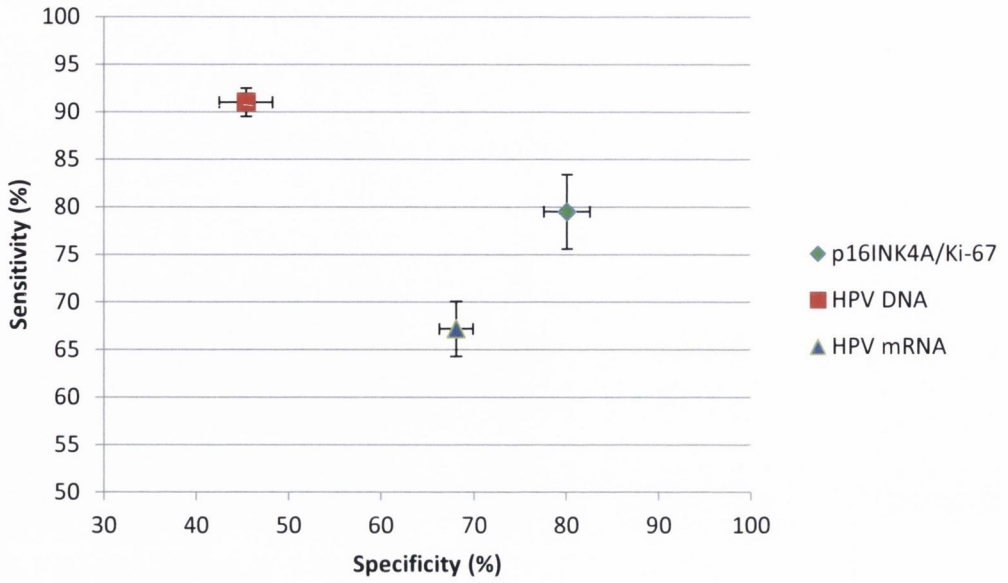


Figure 6-8a p16[INK4A]/Ki-67, HPV DNA and HPV mRNA sensitivity and specificity with 95% CI for detection of CIN 2+ (n=404)

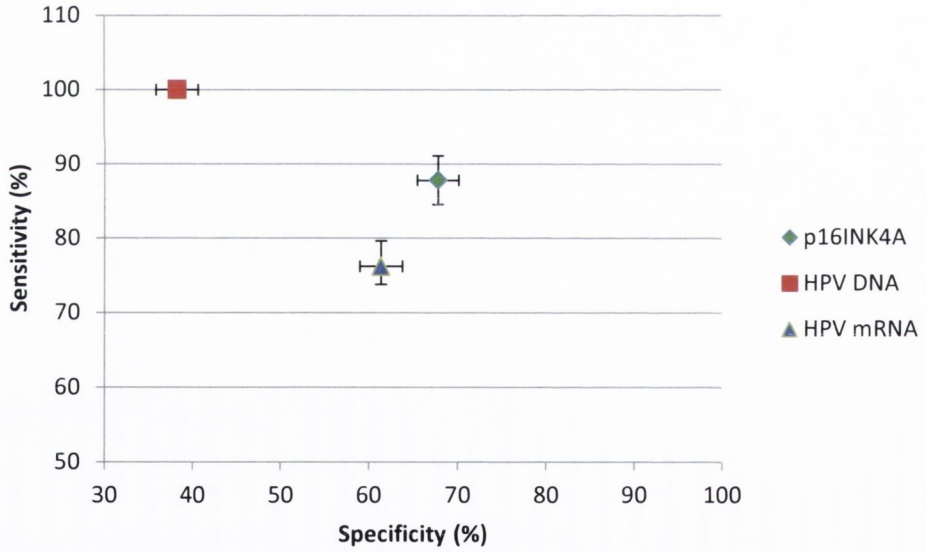


Figure 6-8b p16[INK4A]/Ki-67, HPV DNA and HPV mRNA sensitivity and specificy with 95% CI for detection of CIN 3 (n=404)

The sensitivity of p16^{INK4A}/Ki-67 for detection of CIN 2+ was comparable in both <30 and >30 years at 83.3% (79.6-87.0) and 77.3% (73.4-81.3) respectively. Specificity for detection of CIN 2+ was slightly higher in women over the age of 30 years (74.6% vs 87.1%). LSIL and ASCUS also showed comparable sensitivity, 82.7% (79.4-85.9) and 75.9% (71.1-80.8) respectively and specificity, 81.3% (79.2-83.5) and 83.6% (81.4-85.7) respectively. Across each category, HPV DNA had the highest sensitivity, p16^{INK4A}/Ki-67 and HPV mRNA had similar sensitivity in ASCUS and >30 years only. Otherwise p16^{INK4A}/Ki-67 demonstrated higher sensitivity for detection of CIN 2+. p16^{INK4A}/Ki-67 had the highest specificity and PPV for detection of CIN 2+ in all categories compared to HPV mRNA and HPV DNA, which demonstrated the lowest specificity. NPV remained above 90% for both p16^{INK4A}/Ki-67 and HPV DNA and above 83% for HPV mRNA (Table 6.5).

Table 6.5 Clinical performance of p16^{INK4A}/Ki-67, HPV DNA and HPV mRNA for detection of CIN 2+ stratified by age and referral smears

	Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
All (n=404)	p16 ^{INK4A}/Ki-67	79.8 (77.7-82.6)	82.3 (80.8-83.9)	62.8 (59.2-66.4)	91.6 (90.8-92.5)
	HPV DNA	91.5 (90.1-92.8)	46.7 (44.0-49.3)	39.1 (36.4-41.8)	93.6 (92.7-94.5)
	HPV mRNA	67.2 (63.3-71.1)	69.2 (66.9-71.6)	45.1 (41.5-48.7)	84.9 (83.4- 86.5)
<30 years (n=161)	p16 ^{INK4A}/Ki-67	83.3 (79.6-87.0)	74.8 (71.6-78.0)	57.0 (51.6-62.4)	91.8 (90.4-93.2)
	HPV DNA	94.4 (93.0-95.8)	36.3 (31.7-39.4)	37.2 (33.3-41.1)	94.2 (92.8-95.7)
	HPV mRNA	76.9 (72.1-81.8)	62.8 (58.8-66.8)	45.5 (40.3-50.6)	87.1 (84.8-89.4)
>30 years (n=243)	p16 ^{INK4A}/Ki-67	77.3 (73.4-81.3)	87.1 (85.6-88.7)	68.2 (63.6-72.8)	91.5 (90.4-92.6)
	HPV DNA	89.3 (87.2-91.5)	53.3 (50.0-56.7)	40.6 (36.9-44.3)	93.3 (92.2-94.5)
	HPV mRNA	59.2 (53.5-64.8)	73.5 (70.7-76.2)	44.7 (39.7-49.7)	83.2 (81.2-85.3)
LSIL (n=231)	p16 ^{INK4A}/Ki-67	82.7 (79.4-85.9)	81.3 (79.2-83.5)	63.3 (58.7-67.9)	92.4 (91.3-93.4)
	HPV DNA	94.7 (93.5-95.8)	35.8 (32.5-39.0)	36.4 (33.2-39.7)	94.5 (93.3-95.7)
	HPV mRNA	64.3 (58.9-69.6)	69.9 (67.0-72.8)	42.1 (37.4-46.7)	85.2 (83.3-87.1)
ASCUS (n=173)	p16 ^{INK4A}/Ki-67	75.9 (71.1-80.8)	83.6 (81.4-85.7)	62.1 (56.4-67.8)	90.7 (89.3-92.1)
	HPV DNA	87.0 (84.0-90.1)	60.5 (56.7-64.3)	43.9 (39.3-48.6)	92.9 (91.6-94.2)
	HPV mRNA	71.2 (65.6-76.7)	73.6 (70.4-76.8)	50.0 (44.3-55.7)	87.3 (85.3-89.3)

6.5 Discussion

The aim of this chapter was to evaluate the potential utility of p16^{INK4A}/Ki-67 co-expression for detecting high grade neoplastic lesions in exfoliated cells from samples taken from women referred to colposcopy with LSIL and ASCUS. There is a large body of evidence indicating the utility of individually detecting p16^{INK4A} and Ki-67, however, using a combined approach has now been suggested as a morphology independent marker of cell cycle deregulation and thus a potential triage test for identifying CIN 2+.

In this chapter the p16^{INK4A}/Ki-67 combined test was evaluated in a subgroup of the overall population recruited to investigate triage options for LSIL and ASCUS. A follow period of 6 months was chosen for this analysis as opposed to 18 months used in chapter 5. This was due to the smaller sample number in this population, restricting to 18 months would have reduced numbers further. To maintain meaningful numbers of CIN 2+ for sensitivity analysis, 6 months was used as most women had at least 6 month follow up to date. In chapter 3, section 3.4.6, it was stated that the majority of CIN 2+ was identified at first visit, a total of 94.2% of CIN 2+ cases was identified within 6 months. It is noted that the remaining 5.8% detected after 6 months may result in a minimal effect on specificity.

The data demonstrates co-expression of p16^{INK4A}/Ki-67 in 34.6% of women referred to colposcopy with LSIL and ASCUS cytology. Unlike HPV testing there was no significant difference in the proportion of women with p16^{INK4A}/Ki-67 co-expression between LSIL and ASCUS referred patients (p=0. 593). In this study population 22.5% of women had a normal colposcopic impression at first visit. Within 6 months, CIN 2+ was detected in 8.4% of colposcopy normal women. A positive p16^{INK4A}/Ki-67 result conferred the highest risk of CIN 2+ (34%) in colposcopy normal women, twice that of HPV DNA (14%) and mRNA (12%). Similarly, a high risk of CIN 2+ in p16^{INK4A} positive women has been described by Carozzi *et al* where an initial p16^{INK4A} positive result was significantly associated with subsequent occurrence of CIN 2+ (relative risk 3.74; 95% CI 2.57-5.43) (Carozzi et al., 2012). This represents a small population of women, based on these figures long term follow up studies in colposcopy normal women are warranted in order to determine optimal management of colposcopy normal p16^{INK4A}/Ki-67 positive women.

The p16^{INK4A}/Ki-67 test demonstrated comparable sensitivity of 82.7% (79.4-85.9) and 75.9% (71.1-80.8) and specificity of 81.3% (79.2-83.5) and 83.6% (81.4-85.7) for LSIL and ASCUS respectively. However, women under the age of 30 years had a significantly higher positivity rate of 43.6% compared to those over 30 years (30.6%), resulting in a higher specificity for detection of CIN 2+ in women >30 years (Table 6.5). Regardless, specificity remained higher than that demonstrated by HPV DNA and mRNA across all categories. p16^{INK4A}/Ki-67 testing appeared superior to HPV mRNA testing for the detection of high grade lesions with overall enhanced sensitivity (79.8% vs 67.2%) and specificity (82.3% vs 69.2%). Whereas HPV DNA testing outperformed p16^{INK4A}/Ki-67 with respect to overall sensitivity (79.8% vs 91.5%) but had a lower specificity (82.3% vs 46.7%) for detection of CIN 2+.

A small number of studies have investigated the utility of p16^{INK4A}/Ki-67 dual staining on retrospective study populations (Schmidt et al., 2011, Edgerton et al., 2011, Loghavi et al., 2012). Schmidt *et al* reported sensitivity for detection of CIN 2+ of 92.2% (83.8-97.1) and 94.2% (88.8-97.4) for ASCUS and LSIL respectively and a specificity of 80.6% (75.6-85.1) and 68.0% (62.2-73.4) in 776 women aged 18 and older (Schmidt et al., 2011). A retrospective study by Loghavi *et al* reported on SurePath re-stained specimens, they also reported a high sensitivity of 91% and 100% and a specificity of 61% and 43% for ASCUS and LSIL respectively (Loghavi et al., 2012).

A recent study by Wentzensen *et al* evaluated the use of p16^{INK4A}/Ki-67 in a population of 404 LSIL and ASCUS cases without follow up. The reported overall sensitivity for detection of CIN 2+ in LSIL and ASCUS was 85.5% (77.8-90.9) which is comparable with findings reported here, 79.8% (77.7-82.6) but specificity of 59.4% (53.3-65.1) was lower in comparison to that presented here, 82.3% (80.8-83.9) and what was reported by Schmidt *et al* (Wentzensen et al., 2012). There was a higher positivity rate of p16^{INK4A}/Ki-67 staining in the Wentzensen study 54.5% compared to 35.1% in our study (33.2% in ASCUS and 35.6% in LSIL, Schmidt *et al* reported 34.9% in ASCUS and 52.5% LSIL) matched with a similar proportion of CIN 2+ 30.7% compared to our study 27.2% and Schmidt study 27.6%. The higher proportion of test positive results compared to presence of disease would generate a lower specificity. The authors comment on the lower specificity obtained may be due to the use of extended biopsies allowing them to

"*distinguish true positives from false positives results better than studies where disease is missed because of insensitive colposcopic protocols*" (Wentzensen et al., 2012). In addition p16^{INK4A}/Ki-67 was only reviewed by a trained cytotechnologist without additional pathologist review unlike our study and others (Schmidt et al., 2011).

Previous studies on dual staining have reported p16^{INK4A}/Ki-67 has a sensitivity comparable to HPV DNA testing for detection of CIN 2+ in triage (Schmidt et al., 2011, Wentzensen et al., 2012). These studies have used a process of adjudicated histology diagnosis, including p16^{INK4A} staining (Schmidt et al., 2011) and extended biopsies with up to 4 targeted biopsies (Wentzensen et al., 2012). One small study on 63 samples utilised individual pathologist interpretation and reported findings similar to that reported from this study (Edgerton et al., 2011). Many studies employ a process of adjudicated pathology review of cervical biopsy samples, in some cases p16^{INK4A} has been employed to elucidate difficult cases. In this study no such methods were employed, slides were reviewed by standard clinical methods.

There are a number of advantages associated with the use of p16^{INK4A}/Ki-67 as a biomarker for HPV related cancers. p16^{INK4A}/Ki-67 expression is directly linked to transforming HPV infections due to the activity of the E7 oncogene which is necessary for maintaining malignancy. In addition, the expression of p16^{INK4A}/Ki-67 appears to be independent of the HPV type, obviating the need to detect different HPV types in DNA and mRNA assays and an important property in future screening of a vaccinated population. The combined detection of p16^{INK4A} with Ki-67 eliminates the subjective nature of morphology interpretation and has potential to improve the interobserver variability that exists with cytology particularly in relation to ASCUS (Stoler and Schiffman, 2001). Therefore, the use of dual staining has potential to play a role in stratifying patients to help identify those at true risk. In a meta-analysis by Roelens *et al* it was found that p16^{INK4A} staining in LSIL was limited, while it was more specific, sensitivity was lower than HPV DNA testing (Roelens et al., 2012). The authors could not report on p16^{INK4A}/Ki-67 dual staining at the time there was only one study reporting on dual staining and hc2.

The main concern with HPV DNA triage is the fact that it cannot discriminate between transient and transforming infections resulting in a low specificity.

This results in risk of over referral to colposcopy. The overall positivity rate of HPV DNA was 64.0%, indicating that if this test was used, referral to colposcopy would have been reduced by 36%. In contrast, dual stained p16^{INK4A}/Ki-67 positive in only 34.1%, indicating that colposcopy referral would have been reduced by a substantially larger proportion of over 60% if p16^{INK4A}/Ki-67 was used as a triage test. p16^{INK4A}/Ki-67 had significantly lower positivity rate ($p=<0.05$) in normal, CIN 1 and CIN 2 compared to HPV testing, however it was similar in CIN 3. It is believed that CIN 3 is in fact the true precursor to cervical cancer, as only a proportion of CIN 2 will progress (Castle et al., 2009). The absence of dual p16^{INK4A}/Ki-67 staining in some CIN 2 cases may represent the heterogeneity of these lesions and is perhaps detecting the subgroup likely to progress.

As with all tests p16^{INK4A}/Ki-67 is not without its limitations. The manufacture recommends that consideration must be taken for the presence of dyskaryotic cells without positive dual staining. Hematoxylin is used as a counter stain which has been previously shown to differ from pap staining demonstrating a differences in chromatin appearance most likely due to differences in bluing procedures (Meyer et al., 2007, Edgerton et al., 2011). While it is maintained that the use of a dual stain is morphology independent, there is some level of cell morphology required in order to exclude squamous metaplasia, tubal metaplasia and endocervical cells. A high proportion of positive stained endocervical cells were identified in this study, in particular positive staining by Ki-67. This was demonstrated in figure 6.6 E and F. Thus, a high level of training may still be required for interpretation of p16^{INK4A}/Ki-67 immunostained slides. In addition, inflammation and presence of polymorphs frequently demonstrated Ki-67 positivity, resulting in dense red chromogen reactivity under microscopic field. Consequently, in specimens showing high number of endocervical cells and inflammation, interpretation was more laborious. According to the manufactures instructions, the test is considered positive when one or more cells stains dual positive, it has been suggested that increasing the threshold may offer a better overall trade-off between sensitivity and specificity (Wentzensen et al., 2012).

Despite these issues the p16^{INK4A}/Ki-67 stain performs better than p16^{INK4A} immunostaining alone. Schmidt *et al* compared dual staining with previous p16^{INK4A} only staining (by cytotechnologist review) on identical cases from a

study by Denton *et al* 2010. Sensitivity was identical for dual stain and p16^{INK4A} alone in ASCUS (92.2%) and similar in LSIL 94.2% and 92.0% respectively. However, specificity rates were substantially improved with dual staining, 80.6% and 63.4% for dual stain and p16^{INK4A} alone respectively in ASCUS and 68.0% and 47.1% for dual stain and p16^{INK4A} alone respectively in LSIL (Schmidt et al., 2011). The enhanced specificity rates from a morphological independent method compared to morphology dependent method highlights the issues previously described for using p16^{INK4A} alone. p16^{INK4A} can be found in non-neoplastic cells, while scoring systems can prevent misclassifying positive cases, these are morphology based and subject to individual interpretation. An objective marker that is morphology independent has potential as a more accurate test. In addition, time spend screening a slide based on colourmentric positive/negative result would be shorter than screening when morphological interpretation required. Turnover time could be further improved by applying image based automated screening. A study by Gertych *et al* introduced a novel method for identifying immunoreactive cells in high resolution slide scanner by classifying pixel signatures within the nuclei (Gertych et al., 2012).

In conclusion, the findings from this work demonstrates that dual staining with p16^{INK4A} and Ki-67 offers an improvement on the specificity above HPV DNA testing alone in detection of high grade pre-cancer. Sensitivity however may be an issue, in the next chapter a possible combination of tests is suggested in order to achieve optimal sensitivity and specificity for detection of high grade pre-cancer in women with cytological report of LSIL and ASCUS. The aim is to identify as closely as possible the subset of women harbouring CIN 2+ in those with LSIL and ASCUS cytology.

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-Chapter 7-

Discussion

7 Discussion

Cervical cancer screening has advanced in recent years with the introduction of HPV DNA testing as an adjunct to cervical cytology. However, debate remains over the best management of minor cervical abnormalities. HPV DNA is highly prevalent, particularly in younger women, and for this reason specificity is often an issue in the context of cervical screening. Current efforts to overcome the shortcomings of HPV DNA based assays include the use of biomarkers that specifically distinguish between transient and transforming HPV infections. There is a clear need for an alternative approach for the management of women with minor cytology to stratify those at risk of high grade pre-cancer from those at little or no risk. This will allow resources to be directed towards following those women at high risk, while women with little or no risk can be followed by more conservative, less costly methods. This thesis has assessed the utility of two specific biomarkers, namely HPV E6/E7 mRNA and $p16^{INK4A}$/Ki-67 combined expression for this purpose.

A significant aspect of the work presented in this thesis was focussed on clinical characterisation and epidemiological assessment of the study population (Chapter 3). In summary, the study population was characterised as 59% LSIL referral population, and 41% ASCUS referral, with an overall median age of 31 years. Interestingly, there was a higher prevalence of smokers (38%) in the study population, as compared to the normal Irish female population (22%, according to the office of tobacco control 2010 (www.otc.ie)). Smoking greater than 10 cigarettes per day appeared to contribute to risk of detecting CIN 2+ (OR 1.58; 95% CI 1.00-2.51). The overall rate of subsequent detection of CIN 2+ following minor abnormalities vary widely in the literature, between 2-42% for LSIL (Bolger and Lewis 1988, Cooper, Kirby et al. 1992, Melnikow, Nuovo et al. 1998, Cox, Schiffman et al. 2003, Rana, Marshall et al. 2004, Giannopoulos, Butler-Manuel et al. 2005, Ahmed, Goumalatsos et al. 2008) and 2-30% for ASCUS (Davis, Hernandez et al. 1987, al-Nafussi, Rebello et al. 2000, Edwards, Howat et al. 2002, Emerson, Puzanov et al. 2002, Shanbhag, Roberts et al. 2003, Rana, Marshall et al. 2004). Over an 18 month follow up period the cumulative rate of CIN 2+ was 34% in women referred with LSIL and 26% in women referred with ASCUS. This is slightly high but within the ranges previously described. It should be noted that enrolment for this study coincided with the roll out of the

national screening program, which has likely contributed to a detection of a higher number of underlying high grade lesions in women who were previously unscreened. The majority of CIN 2+ cases (87.0%) were identified at first visit to colposcopy, highlighting the low sensitivity of cytology, which is reported in region of 53% (Cuzick et al., 2006), a figure which varies widely ranging 30% to 87% (Nobbenhuis et al., 2001). Overall, observations noted from chapter 3 indicated that of a population of women referred to colposcopy with LSIL/ASCUS, up to two thirds did not have detectable high grade disease after 18 months follow up, emphasizing the need for improved triage of minor cytology.

Cervical screening and prevention has evolved over the last decade. This is a result of the identification of persistent HR HPV infection as the main etiological agent in development if cervical cancer (Munoz, Bosch et al. 1992, Ho, Burk et al. 1995, Walboomers, Jacobs et al. 1999). Today, it is increasingly and widely recognised that HPV testing can deliver effective cervical screening and aid in prevention of cervical cancer compared to pap cytology which has a modest sensitivity in the region of 30% to 87% and specificity of approximately 86% to 100% (Nobbenhuis, et al., 2001). For this reason a number of HPV DNA based detection methods have populated the market and are now integrated into cervical screening protocols. In chapter 4, HPV DNA based technologies were discussed. The cobas® 4800 HPV (Roche) platform was evaluated against the gold standard hc2 (Qiagen). Findings concluded that the cobas® 4800 HPV test had a high level of agreement with hc2. The clinical performance was in line with what has been previously reported by Lapierre *et al.* Sensitivity and specificity was slightly lower than reported by Stoler *et al.* As discussed in chapter 4 this may have been attributed to the younger age of population based screening in the United States which starts at 21 years compared to 25 years in Ireland, and the higher prevalence of disease in our study population (44% v's 5%). The cobas® 4800 HPV test individually detects genotypes HPV 16 and 18, findings indicated HPV 16 detected by the cobas® 4800 HPV test produce a higher risk of CIN 2+, in agreement with findings from Stoler *et al.* However, HPV 18 did not appear to induce an increased risk to CIN 2+, likely due to the very low prevalence of this genotype in the population and it association with harder to detect lesions of the endocervical canal (de Sanjose, Quint et al. 2010). Overall, findings conclude that the cobas® 4800 HPV test had comparable

sensitivity to hc2 but was slightly more specific for detection of CIN 2+ in women referred with LSIL.

It is widely recognised HPV DNA based tests have suboptimal specificity. Risk stratification is an important element of a screening program. The choice of tests used will determine the level of stratification achieved. The overall aim of this project was to examine three testing methods and identify an approach to best manage women with minor abnormalities on cytology. Based on the findings from chapter 5, HPV DNA was shown to be a sensitive test and HPV mRNA was a specific test for detection of CIN 2+. Findings from chapter 6 indicated $p16^{INK4A}$/Ki-67 demonstrated the highest specificity. However, sensitivity did not match that of HPV DNA based testing. This trade-off between sensitivity and specificity with respect to HPV testing is not uncommon. The clinical performance of a test is important. Clinical sensitivity identifies the proportion of women with disease (CIN 2+) who are correctly identified by a positive test. A lack of sensitivity can result in cases of disease being missed by the test. Clinical specificity refers to the proportion of women with no disease (defined as <CIN2 in this thesis) who are correctly identified by a negative test result. A lack of specificity results in the identification of false positive results that have to be worked up despite being disease free. The PPV and NPV are both related to the test performance and the disease prevalence. A low PPV will be seen where disease prevalence is low. It indicates the proportion of individuals with a positive result that really have the disease. The NPV indicates how well a negative result indicated that no disease is present. Each of these can give an indication to the performance of a test; however, they can be affected by certain factors. For example, high sensitivity can be associated with a high prevalence of, in this case HPV infection, and can result in a compromised specificity. Predictive values tend to depend on disease prevalence in a population. Therefore, in order to assess the utility of HPV tests in a clinical context, the data was applied to the NHS HPV DNA triage model.

The NHS in England, recently implemented HPV DNA testing using hc2 for the triage of LSIL and ASCUS. The triage protocol is shown below in figure 7.1. The remainder of this chapter will focus on applying the HPV DNA, mRNA and $p16^{INK4A}$/Ki-67 data generated throughout this thesis into the NHS HPV Triage

model to examine the impact each test has in the context of triage of minor lesions.

The principles of the NHS HPV Triage protocol are as follows:

1) Both borderline (ASCUS) and mild dyskaryosis (LSIL) should be included.

2) The same protocol applies to the entire screening age range 25-60 years.

3) HR HPV negative women are returned to routine recall.

4) A positive HPV test results in referral to colposcopy.

5) A normal colposcopy impression/biopsy merits return to routine screening.

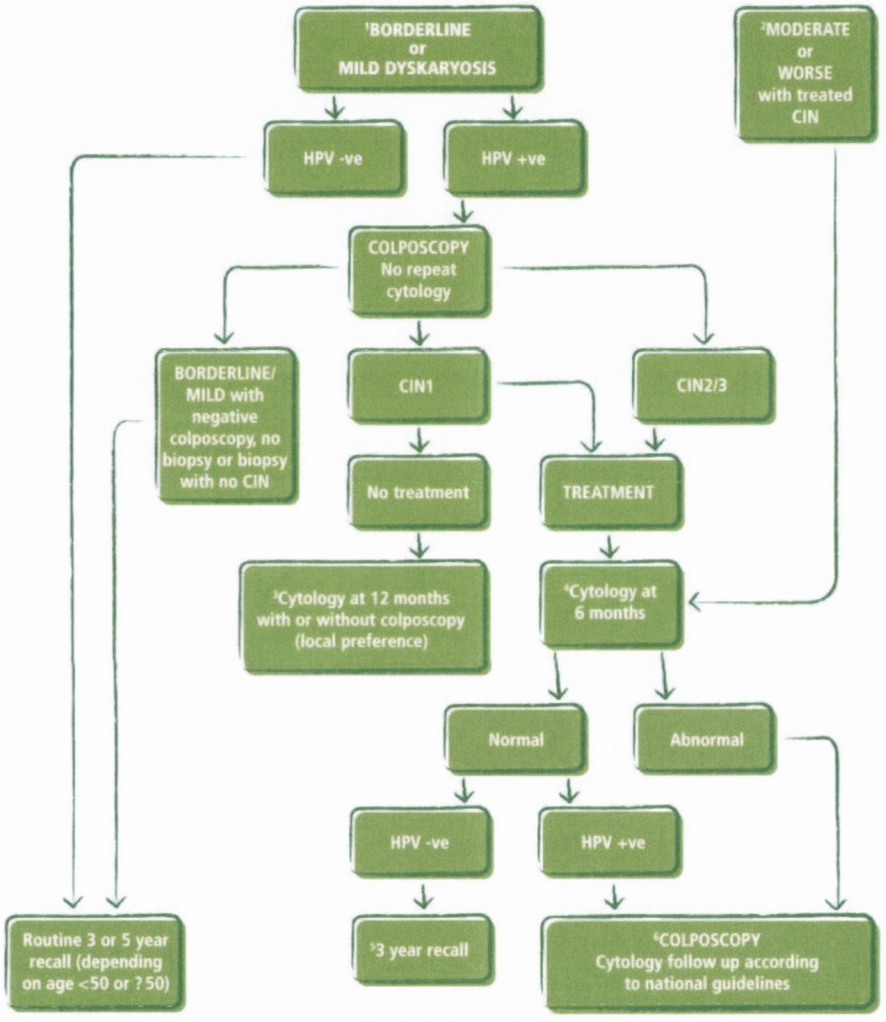
6) An abnormal colposcopy is followed up by standard procedures.

Previous studies have highlighted the limited use of HPV DNA in LSIL and women under 30 years, this is due to the high prevalence of HPV infection in these groups (Arbyn et al., 2006, Ronco et al., 2007). Both the NHS pilot and the sentinel site studies indicated there was an increase in referrals to colposcopy following HPV DNA triage, consequently management strategies have been developed in order to minimize who stays in colposcopy for on-going work up. This is achieved by discharging women who are negative for CIN at first visit.

NHS
Cancer Screening Programmes

HPV Triage and Test of Cure Protocol

For women aged 25 to 64 years



1 If sample is unreliable/inadequate for the HPV test, refer mild and recall borderline for 6 month repeat cytology. At repeat cytology HPV test if Neg/Bord/Mild. If HPV negative return to routine recall, refer if HPV positive. Refer moderate or worse cytology.

2 Untreated CIN1 should be managed as per untreated CIN1 following borderline/mild.

3 Follow up of 12 month cytology only should follow normal NHSCSP protocols.

4 Women in annual follow up after treatment for CIN are eligible for the HPV test of cure at their next screening test.

5 Women ≥50 who have normal cytology at 3 years will then return to 5 yearly routine recall.

6 Women referred due to borderline/mild cytology or normal cytology/HPV positive, who then have a satisfactory and negative colposcopy can be recalled in three years.

N.B Women who reach 65 still require to complete the protocol and otherwise comply with national guidance.

HPV Triage and Test of Cure Protocol April 2010

Figure 7-1 NHS protocol for HPV triage of low grade cytology LSIL and ASCUS

It is important to note that this population differs slightly from the pilot/sentinel site studies as it is based on women with repeat (2+) minor abnormal smears as opposed to first presentation of a minor abnormal cytology. In this instance the data applied to the model represents women with valid results from all three methods under investigation, HPV DNA, HPV mRNA and $p16^{INK4A}$/Ki-67. Detection of CIN 2+ is based on a 6 month follow up period which was chosen for reasons described in chapter 6, largely due to the fact that most women with valid test result had at least 6 months follow up to date. This was supported by the fact that, as stated in section 3.10, almost all (94.0%) cases of CIN 2+ were identified within 6 months.

Applying the HPV DNA data generated in this study (chapter 5) to the NHS HPV triage model, clearly shows the utility of HPV DNA testing and illustrates its benefits to triage women into colposcopy, in particular for HPV positive women (Figure 7.2a) where 65% of women with a positive HPV test had confirmed high grade CIN and in those HPV negative women, 92% were normal on colposcopy (Figure 7.2b).

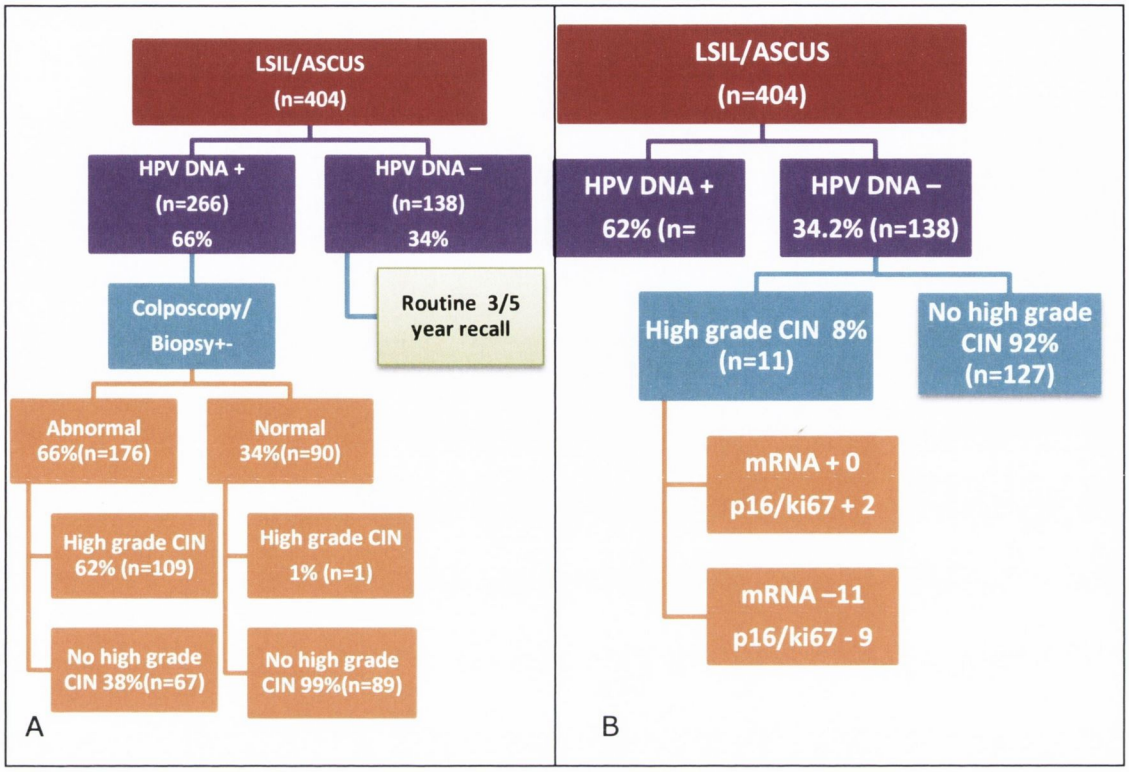


Figure 7-2 Use of HPV DNA test for triage of LSIL/ASCUS

Figure 7.2A focuses on women who had a HPV DNA positive result following repeat minor cytology. As illustrated, 66% of the population were positive for HPV, this would have resulted in 266 out of 404 referrals to colposcopy. At colposcopy, 66% HPV positive women were identified as abnormal at the time of the HPV test. Within a 6 month follow up period, high grade disease was identified in 62%, with no high grade disease detected in the balance. Under the NHS guidelines, the 34% that were normal at first visit to colposcopy would be returned to routine recall at 3/5 years. According to this model 90 out of 266 women would have been referred back to routine screening avoiding on going surveillance at colposcopy. Within 0-6 months 1 of these women had a high grade lesion detected.

Figure 7.1B focuses on women having a HPV DNA negative result following repeat minor cytology. It indicates that 34.2% of women presenting with minor cytological abnormalities were negative for HPV DNA. If a HPV DNA test was performed 138 out of 404 women could have avoided colposcopy reducing referrals by 34.2%. Amongst HPV DNA negative women 92% had no detectable CIN 2+ after 6 months. The remaining 8%, representing 11 women had a high grade lesion detected all of which were CIN 2, 10 of which were detected at the same visit the HPV DNA test performed.

The most recent data available from NHS on the use of this algorithm is from the sentinel site studies. The sentinel site studies have no data relating to women who tested HPV negative as they were returned to routine screening. However, the 2001 HPV triage/LBC pilot studies reported a 1% prevalence of CIN 2+ in HPV negative women and a 96% NPV (Moss et al., 2006). It is evident that HPV DNA testing in our study population would have resulted in a delayed diagnosis of a small number of high grade cases. We report a NPV of 87.6% for HPV DNA testing (chapter 5) and a sensitivity of 85.8%, which is considerably lower than that reported by others. In 2002 data from ALTS reported a NPV of 98.9% and 96.9% for ASCUS and LSIL respectively (Sherman et al., 2002). Similarly, a meta-analysis by Arbyn *et al* reported NPV of 99.0% and 98.2% for ASCUS and LSIL (Arbyn et al., 2006, Arbyn et al., 2010). On the other hand, in 2010, TOMBOLA reported a NPV of 94.5 (92.6-96.0) and 80.5% (75.2-85.1) in ASCUS and LSIL. TOMBOLA also reported that 22% of CIN 2+ cases were negative for HPV (Cotton et al., 2010). A considerably higher value than what is reported in this thesis (8%). A commentary by Arbyn *et al* highlighted differences in the study design of TOMBOLA, which relied on community based histology outcomes (as did this study). Whereas, in many other studies including ALTS, stringent and intense quality control resulted in the reassessment of all histological outcomes by an independent panel (Arbyn et al., 2010). The implications of this will be discussed further on. In brief, this may explain divergences in reported findings of this study and TOMBOLA compared to others.

Results from the sentinel sites studies indicated there was a similar prevalence of HPV DNA in the population of LSIL and ASCUS referral, 64% in the English sites compared to 66% in our study population. However, the prevalence of disease was lower in the sentinel site study where findings indicated 16% of HPV positive women had CIN 2+ compared to a total of 41% of HPV positive women in our population. The consequence of a high prevalence rate of HPV infection among these LSIL/ASCUS referred women with low incidence of high grade disease, as evident from the diagram above, is a substantial proportion of women would be referred to colposcopy unnecessarily. The pilot studies and sentinel site studies reported an increase in colposcopy referral using HPV DNA triage compared to the traditional policy of repeat cytology. However, this was justified by an increase in the amount of high grade CIN detected due to the higher sensitivity of hc2 compared to cytology (Moss et al., 2006, Kelly et al.,

2011). The disadvantage of high colposcopy referrals is the associated high costs of colposcopy in addition to the high levels of anxiety often experienced by women attending colposcopy. The psychological impact for a women attending colposcopy can be quite extensive, studies have reported women express fears of having cancer, future fertility and concerns over relationships and sex (Sharp, Cotton et al. 2011). All of which can be prevented in some women by avoiding unnecessarily referring those who are not at true risk to colposcopy.

The over referral of women to colposcopy is an important shortcoming of HPV DNA testing. Approaches have been suggested to overcome this including increasing the positivity threshold of HPV tests (Arbyn, de Sanjose et al. 2012). For hc2, while the manufacturer's recommend a cut of 1.0 RLU, some studies have reported using a higher cut off (Sargent et al., 2010, Kelly et al., 2011) based on the rationale that slightly higher positivity threshold for hc2 could reduce cross reactivity with non-carcinogenic HPV types and eliminate low viral load infections (Gravitt et al., 2008). Increasing to an RLU of 3.0 has been reported to maintain sensitivity and NPV but increase specificity for detection of CIN 2+ compared with RLU 1.0 (Guyot et al., 2003). Viral load itself has been suggested as a marker viral persistence and risk of CIN (Santos et al., 2003, Origoni et al., 2012). However, studies have reported no correlation between viral load and grade of disease (Keegan et al., 2009) or improved survival in cervical cancer in patients with higher viral load (Biedermann et al., 2004). Our knowledge of HPV infection, in particular productive infections, is that minor lesions characteristically tend to have higher viral load. Whereas in transforming integrated infections, loss of episomal virus, as seen in high grade lesions, leads to lower viral load (Doorbar 2006).

In chapter 5 utility of HPV DNA testing and HPV mRNA testing was compared, findings concluded that HPV DNA testing was more sensitive, whereas mRNA testing was more specific. Women were at a higher risk to CIN 2+ who tested positive for HPV mRNA (Figure 7.3A) compared to women who tested negative for HPV mRNA (Figure 7.3B). Applying the HPV mRNA data generated in this study (chapter 5) to the NHS HPV triage model clearly shows that mRNA testing has the capacity to further reduce the referrals to colposcopy compared to HPV DNA testing (Figure 7.3). There was a reduction of 57%

referrals to colposcopy. However with this came a risk of delaying diagnosis in a small number of women with detectable CIN 2+, n=40, representing 17% of HPV mRNA negative women.

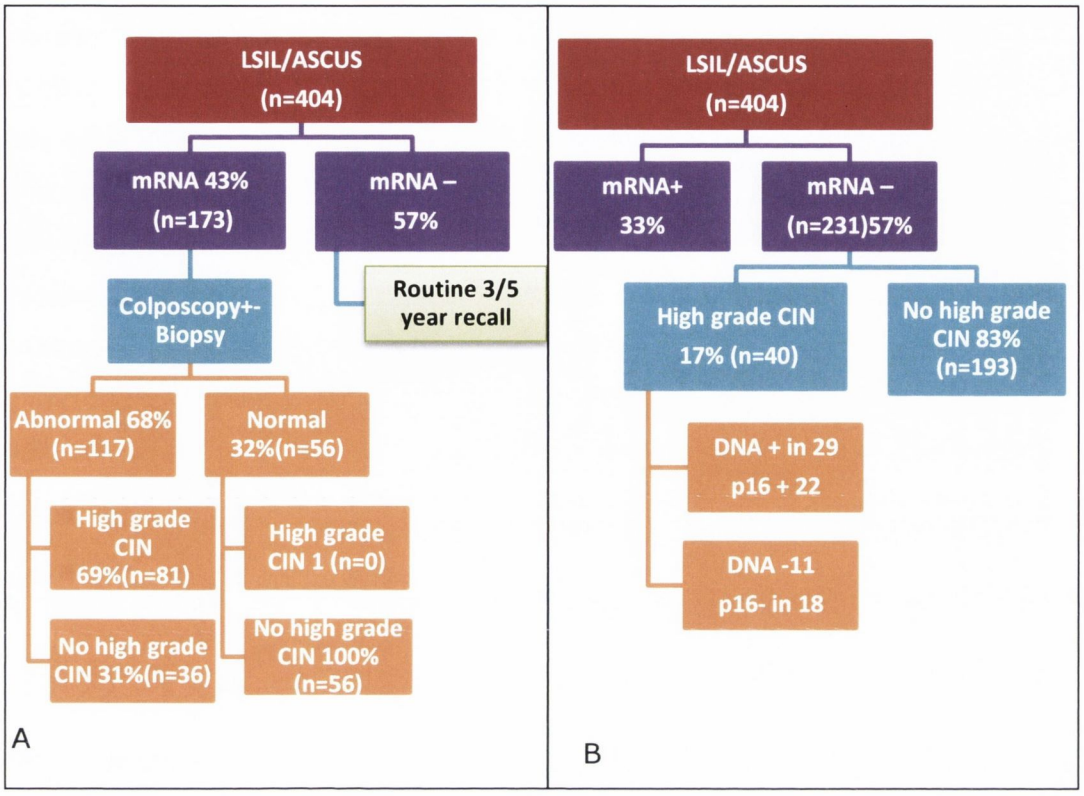


Figure 7-3 Use of HPV mRNA test for triage LSIL/ASCUS

7.3A outlines the events that happened for women who tested positive for HPV mRNA. 43% of women had a positive HPV mRNA test result. At colposcopy, 68% of HPV mRNA positive women had an abnormal colposcopy, this proportion is identical to HPV DNA positive women. However, 69% had high grade CIN 2+ detected compared to 62% of women with HPV DNA positive and abnormal colposcopy. There was no high grade disease detected in women who were normal on first visit to colposcopy and would have safely returned to routine recall. Figure 7.3B outlines the women who had a HPV mRNA negative result. As shown 53% of the population were negative for HPV mRNA, a higher proportion in comparison to HPV DNA testing. No high grade disease was detected over 6 months in 83% of women with a negative mRNA test. However, 17% of women who were negative had a high grade lesion detected. As shown, a HPV DNA test would have picked up an additional 29/40 of these women.

This problem relating to HPV mRNA testing using the PreTect™ HPV Proofer test missing some cases of CIN 2+ is not unfamiliar. In chapter 5, it was discussed that various other studies reported similar findings, and suggested that HPV mRNA negative women should not be returned to routine screening but followed up in one year (Benevolo et al., 2011, Rijkaart et al., 2012). Rijkaart *et al* reported 55 women with ASCUS and LSIL, 29 (53%) were HPV mRNA negative at baseline 13 out of 29 (46%) had a 3 year clinical outcome of CIN 2+. Benevolo *et al* described similar findings on subset of women with LSIL/ASCUS, their findings showed the HPV mRNA test missed approximately one third of CIN 2+ cases. The authors also reported on 187 HPV positive women using hc2 with CIN 2+, 59 were negative for HPV mRNA. Genotyping was performed on 24/59 that were mRNA negative CIN 2+ and found that 10/24 were positive for genotypes other than the five genotypes (16, 18, 31, 33 and 45) detected by the PreTect™ HPV Proofer. Confirming suggestions that detection of CIN 2+ may be improved by increasing the number of genotypes detected by an assay. As discussed in chapter 5, alternative methods such as the ATPIMA HPV assay which detects HPV E6/E7 mRNA from 14 HR HPV types may be an option. A number of groups have proposed $p16^{INK4A}$ immunocytochemistry as a marker of CIN 2+ (Klaes et al., 2001, Wentzensen et al., 2005, Denton et al., 2010). Evidence suggests that $p16^{INK4A}$ correlates with the severity of disease. Data from the Predictors 2 study indicated that $p16^{INK4A}$ staining had a lower sensitivity compared to HPV DNA based methods and the APTIMA HPV test but higher specificity for detection of CIN 2+ (Szarewski et al., 2012). There is wide variation in the literature with respect to use of $p16^{INK4A}$ immunocytochemistry. The limited reproducibility is in part due to the fact that it is morphology dependent and interpretation is poorly standardised (Tsoumpou et al., 2009). More recently a new protocol was designed to simultaneously detect $p16^{INK4A}$ and Ki-67. The benefit of this is that it is morphology and HPV genotype independent based on the premise that co-expression indicates HPV mediated proliferation and deregulation of the cell cycle. Dual expression of $p16^{INK4A}$/Ki-67 has been described as a highly sensitive and specific marker of CIN 2+ in LSIL and ASCUS (Schmidt et al., 2011) as well as triage of HPV positive, pap negative women (Petry 2011). The final test $p16^{INK4A}$/Ki-67 was applied to the NHS Triage model and is shown in figure 7.3A/B

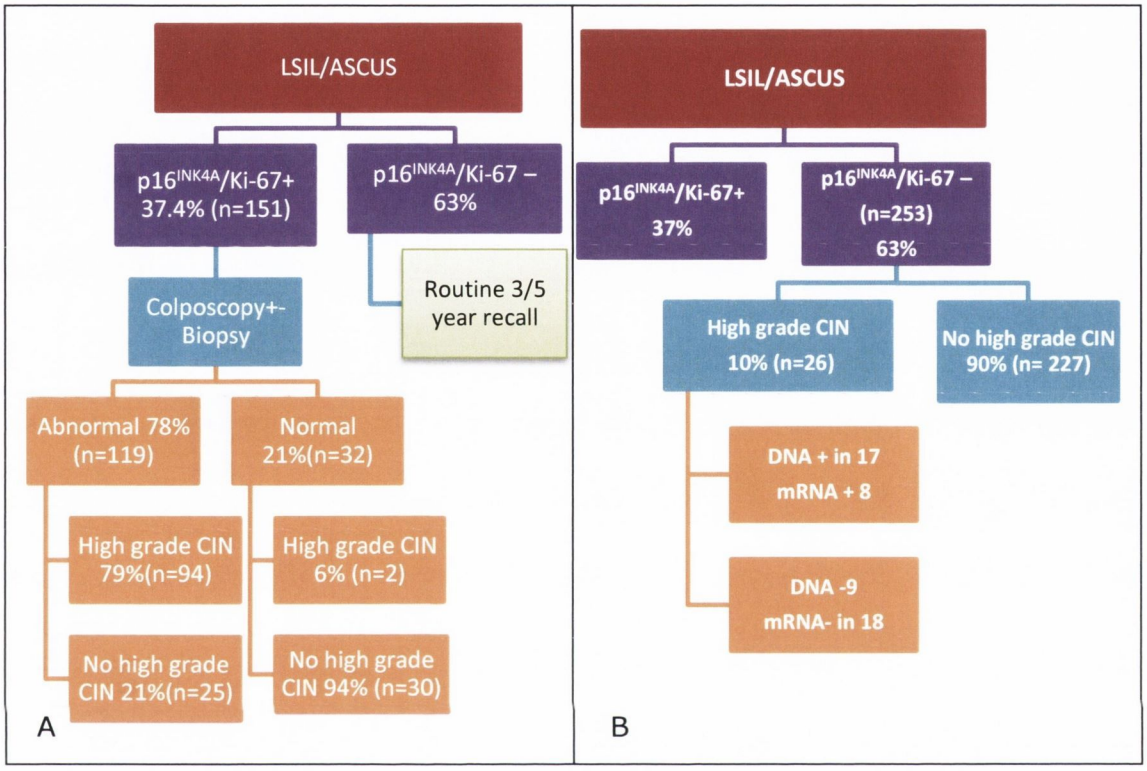


Figure 7-4 Use of p16^{INK4A}/Ki-67 test for triage of LSIL/ASCUS

p16^{INK4A}/Ki-67 had the highest proportion of negative test results when compared with HPV DNA and mRNA tests. 63% of women were negative for the dual stain. After 6 months 90% of p16^{INK4A}/Ki-67 women had no high grade disease detected resulting in 227 women who could have potentially avoided colposcopy. There were a small number of p16^{INK4A}/Ki-67 negative women with high grade CIN, 26 representing 10% of negative tests. A HPV DNA test would have identified 65% (17/26). There were 151 women representing 32% showing positive staining for p16^{INK4A}/Ki-67. At colposcopy a high proportion of these, 78% were abnormal compared to 62% and 68% for HPV DNA and mRNA. Based on histological diagnosis 79% of abnormal colposcopy had high grade CIN detected. 21% had no high grade CIN detected representing 25 women compared to 36 women for HPV mRNA and 67 for HPV DNA. This highlights the high specificity of p16^{INK4A}/Ki-67 staining. A small proportion, 21% (32 women) were normal at colposcopy, 2 of these women had subsequent a high grade lesion detected.

Overall, applying this data to the NHS model suggests that a negative p16^{INK4A}/Ki-67 result would have resulted in 63% of colposcopy referrals being avoided; this represents the highest proportion of referrals when compared to HPV DNA and HPV mRNA. There was a small proportion of women with a negative result that had detectable CIN 2+. The rate of CIN 2+ in p16^{INK4A}/Ki-67 negative cases varies in the literature; studies are limited and based on different study populations. Petry *et al* reported on a population of cytology normal/HPV positive women. The prevalence of CIN 2+ in p16^{INK4A}/Ki-67 women was low at 0.9% (Petry et al., 2011). A study by Schmidt *et al* was based on a population of LSIL and ASCUS smears, based on figures taken from this publication the rate of CIN 2+ in p16 ^{INK4A}/Ki-67 negative women was 3.2% (Schmidt et al., 2011). Wentzensen *et al* reported findings similar to those reported here. This study was based on a colposcopy referral population, based on the figures from Wentzensen *et al* the rate of CIN 2+ was 14% in p16^{INK4A}/Ki-67 negative cases. The major difference in our study and Wentzensen et al 2011 with the two other studies is the use of secondary pathologist review and p16^{INK4A} staining to confirm high grade disease, CIN 2+ on histology (an issue described earlier with respect to HPV DNA testing). The use of p16^{INK4A} staining compared to conventional H&E is discussed further on.

Table 7.1 summarises the findings from negative test results from applying the data to the NHS model. It highlights that number of women who could have potentially avoided colposcopy had a HPV DNA, HPV mRNA or p16^{INK4A}/Ki-67 test been employed prior to colposcopy referral. It also shows the number of cases where a high grade lesion was missed due to a negative test result. p16^{INK4A}/Ki-67 was the most successful test for reducing referrals to colposcopy, with, 253/404 women potentially avoiding colposcopy. While HPV mRNA testing would also have resulted in a high number of women avoiding colposcopy, HPV mRNA testing also represented the highest number cases where there would potentially be a delay in diagnosis of high grade CIN. On the other hand, with HPV DNA testing a substantially lower number of cases would have avoided colposcopy however the upside of this is that less cases of CIN 2+ were missed indicating this is the safest test.

Table 7.1 Outcome of a negative test result

	HPV DNA- (n=138)	HPV mRNA- (n=233)	p16^{INK4A}/Ki-67- (n=253)
Safely avoided colposcopy	31.4% (127/404)	47.8% (193/404)	56.2% (227/404)
High grade disease missed	8.0% (11/138)	17.2% (40/233)	10.2% (26/253)

A higher proportion of women attending colposcopy with a positive HPV DNA test were normal on colposcopy and therefore would have been returned to routine screening compared to the more specific tests whereby a high proportion of women referred were those with clinically relevant lesions. It is evident that there are a number of women positive for HPV DNA referred to colposcopy with no detectable high grade lesions. This was also the case in the sentinel sites study which reports a lower proportion of CIN 2+ (14%) in HPV positive women (Kelly et al., 2011). This is an important consideration with respect to cost effectiveness and the use of resources. A high proportion of women attending colposcopy who are in fact normal put increased pressure on colposcopy clinics. Of all three tests, p16^{INK4A}/Ki-67 detected the highest proportion of CIN 2+ (table 7.2).

Table 7.2 Outcome positive test result

	HPV DNA (n=266)	HPV mRNA (n=173)	p16^{INK4A}/Ki-67 (n=151)
Abnormal colp/biopsy	66.2% (176/266)	67.6% (117/173)	78.8% (119/151)
CIN2+	61.9% (109/176)	69.2% (81/117)	79.0% (94/119)

Despite this it is evident that using a single test alone, for example, HPV mRNA or $p16^{INK4A}$/Ki-67 may still result in a delayed diagnosis of some high grade lesions. The findings from chapter 6 figure 6.7 demonstrated that for the detection of CIN 2+ HPV DNA was the most sensitive test. Adding an ancillary biomarker test to the HPV DNA tests could improve specificity without a loss in sensitivity. Thus, test combinations may help stratify risk more accurately. Figures 7.4, 7.5 and 7.6 show the outcomes of combined testing. The data presented is based on 404 women with valid test result for all three tests and a corresponding histological follow up over 6 months. For illustrative purposes the data presented indicates total proportion of CIN 2+ for each particular test combination, and does not account for women discharged based on normal colposcopy/biopsy at first presentation to colposcopy as described in the NHS models.

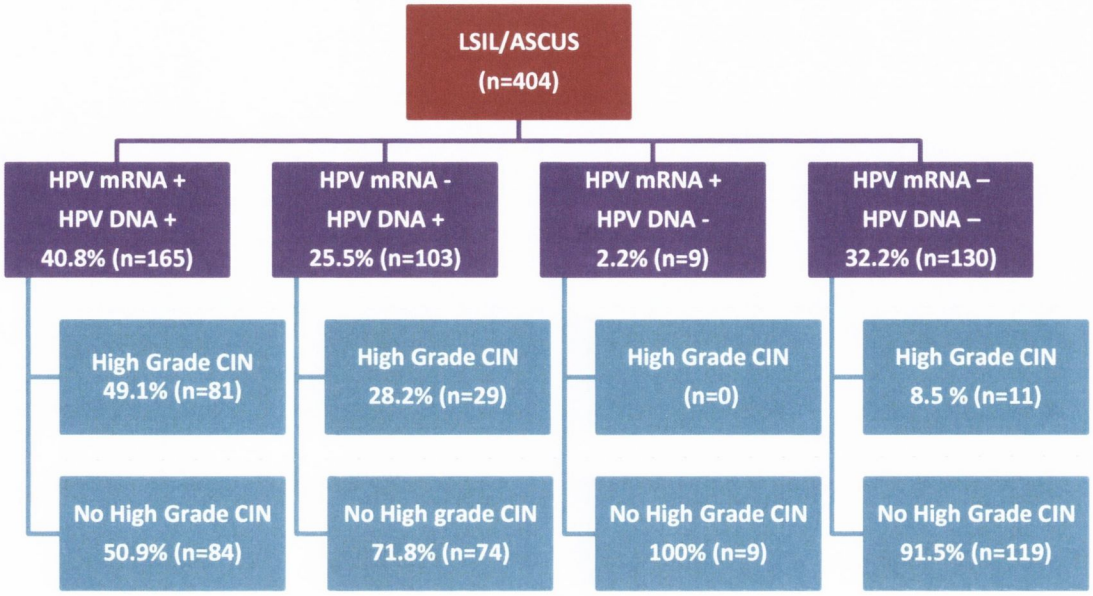


Figure 7-5 Proposed model using combined HPV DNA and HPV mRNA testing

Combining HPV DNA testing with mRNA testing, the referral to colposcopy was similar compared to HPV DNA testing alone. A double negative test did not offer any enhanced protection to women against CIN 2+. A small number of women (n=9) were DNA negative mRNA positive, none of these women had CIN 2+ detected in 6 months and most likely represent false negatives 7/9 of these women were over the age of 30 years. HPV 45 was present in 4/9, HPV 45 mRNA was reported as having no association with CIN 2+ in chapter 5. Combining HPV DNA and mRNA did not offer any overall benefit to triage of LSIL and ASCUS compared to DNA testing alone.

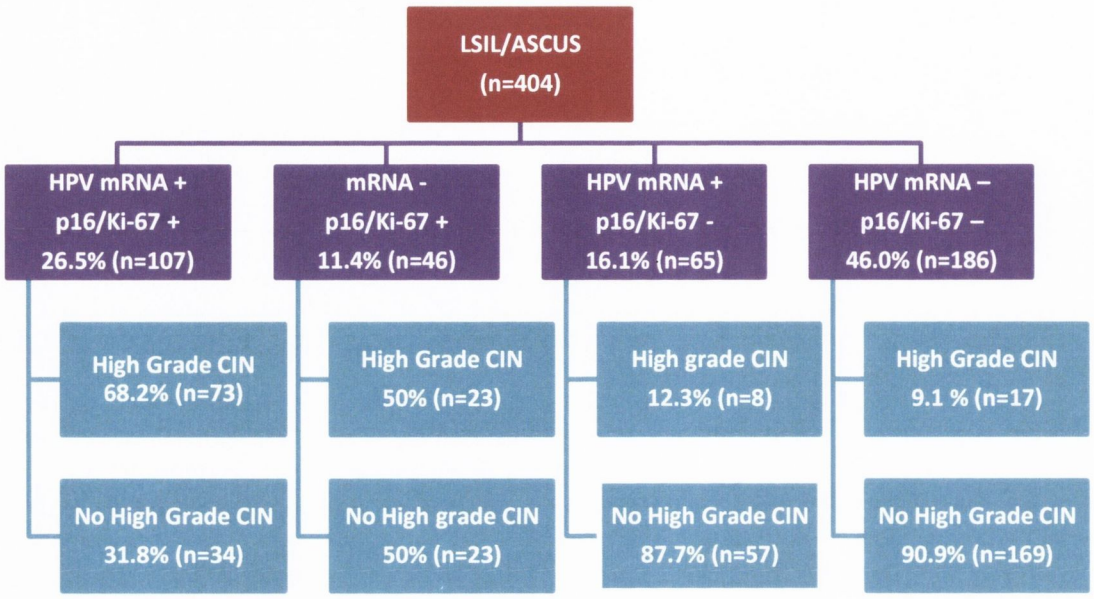


Figure 7-6 Proposed model using combined p16^{INK4A}/Ki-67 and mRNA testing

Combining p16^{INK4A}/Ki-67 with HPV mRNA reduced the number of women being referred to colposcopy to 37.9% (153/404) compared to DNA based methods. The proportion of women with high grade lesions now attending colposcopy was lower than p16^{INK4A}/Ki-67 testing alone (62.7% v's 79%). An mRNA positive test in p16^{INK4A}/Ki-67 negative cased identified 8 out to the 25 women initially with CIN2+. However there was still a small proportion with a double negative result that at risk of CIN2+ (9.1%). Combined p16^{INK4A}/Ki-67 HPV mRNA test to triage did not appear to offer any overall benefit compared to p16^{INK4A} testing alone.

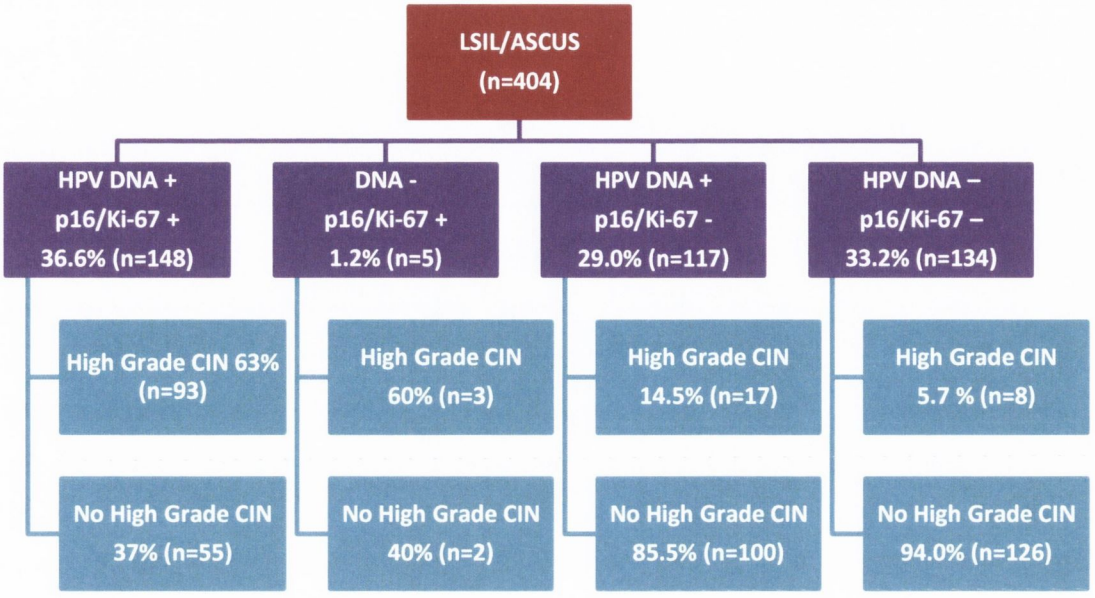


Figure 7-7 Proposed model using combined HPV DNA and $p16^{INK4A}$/Ki-67 testing

Using this model, colposcopy referral is reduced by almost two thirds, similar to $p16^{INK4A}$/Ki-67 testing alone. Combining HPV DNA testing to $p16^{INK4A}$/Ki-67 identified 17 out the 25 CIN 2+ cases missed by $p16^{INK4A}$/Ki-67 testing alone. There remained a small proportion to double negative women with detectable CIN 2+, 5.7%.

It is noteworthy to mention that the 8 HPV DNA -/p16^{INK4A}/Ki-67 – cases were CIN 2, which we know only a subset represent true high grade pre-cancer. 6 out the 8 cases were diagnosed by punch at first visit, 4 out of 8 had a subsequent CIN 1 diagnosed on LLETZ within 3 months of initial CIN 2 diagnosis. A strong correlation between cervical biopsy specimens and loop specimens has been found (Barker et al., 2001). However, a number of studies have previously demonstrated that punch biopsy can over-estimate the degree of dysplasia present (Costa et al., 2003, Skehan et al., 1990). There are several suggestions for subsequent diagnosis of a low grade disease category following a punch biopsy. In the case of smaller CIN 2+ lesions, entire lesion may have been removed by biopsy (Chappatte et al., 1991). In fact, CIN grade 2 or 3 lesions, detected after ASCUS cytology have been shown to be smaller than those detected after HSIL referral (Pinto et al., 2002). Inflammation and wound healing response, induced by biopsy, may aid in clearance of HPV, leading to regression of CIN (Barker et al., 2001). There is also the chance of spontaneous regression of CIN 2 lesions, it could be the case that test negative CIN 2 cases represented those that were likely to regress.

The definition of clinical endpoint is critical in defining test performance. The decision to use CIN 2+ as clinical endpoint in this study is justified by the fact that currently lesions diagnosed as CIN 2+ are considered high grade and subject to excisional treatment. As mentioned previously, in contrast with some other studies, CIN was diagnosed by a pathologist in daily routine practice rather than a reviewed diagnosis. These attributes allowed test performance to be evaluated in a routine population-based setting. However, there are certain limitations associated with this, CIN 2 lesions classified by conventional H&E histopathology have poor reproducibility among pathologists, demonstrating very low kappa statistics, and are often considered equivocal results (Stoler and Schiffman 2001). As a result H&E diagnosis alone has been shown to underestimate sensitivity of screening tests (Zhang et al., 2007).

It is more likely that CIN 2 represents a mixture of low grade CIN 1 and true pre-cancer CIN 3 lesions. Up to 50% of CIN 2 are self-limited and will regress spontaneously (Melnikow et al 1998). However, the remaining are often associated with transforming HPV infections and show features of CIN 3

lesions. Thus, the risk of invasive cancer remains an intermediate risk between CIN 1 and CIN 3. Improved accuracy for distinguishing biopsies with and without true high grade CIN can be achieved by highlighting those that contain transforming HPV infections, indicated by surrogate biomarkers such as $p16^{INK4A}$ (Zhang et al., 2007, Galgano et al., 2010).

The recommendation of a 2-tiered system to harmonise with the biology of HPV associated lesions has been reported (Darragh et al., 2012). In order to put this into practice, additional methods, ie biomarkers such as $p16^{INK4A}$, which will provide a more precise classification, need to be accurately employed. The LAST study made recommendations on the use of $p16^{INK4A}$ IHC for defining HPV associated lesions of the lower anogenital tract and help reduce interobserver variability associated with traditional H&E diagnosis. The study made a number of recommendations regarding use of $p16^{INK4A}$ IHC:

1. Use of $p16^{INK4A}$ when H&E is between CIN2/3 and mimic pre-cancer, for example metaplasia, atrophy, reparative epithelial etc.
2. $p16^{INK4A}$ is recommended to clarify H&E diagnosis of CIN 2, a biologically equivocal lesion representing HPV infection/low grade lesion and pre-cancer.

Strong and diffuse block positive $p16^{INK4A}$ results support a categorization of pre-cancer. Negative or non-block positive staining strongly favours an interpretation of low grade disease or a non-HPV associated pathology.

3. $p16^{INK4A}$ is recommended as an adjudication tool where there is disagreement on histological interpretation between pathologists.
4. Recommends against the use of $p16^{INK4A}$ IHC as a routine adjunct to histological assessment of biopsy specimens with morphologic interpretations of negative, CIN 1, and CIN 3.

    4a. Recommended in high risk cases, such as those with cytology specimens reporting HSIL, ASC-H, ASC-US positive for HPV 16 or AGC.

Any identified $p16^{INK4A}$-positive area must meet H&E morphologic criteria for a high grade lesion to be reinterpreted as such.

In order to fully elucidate the true status of test negative CIN 2 cases in this study, $p16^{INK4A}$ immunohistochemistry could be performed on the biopsy specimens. A positive immune-reactive stain would indicate sampling error with respect cervical smear which HPV tests and immunocytochemistry was performed. Alternatively, a negative immune-reactive biopsy would suggest these were false positives or cases of CIN 2 which were likely to regress. In total there were 68 cases of CIN 2 reported in this study, the use of $p16^{INK4A}$ IHC on these cases would possibly reclassify the original diagnosis, potentially improving the NPV and sensitivity of the molecular tests used. A number of studies report on CIN 3 as clinical endpoint, within this population there were no HPV DNA $p16^{INK4A}$/Ki-67 double negative CIN 3 cases. Indicating all double negative women would be safely returned to routine screening.

One issue encountered in the present study that deserves further attention is the time of collection of cervical smears. Cellularity was noticeably low, this was particularly evident when microscopically evaluating samples. Smears were taken immediately before colposcopy this may have led to more cautious brushing of the cervix to prevent bleeding and therefore lower abnormal cell counts in scrapings and LBC samples. LBC and immunocytochemistry was performed on a subset of samples, a number of these were found to be insufficient smears. Other studies (Overmeer et al., 2011) have reported have similar experience suggesting this may lead to an underestimation of the sensitivity of tests performed.

7.1 Conclusion

Cervical screening has evolved and will continue to improve with biomarker assays that specifically highlight transforming HPV infections. In this project, HPV mRNA and $p16^{INK4A}$/Ki-67 yielded high specificity for detection of CIN 2+ while sensitivity was reduced compared to HPV DNA testing. This led to the investigation of a combined test approach. For the first time, we have shown the addition $p16^{INK4A}$/Ki-67 as an ancillary test to HPV DNA identified one third

of women requiring immediate colposcopy and a further one third at no risk of high grade disease who could potentially avoid colposcopy. Although adding new tests may lead to additional costs, it is conceivable that the reduction in referrals to colposcopy will substantially reduce overall costs in cervical screening programs. This is of particular importance in the Irish screening population where we are currently experiencing an epidemic of minor cytological abnormalities LSIL and ASCUS. As a result, high rates of minor abnormalities are seen at colposcopy, a large proportion of these will not lead to a diagnosis CIN 2+ yet still remain under extensive follow up. Efforts are been made to manage this by extending screening intervals and introducing HPV DNA triage of minor abnormal smears. However, due to the nature of HPV infections HPV DNA testing can still lead to over referral to colposcopy. It is important that we expand on HPV based tests with additional methods more specific methods that will highlight those at true risk of high grade CIN. Based on the findings of this project, I propose co-testing women with minor abnormal smears LSIL and ASCUS using both HPV DNA and $p16^{INK4A}$/Ki-67. Introducing further stratification of minor abnormalities should reduce unnecessary work up and treatment, benefiting women taking part in screening by reducing the psychosocial effects endured by women having repeat abnormal smears in addition to reducing cost associated with colposcopy visits.

Based on the findings from this work and what is presented in the literature a proposed cervical screening algorithm is outlined in figure 7.10.

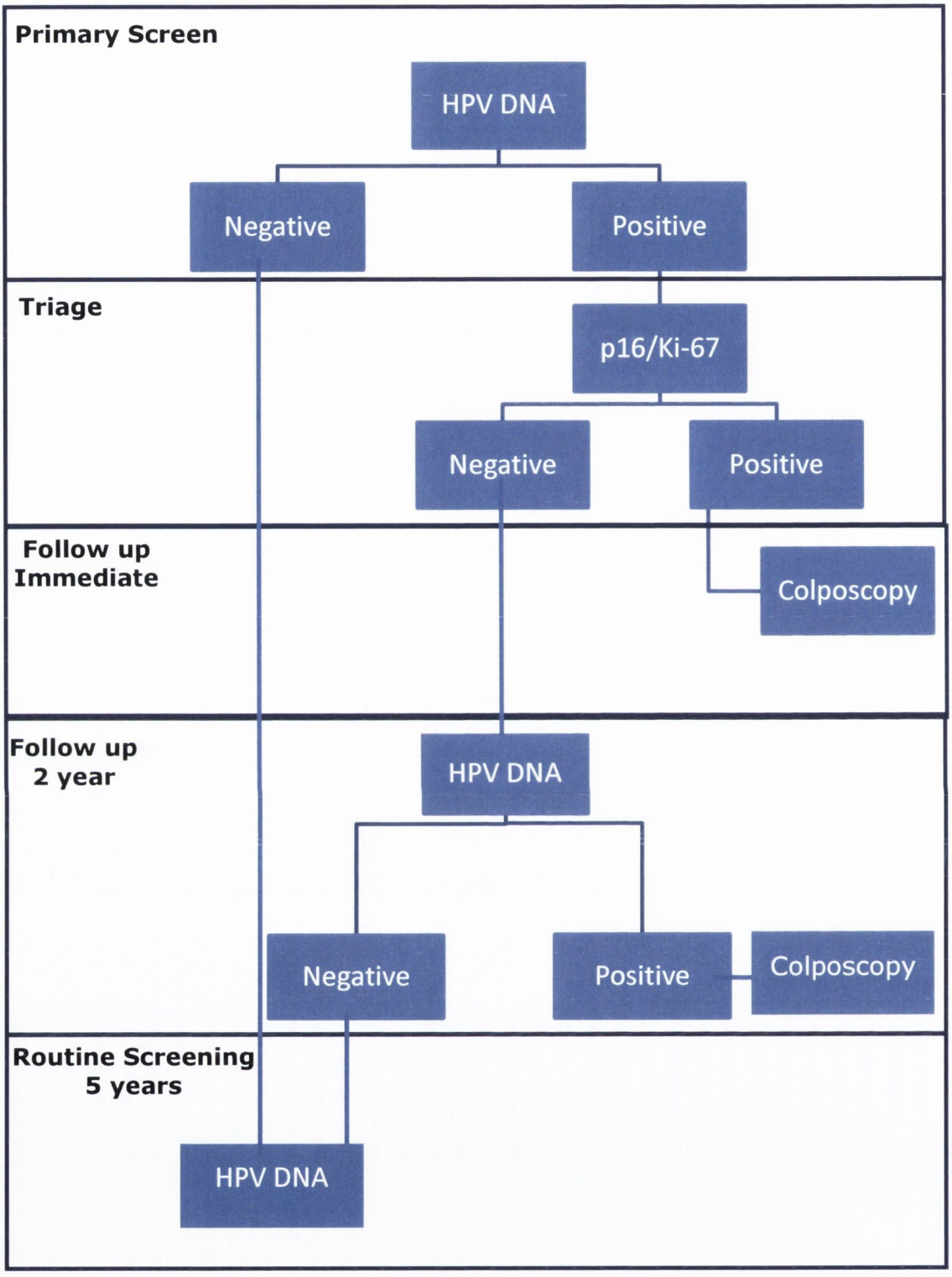


Figure 7-10 Proposed cervical screening strategy

Primary screening with HPV DNA testing has consistently shown to offer improved sensitivity for detection of CIN 2+ compared to cytology (Mesher et al., 2010, Kitchener et al., 2009 , Ronco et al., 2010 Rijkaart et al., 2012a). There are a number of advantages associated with HPV DNA testing for both women taking part in screening and health care providers, the NPV of a single HPV test is extremely high, 99.7% (95% CI 99.6-99.9) and greater than that shown for cytology 99.0% (95% CI 98.7 -99.5) allowing for the safe extension of screening intervals from 3 years to 5 years (Dillner et al., 2008). Furthermore, HPV testing detects more CIN 2+ as there are fewer false negatives compared with cytology screening. The argument for its use is further strengthened by fact that a vaccinated population will be entering screening age in the near future. As cervical abnormalities will become less frequent, the PPV of cytology will be reduced and methods such as HPV testing and biomarkers will be more appropriate.

There is however, one concern with respect to HPV testing. HPV DNA tests detect many transient infections that can spontaneously regress resulting in a low specificity for detecting high grade CIN (Cuzick et al., 2006). To overcome this, co-testing with both HPV and cytology has been evaluated. Findings indicate that co-testing does not offer any enhanced protective effect against high grade CIN compared to HPV DNA testing only (Dilner et al., 2013). Thus, the use of HPV testing alone followed by triage of screen positive cases will be a more cost effective approach to cervical screening (Dilner et al., 2013). In order to select HPV positive women at true risk a triage test with a high specificity must be employed to select which women require immediate colposcopy. LBC allows this test to be performed as a reflex test, avoiding the burden of repeat visits on both women and smear takers.

Strategies to maximise detection of HPV positive women at risk of developing CIN 2+ have been evaluated. Triage with cytology has been reported as effective for the management of HPV positive women (Cuzick et al., 2003). This method has been further improved by findings from the ATHENA (Addressing the Need for Advanced HPV Diagnostics) study which reported an increased accuracy of cytology triage by incorporating HPV genotyping for HPV 16 and 18 (Castle et al., 2011). Recent findings from the NTCC reported that triage of HPV positive screening with $p16^{INK4A}$ is a good predictor of CIN 2+. The authors went on to report a strategy where HPV positive $p16^{INK4A}$ positive

women referred to colposcopy would produce a sensitivity of 91% (Carozzi et al., 2008) compared to 80% by cytology and HPV 16/18 genotyping (Castle et al., 2011). $p16^{INK4A}$ testing has been improved by the addition of Ki-67 as a dual stain. Schmidt *et al* compared $p16^{INK4A}$/Ki-67 dual stained to $p16^{INK4A}$ single stained results on the same specimens. Sensitivity was comparably high, with dual staining offering improved specificity (Schmidt et al., 2011). The clinical relevance of this is would be further reductions in referrals to colposcopy. Based on these findings $p16^{INK4A}$/Ki-67 dual staining was chosen as a triage test for HPV positive women in figure 7.10.

A crucial consideration in the use of the model outlined in figure 7.10 is how to manage women who are HPV positive $p16^{INK4A}$/Ki-67 negative. The NTCC recommend rescreening in 2 years for HPV positive $p16^{INK4A}$ negative women (Carozzi et al., 2012). There have been no large scale studies investigating the utility of $p16^{INK4A}$/Ki-67 as a triage tool following HPV testing. It is likely to be similar to $p16^{INK4A}$ alone, as reported by Schmidt *et al,* thus HPV positive $p16^{INK4A}$/Ki-67 negative women could potentially be reviewed in a similar manor i.e. at a 2 year interval by repeat HPV test with referral to colposcopy in the case of HPV persistence. The result of this will depend on the clearance rate of HPV in $p16^{INK4A}$/Ki-67 negative women, clearance of HPV is thought to occur within 2 years (Rodriguez et al., 2010), Carozzi *et al* proposed that rate of clearance could potentially be more rapid in $p16^{INK4A}$ negative women as the cell cycle has not been disrupted (Carozzi et al., 2012). There will be some level of limitation associated with these methods, while risk of CIN 3 or invasive cancer is low, it is still a consideration in addition to risk of loss to follow up. To address this limitation choosing a primary HPV test with genotyping capability could provide an additional level of stratification and identify those at greatest risk. Data from the ATHENA study comparing 9 screening strategies found that co-testing with HPV and cytology with triage using HPV 16 and 18 genotyping was most effective in maintaining a reasonable sensitivity with only a modest increase in the number of colposcopy visits (Castle et al., 2011). This limitation could be counteracted by the use of more specific methods such as $p16^{INK4A}$/Ki-67 staining.

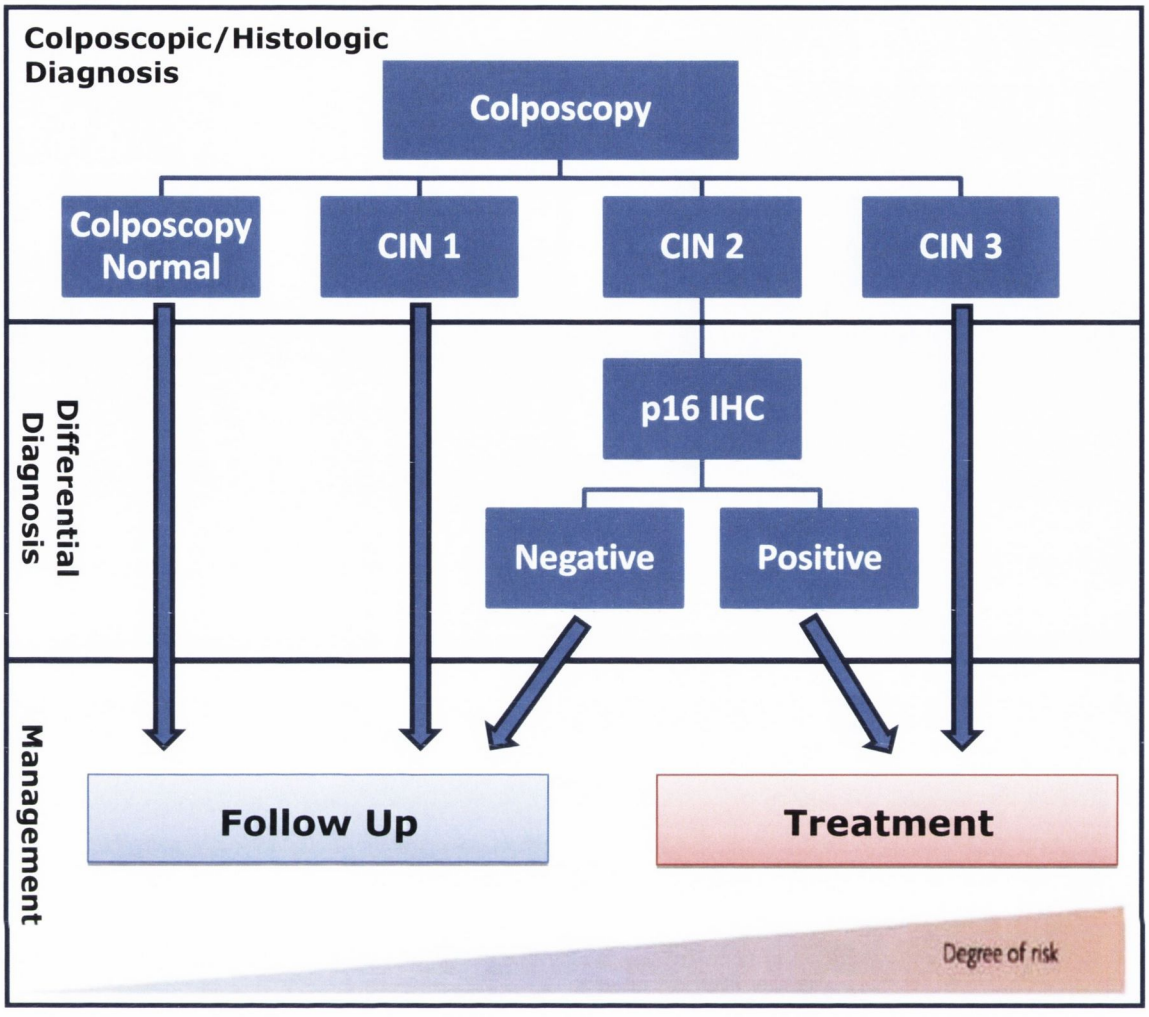


Figure 7-11 Proposed clinical management at colposcopy

Figure 7.11 outlines a potential management algorithm once women enter colposcopy. As CIN 3 is considered a true precursor lesion, excisional treatment is recommended. As discussed above, based on recommendations from the LAST study, differential diagnosis is advised for a histological diagnosis of CIN 2. A positive p16 results supports characterisation of true precursor lesion and treatment is recommended (Darragh et al., 2013). A negative p16 result favours an interpretation of a low grade lesion or a non-HPV associated pathology and follow up is recommended (Darragh et al., 2013). The natural history of CIN 1 and women with inconspicuous colposcopy who are p16 positive is uncertain and requires further study. Hence, while p16 may represent those at higher risk, no recommendation can be made on in such cases. Future investigation in to these populations will be discussed further in section 7.2.

No screening test is 100% effective, thus a balance must be found to minimise the risk of cancer but also prevent over treatment of women without clinically significant disease. This will be at the discretion of health care providers and based on the capacity of available resources. It is clear that no one modality will successfully meet the challenges we face in cervical cancer screening, screening algorithms are likely to change over the coming years as new technologies come available that will stratify risk more accurately. A panel of tests will be an important consideration in the discrimination between high and low grade disease.

7.2 Future direction

HPV vaccination has become a part of cervical prevention strategies. It is not yet known if other HPV types will dominate as a result of vaccination, this could lead to changes in the presentation of cervical pre-cancer. As the prevalence of high grade lesions is predicted to decrease cytology abnormalities will become rarer and more challenging to detect. Screening programs will need to adapt to this, HPV testing will likely become the test of choice for primary screening. RCTs have proven its effectiveness in primary screening by identifying HR HPV types alone or in combination with cytology, in women over 30 years (Kitchener et al., 2009, Mesher et al., 2010, Ronco et al., 2010, Rijkaart et al., 2012). Current screening intervals are 3-5 years depending on age, studies have suggested that a negative HPV result can give protection for up to 6 years (Kitchener et al., 2009). This will be of particular importance in low recourse regions. Collectively, vaccination and HPV testing in primary screening will lead to changes in screening algorithms. There remains debate over best options to triage HPV positive events however it is evident that more specific methods are required. Biomarkers will play a pivotal role in this, the role of $p16^{INK4A}$/Ki-67 dual staining as a specific marker for high grade lesions is of great importance in addition to other methods such as HPV genotyping, APTIMA HPV for HPV mRNA and methylation markers. However evidence remains insufficient and further research is needed to optimise screening protocols (Arbyn et al., 2012).

This study enrolled women undergoing routine follow up for minor abnormal smears, the clinical endpoint was CIN 2+, which was determined by histologic

review, based on a standard protocol outlined in CervicalCheck quality assurance guidelines. In terms of this project, there was a higher than expected rate of HPV DNA negative CIN 2+ cases. A review between HPV negative and HPV positive histologically diagnosed CIN 2 cases supported adjudication with $p16^{INK4A}$ IHC may help in clarifying the true nature of these samples.

This work has established that HPV DNA and $p16^{INK4A}$/Ki-67 may play a role in our cervical screening program. Further studies looking at triage following an initial minor cytological abnormality and in the context of primary screening will be important. Management of HPV DNA positive $p16^{INK4A}$/Ki-67 negative women will need to be addressed. All HPV positive women should be followed up independent of $p16^{INK4A}$/Ki-67 result to investigate whether the presence of $p16^{NK4A}$/Ki-67 overexpression in HPV positive women predicts future development of high grade pre-cancer. This will determine the risk of disease in HPV positive $p16^{INK4A}$/Ki-67 negative women and help predict the intervals for retesting and return to routine screening. As shown in section 6.4.5 of a proportion of women with normal colposcopy p16/Ki-67 positive had a subsequent CIN 2+. However, numbers were small and larger scales studies would be needed to determine the utility of biomarkers in these populations.

This work was carried out under the umbrella of CERVIVA 1, which is committed to research in the area of HPV testing and identification of biomarkers in cervical screening. The recently funded CERVIVA 2, continues to develop and expand emerging research topics of interest to cancer screening in Ireland. Previous knowledge gained on the use of biomarkers will be translated into the context of HPV primary screening. Several biomarker options will be investigated including, HPV E6/E7 mRNA, alterations to methylation patterns of several genes, alterations of viral and host genomes and finally detection of cellular proteins overexpressed in HPV infected cells. In addition, the impacts of HPV vaccination will be explored as well as modelling and cost effective analysis. This work will be of utmost importance, identification of highly sensitive and specific tests will lead improved cost effectiveness in ultimately improves cervical screening.

The role of HPV testing and vaccination in other HPV associated cancers such as anal, vulva and oropharyngeal require further research. The rate of these cancers is increasing however there are no effective screening programs in

place. Screening for anal pre-cancer (AIN) has been proposed to identify high grade AIN. The use of biomarkers including $p16^{INK4A}$ and Ki-67 has been suggested to play a role in diagnosis high grade AIN (Lacey et al., 2006, Kreuter et al., 2010), however larger trials are required. HPV associated head and neck cancer appears to have a significantly better survival outcome (Lacey et al., 2006). Biomarkers for HPV induced malignancies may play a role as prognostic markers and as well as screening and prevention by characterising HPV positive cancerous and pre-cancerous lesions.

Summary

Prior knowledge

HPV DNA testing is an accurate test for the triage of minor abnormalities on cytology. In order to improve specificity research has focused on biomarkers in particular HPV E6/E7 mRNA and $p16^{INK4A}$/Ki-67 expression.

Aim

To further investigate the utility of HPV mRNA and $p16^{INK4A}$/Ki-67 expression. In a population of women referred to colposcopy with minor cytological abnormalities.

Contribution

Findings indicated primary triage of LSIL/ASCUS with $p16^{INK4A}$/Ki-67 followed an ancillary HPV DNA test on $p16^{INK4A}$/Ki-67 negative cases demonstrates the most efficient triage of minor cytological abnormalities. Additionally, a relationship between increasing urinary cotinine concentration and HPV infection, HPV transforming infection and high grade CIN was identified.

Clinical implications

These findings may be useful in identifying modalities to further stratify women at risk of high grade disease. Ultimately reducing referrals of low grade smears to colposcopy clinics and avoiding unnecessary work up of women at little or no risk. Additionally the awareness of smoking history is important which has been shown to contribute risk to CIN 2+.

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Appendix

Figure 1-0 CervicalCheck screening protocol (www.cervicalcheck.ie)

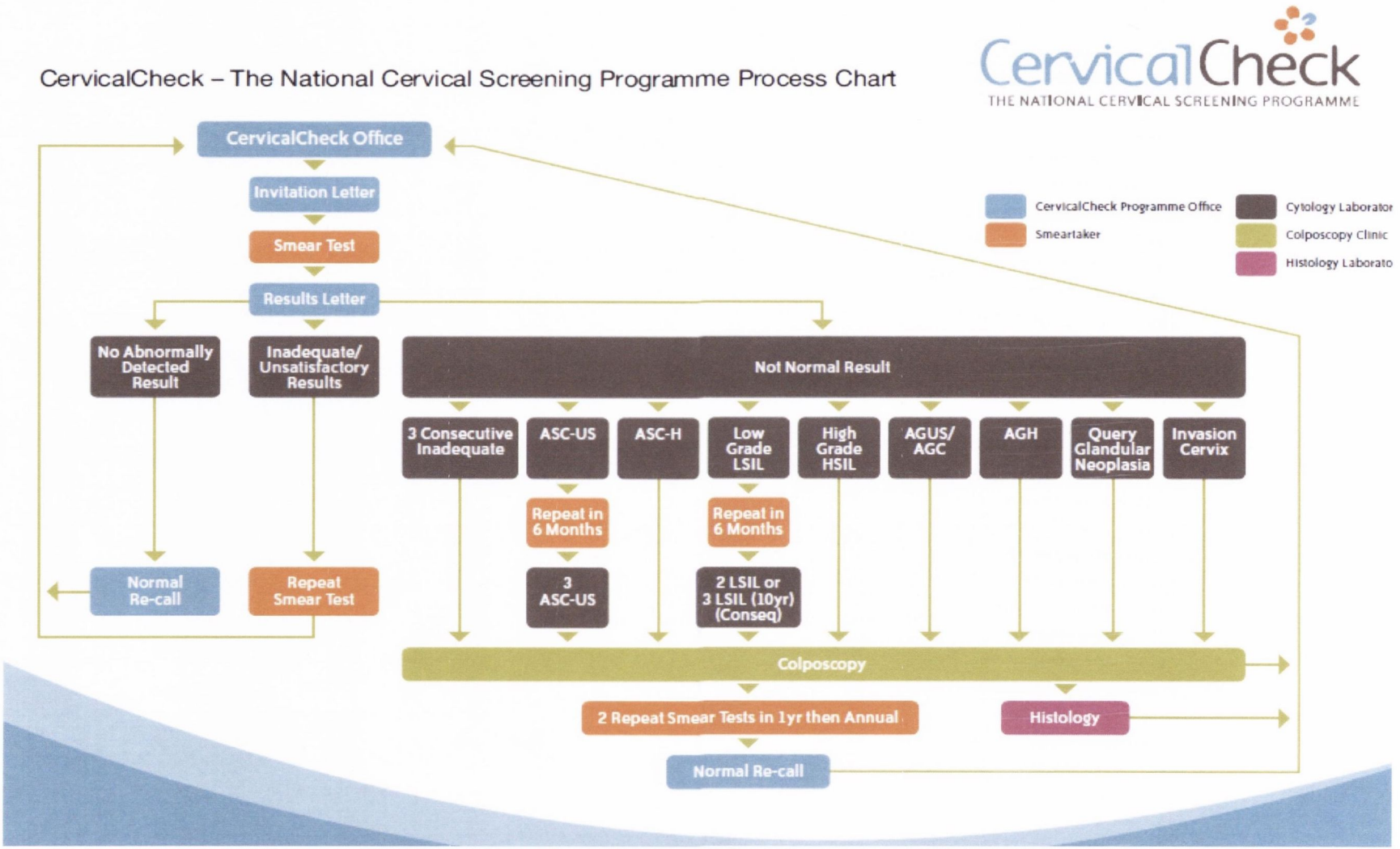

Figure 2-0 Cytology Terminology Translation Tables (www.cervicalcheck.ie)

Service

Office Use	Bethesda Terminology	BSCC Terminology 1986	Office Use	Management Recommendation	Rationale / Recommendation
P1	Unsatisfactory/Inadequate	Unsatisfactory/Inadequate	R6	3 month repeat	Repeat in 3 months
			R7	Refer to colposcopy	3 consecutive unsatisfactory/discretion of pathologist (gynae referral optimal)
					First smear test following a treatment in colposcopy
					Any 3 smear test results that are not normal in the previous 10 years
P2	Negative/NAD (No Abnormality Detected)	Negative/NAD	R1	Exit programme	No further screening required
			R2	3/5 year recall	Routine recall
			R3	12 month repeat	Second and subsequent smear tests following a treatment for HSIL/AGC/AIS
					If HIV+/post organ transplant/DES exposed/renal dialysis
			R4	6 month repeat	Following a result/treatment for ASC-US or LSIL, following a result of AGC
					First smear test following a result/treatment for HSIL/AGC/AIS
					Following hysterectomy & Cervical Intraepithelial Neoplasia (CIN) is completely excised
					Less than 10 years routine recall and no CIN at hysterectomy
			R7	Refer to colposcopy	If suspicious cervix, a gynaecology referral is optimal
P3a	ASC-US (Atypical Squamous Cells of Undetermined Significance)	Borderline Nuclear Abnormalities (BNA) (Squamous)	R4	6 month repeat	First ASC-US or no more than 2 consecutive smear tests showing ASC-US
			R7	Refer to colposcopy	Third consecutive ASC-US smear test
					Previous LSIL (within 18 months)
					Any 3 smear test results that are not normal in the previous 10 years
					First smear test following a treatment for CIN
P3b	ASC-H (cannot exclude High Grade)	BNA-H (cannot exclude High Grade)	R7	Refer to colposcopy	Refer to colposcopy
P4	LSIL (Low Grade Squamous Intraepithelial Lesion)	Mild Dyskaryosis	R4	6 month repeat	First LSIL smear test
			R7	Refer to colposcopy	Second consecutive LSIL smear test
					If previously attended colposcopy and not yet returned to routine recall
					If HIV+/post organ transplant/DES exposed/renal dialysis
					Any 3 smear test results that are not normal in the previous 10 years
P5	HSIL (High Grade)	Moderate Dyskaryosis	R7	Refer to colposcopy	Refer to colposcopy
P6	HSIL (High Grade)	Severe Dyskaryosis	R7	Refer to colposcopy	Refer to colposcopy
P7	Query Squamous Cell Carcinoma	Query Squamous Cell Carcinoma	R7	Refer to colposcopy	Refer to colposcopy
P8a	AGC (Atypical Glandular Cells)	Borderline Nuclear Abnormalities (Glandular)	R7	Refer to colposcopy	Refer to colposcopy
P8b	AGC (Atypical Glandular Cells) Favour Neoplastic Process	Borderline Nuclear Abnormalities (Glandular)	R7	Refer to colposcopy	Refer to colposcopy
P9	Query Glandular Neoplasia /AIS/Adenocarcinoma	Query Glandular Neoplasia /AIS/Adenocarcinoma	R7	Refer to colposcopy	Refer to colposcopy
P10	Broken/Damaged/Expired Vial	Broken or Damaged Vial	R6	3 month repeat	Repeat in 3 months

Glossary

Atypical Squamous Cells of Undetermined Significance (ASC-US)
Atypical Squamous Cells, Favour Neoplastic process (ASC-H)
Atypical Glandular Cells (AGC)
Atypical Glandular Cells, Favour Neoplastic process
Adenocarcinoma In Situ (AIS)
Borderline Nuclear Abnormalities High Grade (BNA-H)
Low Grade Squamous Intraepithelial Lesion (LSIL)
High Grade Squamous Intraepithelial Lesion (HSIL)

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