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Human Papillomavirus Prevalence in the Irish Cervical Screening Population and a Specific Group of HIV Positive Women.

By

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Doctor of Philosophy.

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Under the supervision of

Professor John O'Leary and Doctor Cara Martin

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Declaration

I declare that this thesis is my own work, and has not been submitted previously for a PhD degree at this or any other university. I did not test samples from Antrim Area Hospital, Antrim, Northern Ireland. The clinical, demographic and HPV data was provided as part of a collaboration within the CERVIVA consortium for use in determining a HPV profile for the entire island of Ireland. I agree that the library may lend of copy this thesis on request.

Jamie Mc Inerney

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Summary

Human Papillomavirus (HPV) is the major aetiological agent in the development of cervical pre-cancer and cancer with high-risk HPV (HR-HPV) types 16 and 18 detected in greater than 70% of squamous cell carcinomas of the cervix. Expression of HPV oncoproteins E6 and E7 is necessary for development of malignancy. There is evidence to suggest that women with human immunodeficiency virus (HIV) have a higher prevalence of cervical pre-cancer and cancer. In this project, I aim to determine the prevalence and genotype distribution of HPV in two populations of women; the general cervical screening population and a subgroup of HIV positive women. I have determined a HR-HPV prevalence rate of 19.2% in the Irish cervical screening population (n=3193, median age 39.1, age range 17-89). HPV 16 was the most prevalent HPV genotype detected, followed by HPV 31/HPV 52, HPV 18, HPV 51 and HPV 39/HPV 66. I have also determined the prevalence and persistence of HPV in a cohort of HIV positive women on anti-retroviral treatment (n=321, median age 33, age range 17-71). The HR-HPV DNA and mRNA prevalence rates in this population 51.1% and 21.8% respectively. Among women with cytological abnormalities 82.6% were positive for HR-HPV DNA with 21.8% positive for HPV E6/E7 mRNA. HPV 45 was the most predominant genotype followed by HPV 33, HPV 16, HPV 18 and HPV 31. The rate of multiple HPV type infections was 24.3%. In women with follow-up smears prevalence rates for HPV DNA 42% at baseline and 34.1% at ~6-18 month follow-up. In relation to HPV mRNA prevalence rates were 16.9% at baseline and 23.4% at ~6-18 month follow-up. I found that women receiving anti-retroviral therapy had a lower incidence of HPV DNA, with no effect noted in HPV mRNA prevalence. I also report a higher incidence rates of HPV DNA and mRNA prevalence in women with low CD4 counts (<200 x 10 6/L).

Abbreviations

°C	Degrees Celsius
AB 9700	Applied Biosystems Gold-plated 96-well GeneAmp PCR System 9700
ADAT1	Adenosine deaminase 1
AGUS	Atypical glandular cells of undetermined significance
AIDS	Acquired immunodeficiency syndrome
AL	Lysis Buffer
ALTS	Atypical squamous cells of undetermined significance and Low-grade squamous intraepithelial lesion HPV triage study
ASCUS	Atypical squamous cells of undetermined significance
ATL	Tissue Lysis Buffer
AW2	Wash Buffer 2
BSCCP	British Society for Colposcopy and Cervical Pathology
CAR	Carrier RNA
CC	Cervical cancer
CDC	Centers for Disease Control and Prevention
CE	**C**onformité **E**uropéene
CIN	Cervical intraepithelial neoplasia
CIT	Citrate Concentrate
CLM	Column
CO	Cut-off
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytosine triphosphate
dGTP	Deoxy Guanine triphosphate
DNR	Denaturation Reagent Mixture

dTTP	Deoxy Thiamine triphosphate
dUTP	Deoxy Uracil triphosphate
CDK	Cyclin dependant kinase
DNA	Deoxyribonucleic acid
E	Early Region
ELISA	Enzyme-linked immunosorbent assay
ETL	Elution tube
FDA	Food and drug administration
FRET	Fluorescence resonance energy transfer
GP	General Practitioner
GUIDE	Genitourinary and infectious disease clinic
H	Hour
HART	HPV in addition to routine testing
Hc	Hybrid capture
hc2	Hybrid Capture II test
HRP	Horseradish peroxidase
HIV	Human immunodeficiency virus
HSE	Health service executive
HSIL	High-grade squamous intraepithelial lesion
HPV	Human Papillomavirus
HRC	High-Risk Calibrator
HR-HPV	High-risk HPV
L	Late region
L	Litre
LSIL	Low-grade squamous intraepithelial lesion

LR-HPV	Low-risk Human papillomavirus
Mg^{2+}	LINEAR ARRAY HPV Magnesium Solution
Mins	Minutes
mL	Millilitre
mM	Millimolar
MMX	LINEAR ARRAY HPV Master Mix
mRNA	Messenger RNA
MW	Elution Buffer
n	Cohort number
NASBA	Nucleic Acid Sequence Based Amplification
NC	Negative Calibrator
oligo	Oligonucleotide
PCR	Polymerase chain reaction
PV	Papillomavirus
QC1-LR	Quality Control 1 Low-Risk
QC2-HR	Quality Control 2-High-Risk
RBS35	RBS35 Tray Cleaning Solution
RCF	Relative centrifugal force
RLU	Relative light unit
RLT	Tissue Lysis Buffer
RNA	Ribonucleic acid
RPE	RPE wash buffer
RT	Room Temperature
SA-HRP	Streptavidin-Horseradish Peroxidase Conjugate
SDS	20% Sodium lauryl sulphate and 1% ProClin® 150

SIL	Squamous intraepithelial lesion
SPSS	Statistical Package for the Social Sciences
SSPE	Sodium phosphate solution, sodium chloride, EDTA and 1% ProClin® 150 preservative
STI	Sexually transmitted infection
STM	Specimen Transport Medium
STM/DNR	Specimen Transport Medium/Denaturation Reagent Mixture
Strip	Linear Array HPV Genotyping Strips
TBS	The Bethesda System
TMB	Tetramethylbenzidine
TNA	Total Nucleic Acid
U1A	Human U1 small nuclear ribonucleoprotein specific protein A
μ	Micro

Posters and Presentations

Data from this thesis has been presented on an ongoing basis throughout the course of the studies, with updated data presented at each venue. Posters (P), Oral Presentations (O) and Abstracts (A)

Posters and presentations relating to Chapter 4

Human papillomavirus prevalence and genotype distribution in Irish women,

7th international Cancer Conference 2009 University College, Dublin, May 2009 (P)

Institute of Molecular Medicine, Trinity College, Dublin, May 2009 (P)

Friends of the Coombe Research Symposium, Coombe Women and Infants Hospital, December 2008 (O)

EUROGIN November 2008 - Joining forces for cervical cancer prevention. (O)

British Society for Clinical Cytology Annual Meeting, Dublin 2008. (O)

Institute of Molecular Medicine 10th Annual Meeting, St. James Hospital, Dublin 2007. (P)

Human papillomavirus DNA and mRNA prevalence and persistence in a cohort of human immunodeficiency virus positive women in Ireland.

USCAP Washington DC, USA March 2010 (P)

Eurogin Monaco, February 2010 (O)

7th international Cancer Conference 2009. Institute of Molecular Medicine, Trinity College, Dublin, May 2009 (P)

The British Society for Colposcopy & Cervical Pathology. Annual Scientific Meeting, University College, Dublin, May 2009 (P)

Friends of the Coombe Research Symposium, Coombe Women and Infants Hospital, December 2008 (A)

EUROGIN November 2008 - Joining forces for cervical cancer prevention. (A)

Chapter 1

General Introduction

1.0 General Introduction

1.1. Overview

Cervical cancer (CC) is the third most common cancer in women worldwide accounting for 275,000 deaths in 2008, 88% of which occur in developing countries (Ferlay *et al.*, 2010). Cytological screening has reduced the incidence and mortality of CC worldwide, especially in countries with developed cervical screening programmes (van der Aa, 2008, Gunnell, 2007). HIV positive individuals are at greater risk (up to seven time greater) of developing CC (Boshoff, 2002, Stern, 2005).

In Ireland there are three significant bodies working on CC prevention, A national cervical screening programme (CervicalCheck), a HPV vaccination programme and a research consortium (CERVIVA). CervicalCheck the National Cervical Screening programme launched nationwide in September 2008. Before the introduction of CervicalCheck, screening for CC was opportunistic, with smears taken from women via their local GP office, sexually transmitted disease clinics, "Well Woman" and family planning clinics and following childbirth. CervicalCheck provides free smear tests through primary care settings to 1.1 million eligible women aged 25-60 years. The Irish Health Service Executive (HSE) introduced a HPV Vaccination Programme which commenced September 2010. All girls who are starting 1st and 2nd year in secondary schools will be offered the GARDASIL® HPV vaccine free of charge. CERVIVA (The Irish Cervical Screening Research Consortium) was set up in 2005, consisting of several 7 Irish academic institutions, 8 hospitals and 10 commercial diagnostic or biotechnology companies. The ultimate goal of the consortium is to advance high quality peer-reviewed research programmes that provide the best possible information and guidance in the

delivery of cervical screening services to Irish women. This thesis is the basis of work underway within the consortium. It is well established that persistent infection with Human Papillomavirus (HPV) is necessary for the development of CC, with HPV present in 99.7% of CC's (Walboomers *et al.*, 1999, Munoz, 2000). It is also known that HIV positive men and women are at increased risk of HPV infection and associated disease (Palefsky, 2006a).

In this chapter the key areas that will be addressed are:

1. The role of HPV in development of CC and pre-cancer
2. The prevalence and genotype of HPV in different populations
3. The role of HPV in CC in HIV positive populations

1.2. Cervical cancer

CC is a carcinoma (a malignant tumour of epithelium) that presents in the cells of the cervix. The aetiological cause of CC is infection with HR-HPV detailed in sections 1.4.2 and 1.4.3 of this chapter. However, there are several other risk factors involved in the development of CC. These risk factors include smoking history (with possible increased risk relative to the amount of tobacco consumed), HPV co-infection with Chlamydia, the use of hormonal contraception, parity, as well the early onset of sexual activity and pregnancy (Deacon *et al.*, 2000, Moreno *et al.*, 2002, Munoz *et al.*, 2002). It has also been stated that there may be a possible genetic predisposition to developing CC as infection with HPV is quite common, while CC is relatively rare (Martin *et al.*, 2006).

The disease progresses from initial infection with HPV to the development of cancerous cells that can invade surrounding tissues and organs, which can lead to the formation of

metastatic disease. CC is a unique cancer, in that is has a well defined pre-malignant lesion. The natural progression of cervical disease is from mild Cervical Intraepithelial Neoplasia I (CIN I) or Low-grade squamous intraepithelial lesion (LSIL) to moderate and more severe degrees of neoplasia Cervical Intraepithelial Neoplasia II/ Cervical Intraepithelial Neoplasia III (CIN II/CIN III) or High-grade squamous intraepithelial lesion HSIL. In glandular lesion the progression is from Low-grade Cervical Glandular Intraepithelial Neoplasia to High-grade Cervical Glandular Intraepithelial Neoplasia, to invasive Adenocarcinoma. Additionally, Atypical squamous cells of undetermined significance (ASCUS) and Atypical Glandular cells of Undetermined Significance (AGUS) diagnosis can occur when the disease cannot be classified as either negative or mild, moderate or severe disease.. Cervical pre-cancer and cancer develop in the epithelium of the transformation zone of the cervix.

The rates of cervical abnormalities in women in Ireland in the general screening population have been reported between ~11-15% (Keegan *et al.*, 2007, CervicalCheck., 2010). The initial report from the CervicalCheck programme found that the incidence of ASCUS in women currently being screened as part of the CervicalCheck programme in Ireland is 9.2%, with 4% of women presenting with LSIL, and 1.4% with HSIL. The rates of CC are relatively low at ~10 cases/100000 people (NCRI, 2009, CervicalCheck, 2010). The rate of progression from LSIL to CC is a slow process, with many low grade abnormalities spontaneously regressing (Cuschieri *et al.*, 2004b), thus CC is different from other cancers, and early detection of pre-cancerous lesions has significantly reduced the risk of CC worldwide, especially in countries with CC screening programs (van der Aa, 2008). As mentioned in section 1.1, CC is the third most common cancer found in women worldwide; this is relatively higher than the burden of CC in an Irish context,

where CC is the eight most common cancer affecting women. However, despite the relatively lower prevalence of the disease in Ireland there are still over 200 cases diagnosed per annum, with a five-year relative survival rate of those women diagnosed with CC reported at 57.2% (NCRI, 2011). In 2006 there where 232 cases of CC in Ireland with 83 women dying from the disease (NCRI, 2009). National Cancer Registry Ireland (NCRI) data from 1994-2003 suggests cervical carcinoma in-situ and CC have incidence hotspots with increased risk of disease in the east and south of the country (Figure 1.1).

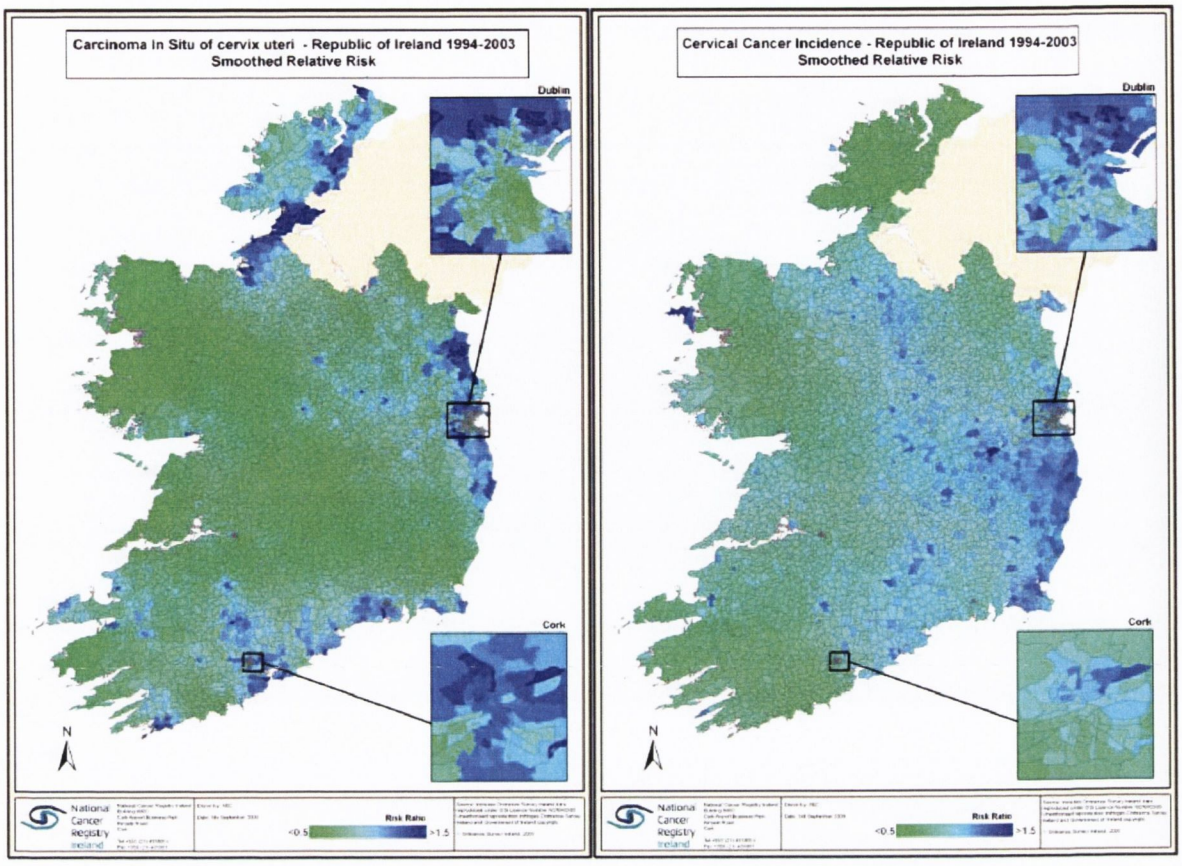


Figure 1.1: 1994-2003 Cervical Carcinoma in-situ and CC incidence map. The areas shaded in green represent the parts of the country where the relative risk of developing the corresponding disease is less than the national average. The areas shaded in blue represent the parts of the country where the relative risk of developing the corresponding disease is greater than the national average. In both maps it can be seen that certain areas of the midlands, the south and the east contain hotspots of increased risk of disease. [produced by Anne-Elie Carsin (NCRI, 2009)]

1.3. Cytological screening for CC

Cytological screening has reduced the incidence and mortality of CC worldwide, especially in countries with developed cervical screening programmes (van der Aa, 2008, Gunnell, 2007). The Pap test initially described by Georgios Papanicolaou in 1928 showed how cells from the cervix removed using a spatula, smeared onto a glass slide could be stained to highlight cellular abnormalities. It was this breakthrough that lead to the development of cervical cytology.

To date, cytology along with histopathology remain the most common methods for the early detection of cervical disease and CC. Cytology is used to determine the level of pre-cancerous cellular changes by using the pap smear test to determine the grade of disease in a sample. Cervical intraepithelial neoplasia (CIN) is the terminology adopted by the British Society for Colposcopy and Cervical Pathology (BSCC) for the classification of pre-cancerous squamous epithelial lesions of the cervix. Depending on the severity of neoplastic change a sample is graded as mild dysplasia (CIN1) moderate dysplasia (CIN2) or severe dysplasia (CIN3), however it has been recommended that all systems should be translatable into The Bethesda System (TBS) for reporting of cervical cytology (Herbert *et al.*, 2007). Under TBS squamous intraepithelial lesions (SIL) are graded as either LSIL or HSIL (Solomon *et al.*, 2002).

The major downfall of cytology appears to be the number of false negative cases. This can be related to the fact that up to 80% of the sample in conventional smears can remain on the collection spatula (Malle, 2003). In addition, problems in slide preparation whereby material is distributed on the slide surface in differing densities can make the diagnosis more difficult. To combat these problems a number of liquid pap based

technologies have been developed. The liquid based systems involve suspension of the cellular material in an alcohol based solution. A slide with a monolayer of cells is then prepared from this suspension allowing for an even distribution of the sample on the slide surface, avoiding areas of high cell density which are more difficult to diagnose. There a currently a number of pap based technologies available, ThinPrep® (Cytyc Corp., Massachusetts, U.S.A.), SurePath™ (TriPath Imaging®, Inc., North Carolina, U.S.A.), DNACITOLIQ (Digene Brazil, Sao Paulo, Brazil) and AutoCyte PREP (TriPath Imaging, Inc., North Carolina, U.S.A.) of which only ThinPrep® has received Food and Drug Administration (FDA) approval.

The advantages of liquid based cytology in comparison with conventional cytology include; slides that are produced are easier to read as they have reduced blood and in particular inflammatory content, repeat/ancillary tests can be carried out on the remainder of the specimen as only a small proportion of the suspension is used to create a slide (depending on cellularity), faster screening of slides (Bergeron, 2001) and automation of slide preparation and screening (Bolger *et al.*, 2006).

The development of liquid based cytology reduces the incidence of unsatisfactory cytological results compared to tradition cytological methods. Additionally it has increased the sensitivity and specificity of cytological diagnosis while enabling ancillary testing (Bundrick *et al.*, 2005). However liquid based cytology still has limited sensitivity and specificity for the detection of cervical disease. This results in multiple screening events and referral to colposcopy in low grade or borderline cases (Wright, 2007).

1.4. Human papillomavirus

Papillomaviruses (PV), including HPV, are members of the Papillomaviridae. PV's are DNA viruses that infect epithelium or mucosal surfaces of a wide variety of animals, including humans. Over 120 types have identified in humans (HPV), of which nearly 50 infect the genital mucosa (de Villiers *et al.*, 2004). HPV infection of the genital tract results in what can be considered 2 diseases, condylomas (warts) and neoplasia (pre-cancerous lesions). Cutaneous HPV types cause warts or verrucas common to hands and feet and can develop when the viral DNA is a separate self-replicating unit not integrated into the host genome. Mucosal HPV types can cause flat warts, pre cancerous lesions and pre-cancers. Infection with both low and high-risk HPV types along with associated lesions can regress without the need for treatment (Cuschieri *et al.*, 2004b). HPV types can be sub-divided into 2 groups; low risk HPV (LR-HPV) and high risk HPV (HR-HPV) depending on the propensity for malignant progression of their associated lesions. LR-HPV types are so called as they are rarely if ever found in invasive cervical carcinomas, with HR-HPV types so called due to their association with pre-cancer and cancer (de Villiers *et al.*, 2004) (Table 1.1). A reclassification of these HR-HPV types has been published recently with HR-HPV being split into 4 groups (Schiffman *et al.*, 2009) (Table 1.1). This reclassification was based on HPV phylogenetic data along with meta-analysis data outlining the prevalence of HPV genotypes in cytologically normal women, and women with CC (Schiffman *et al.*, 2009). The HR-HPV groups were shown to exist as a single phylogenetic clade in the HPV evolutionary tree. The first group is made up of the most common types found in cancers. The second HR-HPV group is made up of other carcinogenic types generally found in CC cases worldwide. The remaining groups were formed from probable carcinogenic and possible carcinogenic HPV types. These groups were determined due to there reported rare association with CC. HPV 68 was considered

probably carcinogenic due to its phylogenetic position and suggestive yet limited experimental data on its association with cervical disease. The possible carcinogenic group was determined due to the extremely rare occurrence of the genotypes alone in CC despite the relatively common population prevalence of the individual genotypes (Schiffman *et al.*, 2009). Originally it was presumed that low grade cervical disease was mostly caused by the LR-HPV types. However it has been confirmed that HR-HPV types are present in >80% of CIN 1 cases (ALTS, 2003). HR-HPVs are also present in 99.7% of CC's (Walboomers *et al.* 1999). Lesions caused by both LR and HR- HPV types can regress without the need for treatment, with genital infection by HR-HPV types even being asymptomatic.

Table 1.1: Classification of HR-HPV types.

2004 HR-HPV classification	2009 HR-HPV classification			
HR-HPV types[a]	Most common Carcinogenic HPV types[b]	Other Carcinogenic HPV types[b]	Probable Carcinogenic HPV type[b]	Possible Carcinogenic HPV types[b]
16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70	16, 18, 45, 31, 33, 35, 52 and 58	51, 56, 39 and 59	68	26, 53, 66, 67, 70, 73, and 82

[a](de Villiers *et al.*, 2004), [b](Schiffman *et al.*, 2009)

1.4.1. Human papillomavirus structure and genome

Members of the Papillomaviridae are non-enveloped viruses with a ~55nm diameter icosahedral capsid, composed of 72 capsomers. The main capsid is made up of the L1 gene product (Baker *et al.*, 1991). In addition to the L1 protein each capsid contains the L2 protein, which participates in encapsidation of the genome and viral entry into the host cell (Holmgren *et al.*, 2005). Within the capsid is a circular double stranded DNA genome

of ~8kb that contains one coding sequence (Longworth and Laimins, 2004). The genome can be divided into three different functional regions; the Early region (E), the Late region (L) and the Long Control Region (LCR). The LCR regulates HPV genome expression through the action of enhancer and silencing sequences. The early region encodes 6 non-structural genes, E1, E2, E4, E5 E6 and E7, which are required in the early stages of the life cycle (Figure 1.2) (Desaintes and Demeret, 1996). The late region encodes the genes L1 and L2 structural genes (Baker *et al.*, 1991, Holmgren *et al.*, 2005).

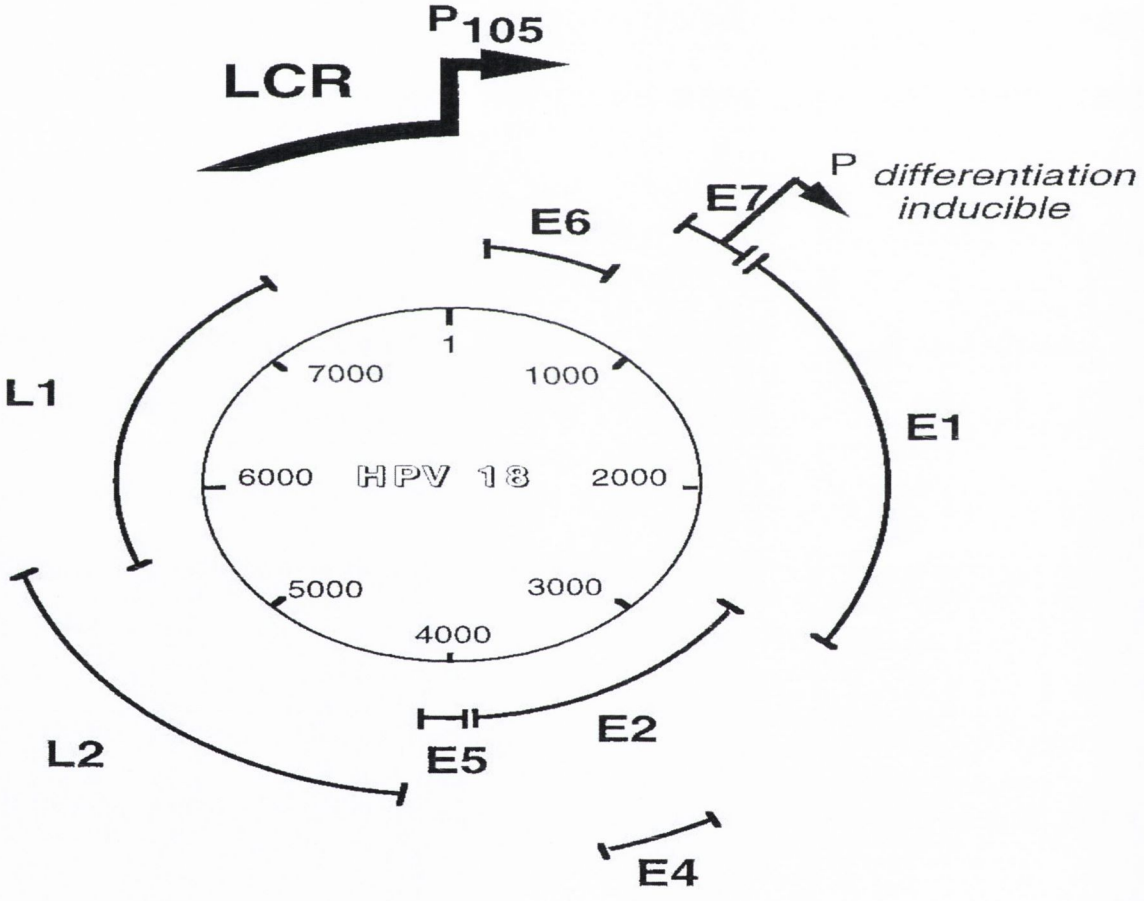


Figure 1.2: Structure of HPV 18 genome. The HPV 18 genome exhibits an organization similar to all papillomaviruses. It consists of two regions coding for early and late genes on the same DNA strand. A non-coding 800 bp fragment, called long control region (LCR), located upstream of the E6 gene, controls both viral transcription and DNA replication. (Taken from Desaintes and Demeret, 1996).

1.4.2. Human papillomavirus life cycle

Initial genital HPV infection occurs when the virus gains access to epithelial basal cells via micro traumas, which, can develop through sexual intercourse or during metaplasia of the transformation zone. In the epithelial basal cells the viral genome remains at low copy numbers only replicating its DNA once per cell cycle (Flores, 1997). HPV genome replication is achieved through the activities of the E1 and E2 proteins. The El and E2 proteins are the only viral proteins that are directly involved in viral genome replication, thus HPV require host cell factors normally only expressed in S-phase to complete DNA replication (Munger, 2002). The E2 protein recruits the E1 protein to the origin of replication, where E1 protein catalyses DNA unwinding and is involved in recruiting host DNA polymerase, allowing the initiation of viral replication.

As virally infected cells move into the suprabasal layer of the epithelium, genome amplification occurs (Peh *et al.*, 2002). In infected suprabasal cells E6 and E7 are expressed, causing the retardation of the normal terminal differentiation by driving cells into unscheduled re-entry into the S-phase of the cell cycle (Sherman, 1997, Martin *et al.*, 2006). Both E6 and E7 proteins have functions that stimulate cell cycle progression through their association with host cell cycle regulators (Munger, 2001). The E7 protein association with host molecules such as retinoblastoma gene product (pRb) prevents the binding of E2F to pRb and allows for the expression of proteins conducive to viral genome replication. Genome amplification does not only require increased expression of E6 and E7 but also all other early genes (Peh, 2004, Fehrmann, 2003, Genther, 2003).

As discussed in section 1.4.1 the HPV genome encodes two structural proteins, L1 and L2. These proteins only become expressed in the superficial layers of the epithelium following viral genome amplification (Ozbun, 1998). Following encapsidation of the genome by L2, L1 is expressed allowing for the assembly of infectious particles in the upper layers of the epithelium (Florin, 2002, Holmgren *et al.*, 2005). The HPV virions are released when the infected cells reach the epithelial surface (Woodman, 2007). HR-HPV infection of the cervix is shown in Figure 1.3.

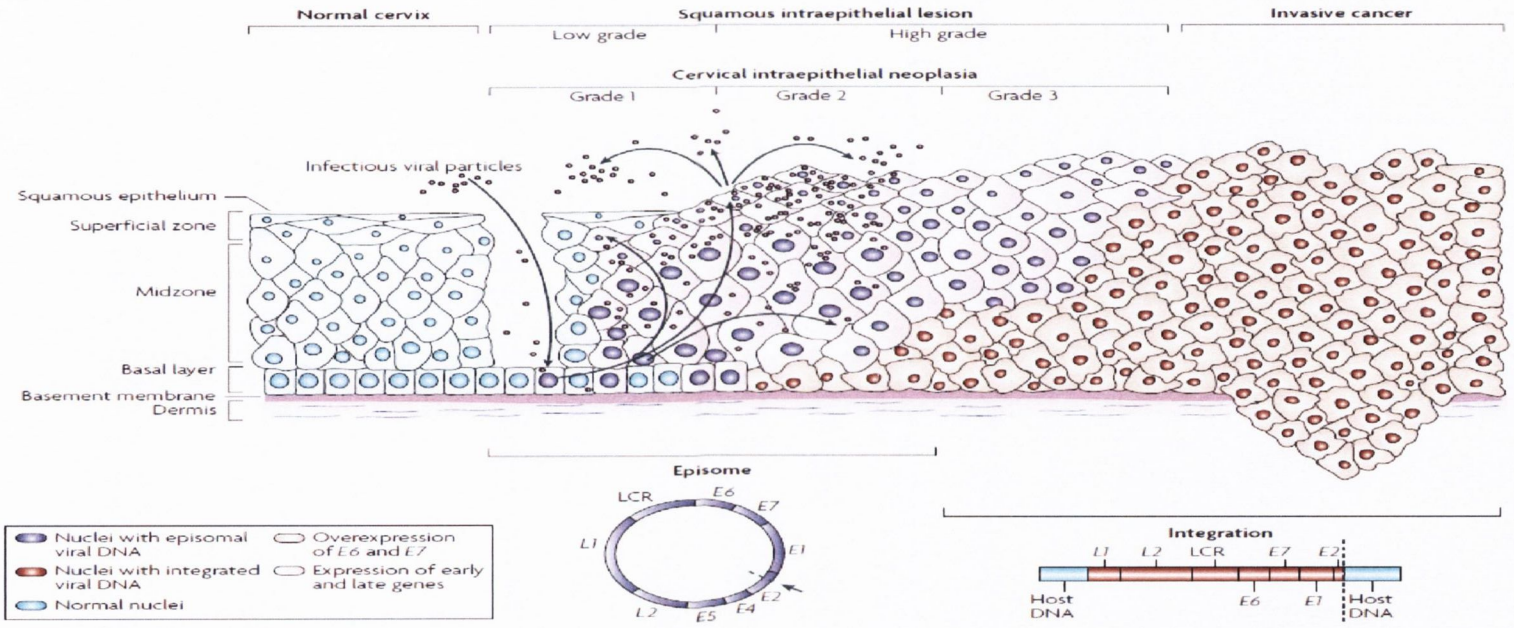


Figure 1.3: HPV-mediated progression to CC. HPV infects the basal cells of the epithelium via micro-traumas. Following infection, the early HPV genes E1, E2, E4, E5, E6 and E7 are expressed and the viral DNA replicates from episomal DNA. As the cells progress through the epithelium, the viral genome is replicated further. The late genes L1 and L2, and E4 are expressed near the surface of the epithelium allowing the shedding new infectious virus. LSIL support productive viral replication, however a number of high-risk HPV infections progress to HSIL. The progression to CC is associated with integrated HPV infection and subsequent over expression of the HPV E6 and E7 oncogenes (taken from Woodman, 2007).

1.4.3. HPV and carcinogenesis of the cervix

HPV DNA integration frequently consists of a partial deletion of the E4, E5, L1 and L2 gene, as well as the disruption of E1 and E2 leading to the over expression of E6/E7, and it is this process that results in the oncogenic properties of HR-HPV types (Munoz, 2006, Woodman, 2007). As E6 and E7 proteins are produced in the cells, tumour suppressor proteins, cell cyclins and cyclin-dependant kinases become deregulated, increasing immortalisation capacity (Southern, 2000). Both E6 and E7 are needed to produce and maintain a transformed phenotype, particularly due to their effects in interfering with apoptosis and cell cycle control (Munger, 1989, Watanabe, 1989). E7 binds to pRb preventing its binding to E2F, thus allowing for the expression of proteins involved in cellular proliferation. E7 is also known to interact with several other host proteins involved in cell cycle regulation including histone deacetylases components of the AP-1 transcription complex, and the cyclin-dependent kinase inhibitors p21 and p27 (Doorbar, 2005). The ultimate result of E7 activity is uncontrolled cellular proliferation (Figure 1.3). HPV E6 facilitates the effects of HPV E7 by relaxing cell cycle checkpoint controls (Martin *et al.*, 2006).

As a result of the HPV E6 and E7 induced uncontrolled cellular proliferation and the prevention of host cell apoptosis, genetic alterations that occur during the cell cycle are not repaired as efficiently and mutated DNA can accumulate, which leads to fully transformed cells and invasive cancer (Steenbergen *et al.*, 2005).

1.4.4. HPV epidemiology

Surveys to determine HPV prevalence are being undertaken in several countries to determine factors associated with HPV persistence, clearance, and recurrent infections. It is important that individual populations have information on the age- and type-specific prevalence of HPV infection. It is thought that around 80% of sexually active women will be infected with HPV within their lifetime (Jenkins, 1996). The prevalence of HPV is known to vary greatly in populations worldwide from as low as ~3% in Southern Europe to as high as ~44% in certain parts of Africa (Bosch and de Sanjose, 2003) Infection with HPV 16 is the most common type of HPV infection followed by HPV 18, 31, 45, 35 and 58 worldwide (Munoz, 2003). Table 1.2 (modified from De Vuyst *et al.*, (2009) meta-analysis) shows the HR-HPV prevalence rates in several populations throughout Europe. The lowest HPV population prevalence reported is 3% (HR-HPV 2.2%) in a cohort of Spanish women (n=973, average age 43 years) (de Sanjose *et al.*, 2007). De Sanjose *et al*, (2003) reasoned that the very low prevalence of HPV was due to the fact that the population was largely monogamous. Data relating to HPV prevalence and type distribution data in the Irish population is confined to 2 studies (Menton *et al.*, 2009, Keegan *et al.*, 2007). The Menton *et al.*, (2009) study was carried out on a population of self referred Irish women with visible external genital warts presenting to a sexually transmitted disease clinic (not a screening population). The authors reported a HPV prevalence rate of 88% and a genotype distribution with HPV 6 being the most common, followed by HPV 11, HPV 16, HPV 18, HPV 33 and HPV 53. The study used the MY09/11 HPV primer set for the detection of HR-HPV, further details on this set of primers is discussed in Chapter 2 Section 2.0. The samples were genotyped for 16 HPV type, using a reverse hybridisation assay INNOGENTICS HPV genotyping assay (INNOGENTICS, Belgium), with version 2.0 (n=24 genotypes) of the assay being used

in cases where the original assay could not detect a HPV genotype. A recent study conducted in Ireland, from general practioners in the Dublin area found an overall total HPV prevalence of 19.8% in women attending routine cervical cytological screening (Keegan *et al.*, 2007). The prevalence of HPV infections was highest among young women and fell with age (Keegan *et al.*, 2007) a well established HPV infection trend (Schiffman, 1992). There is data however, suggesting that HPV virus can remain latent, reactivate or even be persistent as some studies have shown that there can be a second peak in HPV prevalence in women over the age of 35 (Giuliano *et al.*, 2005, Rassu, 2005).

It should be noted that HPV prevalence varies greatly (nearly 20 times difference) in different populations (Clifford *et al.*, 2005). Studies from the United Kingdom show that HR-HPV prevalence can vary greatly in different sub-populations, from 7.1-15.7% (Peto *et al.*, 2004, Cuzick *et al.*, 2003, Hibbitts *et al.*, 2006, Sargent *et al.*, 2008, Cuschieri *et al.*, 2004a). Some of the differences can be attributed to the age distribution of the populations. It has been suggested that there is sufficient evidence from population based studies to suggest that HPV epidemiology is required on a population by population basis (Cuschieri *et al.*, 2004a). For this reason table 1.2 has been modified and updated from the De Vuyst *et al.,* (2009) meta-analysis of HPV prevalence in Europe to show HR-HPV prevalence data from studies carried out using the Hybrid Capture 2 (Qiagen Ltd.) assay. Table 1.3 also shows HPV prevalence data from Europe where other HPV testing technologies have been used. The data shown in table 1.2 and table 1.3 further enhances the need for population by population HPV testing as HPV prevalence is seen to vary in populations sampled using the same technologies. In addition, the overall HPV prevalence between different continents worldwide appears to vary. Clifford *et al* (2005)

compared HPV distribution and HPV type distribution in cytologically normal women using published data representative of populations in Africa, Asia, South America, and Europe, concluding that HPV infections are most prevalent in Africa (25.6%) and South America (14.3%) when compared to Asia (8.4%) and Europe (5.2%). This corresponds to data relating to CC rates, which are significantly higher in developing countries when compared to developed countries, with ~88% of CC related deaths yearly occurring in the developing world (Ferlay *et al.*, 2010).

Table 1.2: HPV prevalence from the European Union and Switzerland using Hybrid Capture 2 (hc2).
(Modified from De Vuyst *et al.*, 2009)

Country	Reference	Study period	Age range (mean age)	Population source (exclusion criteria)	Women screened (N)	HPV test	HPV prevalence		
							HR-HPV %	HPV 16 % of HR-HPV pos	HPV I8 % of HR-HPV pos
England	(Sargent *et al.*, 2008)	2001-2003	20-64 (40)	Screening trial	24470	hc2[a]	15.5	31.2	12.3
England	(Cuzick *et al.*, 2003)	1998-2001	30-80 (42)	Screening trial	10358	hc2	7.6	-	-
Finland	(Leinonen *et al.*, 2008)	2003-2004	2S-65 (45)	Screening trial	16895	hc2	7.5	-	-
Denmark	(Nielsen *et al.*, 2008)	1991-1993	20-29/40-50	Screening trial	10544/1443	hc2[b]	17.9/4.4	29.0/19.0	11.9/0.0
Denmark	(Kjaer *et al.*, 2008)	2004-2005	15-93 (36)	Organised screening	11600	hc2[b]	22.8	26.2	11.9
Germany	(Klug *et al.*, 2007)	1998-2000	(42.7)	Organised screening	8101	hc2[a]	6.4	31.4	9.0
France	(Clavel *et al.*, 2001)	1997-2001	15-76 (34 median)	Organised screening	7932	hc2	15.3	-	-
Switzerland	(Petignat *et al.*, 2005)	2001-2002	13-96 (42)	Organised screening	7254	hc2	11.4	-	-
Italy	(Ronco *et al.*, 2008)	2003-2004	25-60 (42 median)	Screening trial	46900	hc2	8.4	-	-
Italy	(Rossi *et al.*, 2011)	2007-2008	25-64	Organised screening	3410	hc2	9.5	30.8	4.3

The hc2 assay detects HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and is not designed to detect LR-HPV types. [a]Genotyping on all hc2-positive samples using PGMY09/11. [b]Genotyping on all hc2-positive samples using LIPA.
CIN: cervical intraepithelial neoplasia; hc2: hybrid capture 2; HPV: HPV; HR-HPV: high-risk HPV; pos: positive.

Table 1.3: HPV prevalence from the European Union. (modified from De Vuyst *et al.*, 2009)

Country	Reference	Study period	Age range (mean age)	Population source (exclusion criteria)	Women screened (N)	HPV test	HPV prevalence		
							HR-HPV %	HPV 16 % of HR-HPV pos	HPV I8 % of HR-HPV pos
Wales	(Hibbitts *et al.*, 2006)	2004	20-65 (38)	Organised screening	9079	GP5+/6+	11.2	31.4	21.7
Scotland	(Cuschieri *et al.*, 2004a)	2000	16-78 (37)	Organised screening	3444	GP5+/6+	15.7	41.1	14.3
Ireland	(Keegan *et al.*, 2007)	2004-2005	16-72 (35)	Organised screening	996	MY09/11	19.8[a]	-	-
Sweden	(Naucler *et al.*, 2007, Silins *et al.*, 2005)	1997	32-38	Screening trial	6089	GP5+/6+	7.1	30.9	8.5
Netherlands	(Coupe *et al.*, 2008)	1999-2002	18-65 (43)	Screening trial	45362	GP5+/6+	5.6	32.5	9.9
Spain	(de Sanjose *et al.*, 2003)	1998-2000	14-74 (43 median)	Population-based Sample	973	GP5+/6+	2.2	42.9	0.0
Poland	(Bardin *et al.*, 2008)	2006	18-59	Population-based Sample	897	GP5+/6+	11.3	33.0	6.4
Greece	(Agorastos *et al.*, 2004)	2000-2001	17-67 (43)	Organised screening	1296	PGMY09/11	2.5[a]	18.7	
Belgium	(Arbyn *et al.*, 2009)	2006	14-97 (42)	Organised screening	9297	RT-PCR[b]	15.2	24.4	10.2
Spain	(Martorell *et al.*, 2010)	2007	18-64	Organised screening	1956	GP5+/6+ and MY09/11	9.3%	3.9	0.6

[a]Overall HPV prevalence, as it was not possible to separate low-risk from high-risk HPV infections. [b]Multiplex TaqMan-based real-time quantitative PCR. CIN: cervical intraepithelial neoplasia; HPV: HPV; HR-HPV: high-risk HPV; pos: positive.

The reported HPV prevalence in North America (USA and Canada) also varies, possibly due to differing study guidelines and region specific differences in sexual behaviour. The Centers for Disease Control and Prevention (CDC) study, the National Health and Nutrition Examination Survey (NHANES) reported a HPV DNA prevalence rate of 26.5% using the line blot assay, (LIPA, Roche diagnostics) on self-collected cervical swab samples from a screening population of women in the United States aged 14-59 years (Dunne *et al.*, 2007). An earlier study on HPV DNA prevalence (MY09/11) in women attending for routine testing, showed a very high rate of 39.2%, however this study had a young age profile as it only tested women 18-40 years (Peyton *et al.*, 2001).

It is therefore important to take care when comparing HPV DNA prevalence rates as variables such as, age profile of the populations examined, the HPV tests used, and social factors, can cause differences in HPV rates and must be taken into account. In relation to the HPV tests, it is interesting to note that due to the reclassification of HR-HPV genotypes care must be taken in the development of HPV tests for use as a tool in primary screening (Castle, 2009). Castle (2009), questioned whether HPV testing technologies for use in CC screening should include the group of possible carcinogens outlined in the Schiffman *et al* (2009) paper. It was also suggested that future technologies for the detection of HPV in a primary setting may need to be tailored to detect genotypes specific for different populations (Castle, 2009).

Not only does prevalence vary in different populations, it has been shown that HPV genotype distribution can also vary greatly in different populations. The meta-analysis by Clifford *et al*, (2005), listed the top genotypes (HPV 16, 42, 58, 31, and 18) found worldwide in cytologically normal women. Worldwide there are differences in the

relative percentage of each HPV genotype. However, the same HPV genotypes occur in the top few types found in different areas worldwide; Africa; HPV 42 (11%), HPV 16 (8%), and HPV 35 (8%). Asia; HPV 16 (14%), HPV 33 (6%), and HPV 18 (5%). South America; HPV 16 (15%), HPV 58 (7%), and HPV 18 (5%). Europe; HPV 16 (21%), HPV 31 (9%) and HPV 18 (5%) (Clifford *et al.*, 2005). The HPV prevalence study carried out in Dublin stated that HPV 16 followed by HPV 18, 66, 33, 53, 31 and 58, were the most commonly detected HPV types (Keegan *et al.*, 2007).

Data relating to the expression of messenger RNA (mRNA) from oncogenic HPV E6/E7 may allow for the detection of those women most at risk of CC (Molden *et al.*, 2005a). To date there have been few papers detailing HPV mRNA prevalence, with the majority of papers focused on high grade disease in a bid to determine possible uses of mRNA test technologies. A study carried out on women less than 30 years showed that HPV mRNA prevalence (14.5%) was significantly lower that HPV DNA (32.2% and 20.8%) in a population without any HSIL (Molden *et al.*, 2006). HPV mRNA prevalence has also been detailed in women over 30 years with the prevalence reported at 3.0% with no significant difference when compared to type specific PCR (~4%), and lower than consensus PCR results (~10%).

Several commercially available tests have been developed to test for HPV in cervical cytology samples, many of which are responsible for the data provided in this section and a review of HPV testing technologies is discussed in Chapter 2 of this thesis and will be further discussed there.

1.4.5. HPV testing in cervical screening

There are three commonly suggested scenarios for the use of HPV testing in the detection of cervical disease; HPV testing as an adjunct to cytology for the triage of ASCUS and LSIL women, HPV testing as an adjunct to cytology for the triage of older women and HPV testing as a test of cure following treatment in a colposcopy setting.

The role of HPV testing in the management of women with LSIL and ASCUS was determined in ALTS studies (ALTS, 2003a, ALTS, 2003b). In the LSIL study HPV positivity rate was greater than 80% and therefore the utility of HPV testing in LSIL was considered impractical. Thus a separate strategy for the management of this group of women would need to be produced (ALTS, 2003a). HPV DNA testing in ASCUS was shown to detect 92.4% of HSIL cases, thus proving effective as triage for detecting HSIL, while reducing the number of women presenting to colposcopy by half (ALTS, 2003b).

HPV DNA testing has already met approval as an adjunct to cytological screening in women ≥30 years in the USA, allowing women who test negative for both cytology and HPV DNA to be tested with less frequency (Wright, 2004). In the UK, the HART (HPV in Addition to Routine Testing) study showed that HPV testing could be used for primary screening of women over the age of 30 years, with cytology used as triage on those who test HPV positive. However they noted problems with testing women under the age of 30 due to the high infection rates in younger women, and the relatively high costs of HPV testing as opposed to cytology (Cuzick, 2003). Similar results have been shown in the ARTISTIC trial in the UK where it was determined that HPV testing was practical in triage of women ≥30 years but that the use of testing in women <30 years would be impractical (Kitchener *et al.*, 2006). HPV testing plays a role in the management of

women following colposcopy. It has been shown that following treatment for high grade disease HPV testing with Hybrid Capture II (hc2) (Qiagen Ltd) in conjunction with cytology could be used to determine women at increased risk of recurrent disease (Zielinski, 2003). The authors showed that those women who test negative by both cytology and HPV DNA testing at 6 months post treatment appeared to be at no more risk than normal women in the general population.

It has been suggested that testing for integrated HPV infection through detection of HPV E6/E7 mRNA may provide better results when used under the above scenarios. Several studies suggest that HPV mRNA testing is more specific than HPV DNA testing for the detection of HSIL and high grade disease (Keegan *et al.*, 2009, Molden *et al.*, 2005a, Molden *et al.*, 2005c). Due to the high prevalence of HPV DNA in younger women HPV mRNA testing may be a more useful tool for HPV screening in this population (Molden *et al.*, 2006).

There is also evidence that screening could be stopped in women over the age of 50. It is noted however that this is only in countries with screening programs and where the women's HPV history is well documented. Before this could occur however, countries would have to include HPV testing in cervical screening programmes and the nature of HPV infection in older women would need to be better understood (Baay *et al.*, 2004).

1.5. HPV vaccination strategies

Currently, there are two prophylactic HPV vaccines which have been licensed for use in CC prevention; GARDASIL® (Sanofi Pasteur MSD) for the immunisation of women

between the ages of 9-26 against HR-HPV types HPV 16 and HPV 18 and against LR-HPV types HPV 6 and HPV 11, and the Cervarix vaccine (GlaxoSmithKline Biologicals) against HPV 16 and HPV 18 (FDA, 2010). In Ireland both vaccines have been given a license for public distribution, and a course of vaccinations lasts 6 months in which the individual receives three injections (HIQA, 2008). In September 2010 the National Vaccination Programme was launched in school to provide all girls entering first and second year in the secondary school system the vaccine course free of charge. To date therapeutic vaccines have not been developed with sufficient efficacy to be considered for immunisation programmes (Roden, 2006).

The currently available vaccines as mentioned confer immunity against the 2 most common HR-HPV types, HPV 16 and 18 responsible for approx 70% of invasive cancers (Smith *et al.*, 2007). Thus the introduction of HPV vaccines will not eliminate the need for CC screening programmes.

1.6. Human immunodeficiency virus

Human immunodeficiency viruses are lentiviruses of the family Retroviridae. There are 2 major forms of HIV, Human immunodeficiency virus type 1 (HIV-1) and Human immunodeficiency virus type 2 (HIV-2). Infection with HIV-1 is the more common of the 2 infections with HIV-2 infection being mostly confined to areas of Western Africa (Reeves and Doms, 2002). It is also known that the HIV-1 is more virulent and transmissible than the HIV-2 virus, with lower virus loads in asymptomatic infection and slower disease progression being noted in the latter (Reeves and Doms, 2002). HIV infection is known to lead to acquired immunodeficiency syndrome (AIDS). AIDS is the

endpoint of HIV infection where the virus has caused the lowering of host immunity by reducing the number of immune cells in the body. HIV infects the CD4 T cells, macrophages and dendritic cells of the human immune system. AIDS is defined by the failure of the host immune system which allows for opportunistic infections which can lead to the death of the host.

1.6.1. The role of HPV and CC in HIV

HIV positive individuals are at greater risk of developing various cancers such as CC and lymphomas (Boshoff, 2002). HIV positive women are up to seven fold more at risk of developing CC in comparison to the general population. This is most likely related to persistent HPV infection (Stern, 2005). Indeed, in HIV infection, immunosupression is associated with higher rates of LSIL progression to HSIL, although there is no evidence of an association with HSIL progression to cancer (Palefsky, 2007). HPV infection is higher in HIV positive populations compared to HIV negative populations of a similar nature. It has been noted that HPV DNA prevalence is higher in HIV-positive women from Africa (56.6%) and South/Central America (57.3%) when compared to data on HIV positive cohorts from Europe (32.4%), Asia (31.1%) and North America (31.4%), a pattern seen in general female populations from the corresponding regions (Clifford *et al.*, 2006). HPV 16 is relatively less prevalent in HIV positive women (Clifford *et al.*, 2006).

HIV-positive women with high grade cervical disease have a greater proportion of HPV types other than HPV 16 with increased multiple HPV infection rates (Clifford *et al.*, 2006). Several studies have shown that HPV DNA prevalence is higher in HIV positive

populations compared to general populations (Luchters *et al.*, 2010, Tornesello *et al.*, 2008).

The widespread use of anti-retroviral therapy has led to a decrease in many opportunistic viral related malignancies including Kaposi's sarcoma, however this phenomenon has generally not been seen in cervical malignancies (Adler, 2010, De Vuyst *et al.*, 2008). This may be related to the longer life expectancy in HIV positive patients provided by anti-retroviral treatment, the increased cumulative exposure to HR-HPV and increased accumulation of genetic mutations required for oncogenesis could lead to an increase in CC in HIV positive cohorts (Sirera *et al.*, 2007). However, anti-retroviral treatment has been shown to reduce the incidence of SIL and of HPV infection (Minkoff *et al.*, 2010). A correlation has also been shown between high rates of HPV DNA in severe cases of immunosupression (Levi *et al.*, 2002).

There are several mechanisms to explain the increased prevalence and aggressiveness of HPV infection is HIV positive patients. These include the interaction between the two viruses, the weakened immune response, and genetic instability. It is clear that the immune response to HPV in immunosupressed patients is weak. This may in part be due to the fact that HPV virus tends to infect epithelial cells, while HIV is systemic and may alter the immune response to HPV.

Secondly the HIV virus may interact directly with HPV virus. It has been demonstrated that the HIV-1 Tat protein can increase HPV 16 E6 and E7 oncogenic expression in HPV 16 positive human cells (Kim *et al.*, 2008). The HIV-1 Tat protein can also enhance the

proliferative capacity of HPV 16 positive cells in vitro (Kim *et al.*, 2008). This result suggests that HIV could play direct role in HPV related disease in HPV positive patients.

Thirdly, genetic instability can be a major contributing factor to HPV infection and CC progression (Martin, 2007). HPV viral integration into the host genome induces genomic instability through the interaction of its viral oncoproteins E6 and E7 with a number of cell cycle regulatory proteins, including p53 and retinoblastoma protein (pRb) respectively. While HPV E7 expressing cells tend to show numerical chromosomal abnormalities, HPV E6 expression is more commonly associated with structural alterations such as translocations or deletions. A number of the documented chromosomal abnormalities in CC have either been associated with the site of HPV viral integration or located within fragile sites (Martin *et al.,* 2006). Among the most commonly detected genetic aberration in CC is gain of chromosome 3q (Martin *et al.,* 2006).

1.6.2. HIV structure and genome

HIV is approximately 120 nM in size, composed of two copies of the single-stranded RNA genome surrounded by a cone-shaped core and a spherical capsid made up of 2,000 copies of the viral protein p24 (Gelderblom, 1991, Welker, 2000). The capsid is surrounded by a matrix of p17 viral protein and the viral envelope (lipid membrane) (Figure 1.4). The Env glycoprotein complex allows for the interaction of the virus with host cells and is embedded in the viral envelope. Inside the capsid genomic RNA is bound to the proteins required for replication (Figure 1.4). The HIV genome exists as a single-stranded RNA genome approximately 9 kb. The genome contains 9 genes that encode 15 different proteins, the structural proteins (Gag, Pol, and Env), regulatory proteins (Tat and

Rev), and accessory proteins (Vpu, Vpr, Vif, and Nef). The Gag region of the genome encodes the p55 protein which is cleaved to p17, p24, p7, and p6 proteins (Freed, 1998). The Pol region of the genome encodes the viral enzymes protease, reverse transcriptase, RNAse, and integrase used in the early stages of the life cycle allowing integration into the host genome. The Env region produces the glycoprotein's found on the surface of virions. A precursor gp160 is produced and processed into the external glycoprotein gp120 and the transmembrane glycoprotein gp41 (Freed, 1989). The Tat and Rev regions encode proteins responsible for regulation of HIV gene expression. Tat has two forms, Tat-1 and Tat-2 proteins while Rev encodes one 19kDa phosphoprotein. The Vif encodes for a protein that promotes the infectivity viral particles (NIAID, 2008).

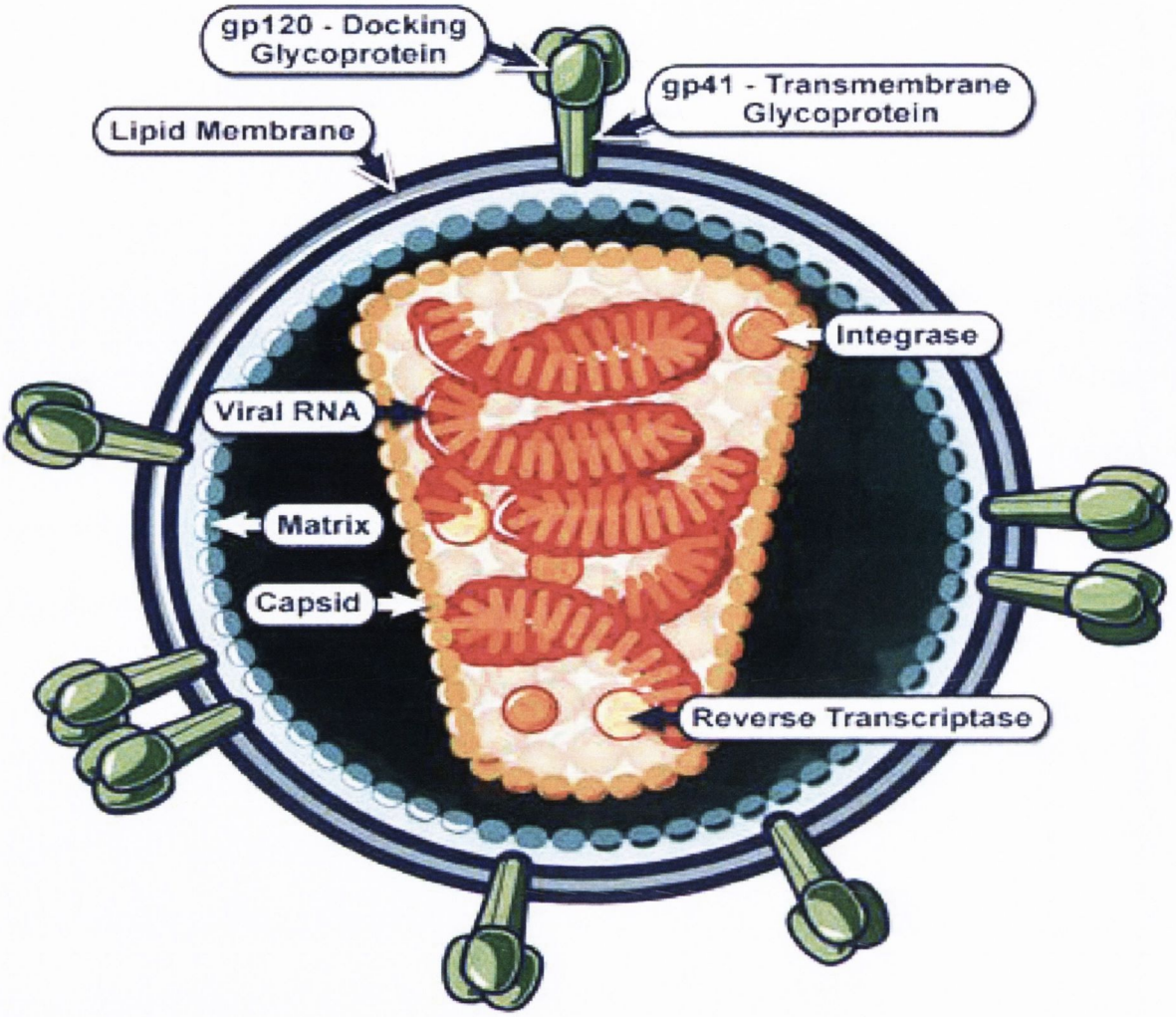


Figure 1.4: Structure of the Human immunodeficiency virus. The HIV virus is a RNA retrovirus. The viral RNA is surrounded by a cone and spherical shaped protein capsid. The enzymes required for the replication of the RNA genome is contained within the capsid. The Spherical capsid is surrounded by a Lipid membrane. (Taken from The United States Department of Health, National Institute of Allergy and Infectious Disease website, www.niaid.nih.gov, accessed 15-08-2010)

1.6.3. HIV life-cycle

HIV is transmitted through exposure to bodily fluids of an infected individual (Hladik and McElrath, 2008). Once inside the host, the HIV particles gain entry to macrophages and CD4 T-cells by the interaction of the viral surface glycoprotein complex gp160, which contains binding sites for receptors (CD4 and chemokine receptors) on the target cells. The viral membrane fuses with the cell membrane and the capsid is released into the target cell (Chan, 1998, Wyatt, 1998). The genomic RNA is then transcribed into double stranded viral DNA (vDNA) by the activation of reverse transcriptase. The vDNA is transported to the nucleus of the host cell where it is integrated into the host genome by the activation of integrase. The integrated DNA is then copied to mRNA; the host cell enzymes splice the mRNA producing the template for the Tat and Rev proteins. As mentioned Tat and Rev are regulatory proteins and their activation inhibits further splicing. The inhibition of this splicing is necessary to allow the transcription of Gag and Env from the full length mRNA. The mRNA then binds to the Gag protein and forms the new virion. The produced Env protein is cleaved by the host into the gp41 and gp120 proteins. At the surface of the host cell the viral proteins along with the HIV genomic RNA gather to form a new virion which buds from the host cell (Benaroch *et al.*, 2010). HIV proteases then cleave polyproteins forming, proteins and enzymes required for infection, reverse transcription and integration of another cell (Figure 1.5).

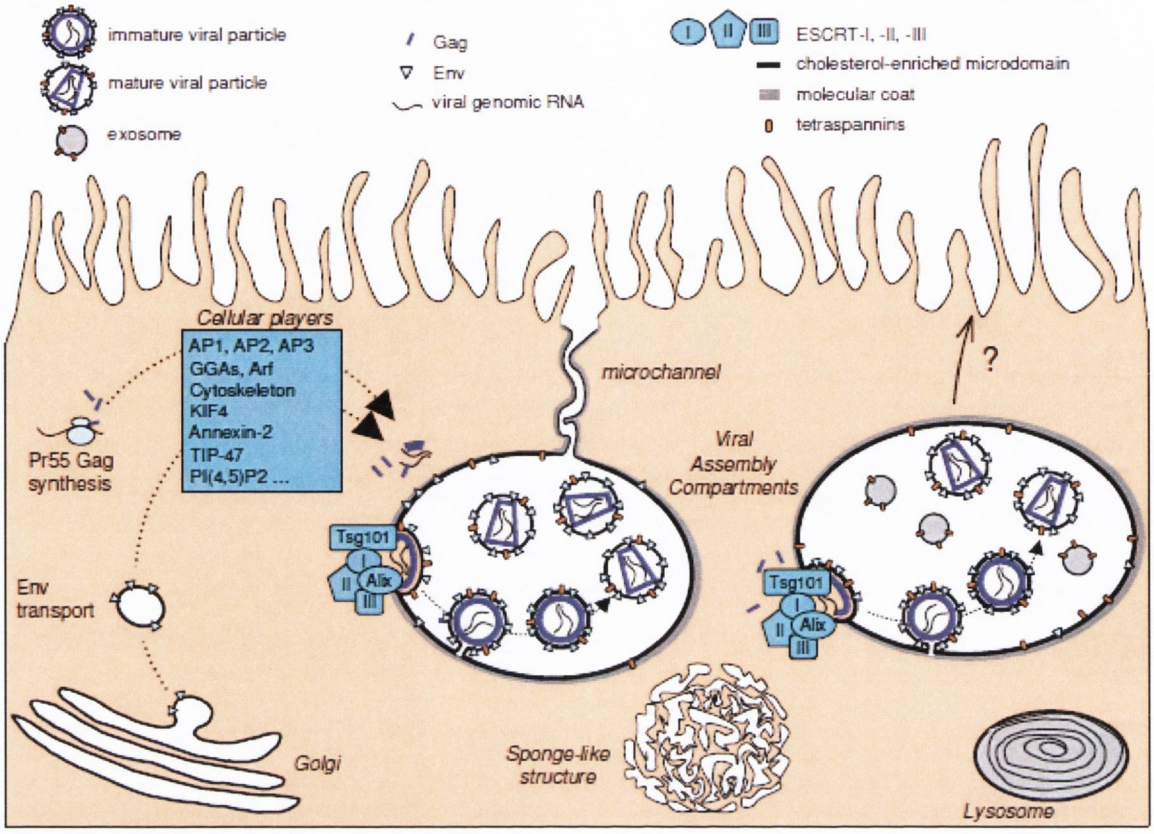


Figure 1.5: HIV assembly in macrophages. The viral genomic RNA transcribed in the nucleus is exported to the cytoplasm. The transmembrane envelope (Env) protein is produced in the endoplasmic reticulum and transits through the Golgi apparatus while Gag is synthesized on free cytosolic ribosomes. Both Env and the Gag precursors are targeted to the assembly site through unidentified pathways. The main cellular factors suspected to play a role in these trafficking events are indicated; nevertheless most of the time their roles have still to be established in macrophages. The assembly process requires the hijacking of host cell factors. The assembly compartment can be connected at least transiently to the plasma membrane through thin microchannels that do not allow virion passage (reproduced from Benaroch *et al.*, 2010).

1.6.4. HIV virus epidemiology

The 2008 World Health Organisation (WHO) and The United Nations Joint Programme on HIV/AIDS (UNAIDS) estimate that 33 million people are living with HIV infection. The vast majority of HIV cases are found in sub-Saharan Africa (22 million cases) representing two thirds of cases worldwide, followed by south-east Asia (4.2 million) (WHO/UNAIDS, 2008) (Figure 1.6). The incidence of new HIV infections is occurring at a rate of approximately 2.7 million cases each year which is up from the 2007 estimates of 2.5 million new cases per year. The majority of new HIV cases (96%) each year occur in developing countries with 45% of all infections occurring in those aged 15 to 24 years (WHO/UNAIDS, 2008). It has been speculated that a large proportion of those infected with HIV (especially in sub-Saharan Africa) (24-27% in the USA) may not know they are HIV positive. (Glynn and Rhodes, 2005).

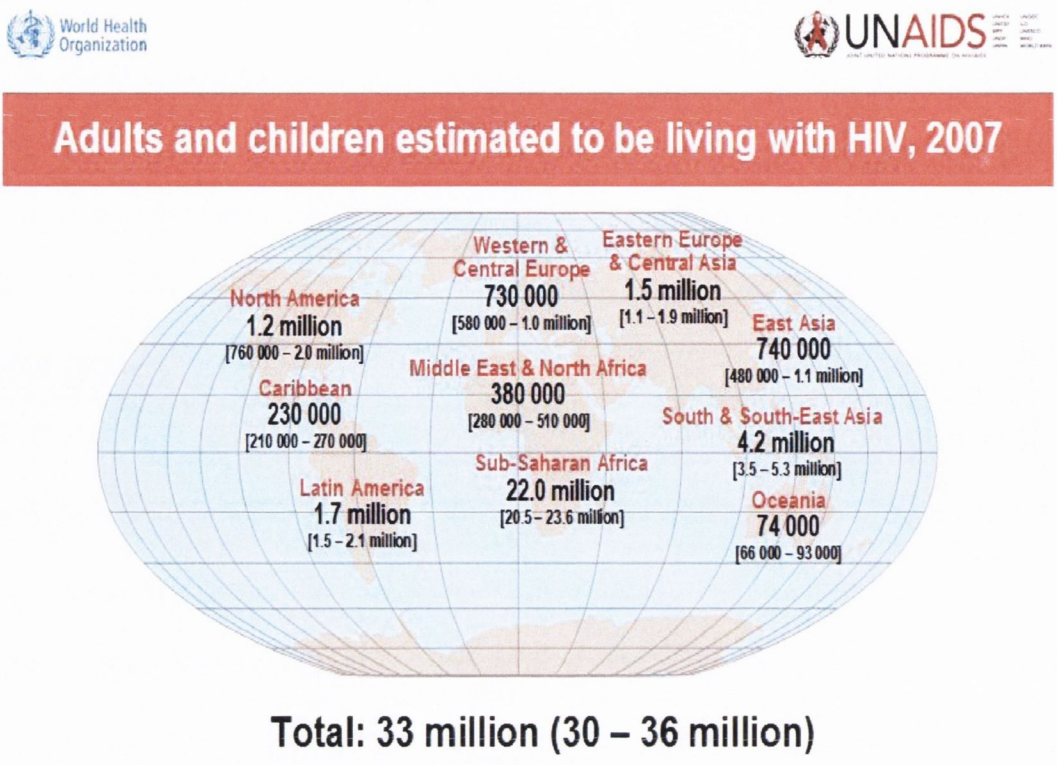


Figure 1.6: HIV positive adults and children estimated to be living with HIV. The prevalence of HIV is highest in sub-Saharan Africa with ~66% of the total sufferers worldwide (WHO/UNAIDS, 2008).

1.7. Hypothesis

HPV DNA prevalence in Ireland will be similar to that in the UK and the rest of Europe, even in the context of the lack of an established screening programme. The specific HPV genotypes distribution may differ somewhat within the island of Ireland and may be region specific. HPV DNA and mRNA prevalence rates in HIV positive women residing in Ireland will be higher than in general populations. Anti-retroviral therapy will reduce the prevalence and persistence of HPV infection in HIV positive women.

1.8. Aims

- To review the current HPV molecular testing technologies.
- To determine HPV DNA prevalence rate in the Irish cervical screening population.
- To determine HPV genotype profile in the Irish cervical screening population.
- To determine regional/population specific differences if any in the Irish cervical screening population.
- To determine the prevalence and persistence of HPV DNA and mRNA in HIV positive women residing in Ireland.
- To determine the effects of anti-retroviral therapy on HPV DNA and mRNA infection.

Chapter 2

HPV Molecular Testing Technologies

2.0 HPV Molecular Testing Technologies

HPV molecular testing has focused traditionally on two main areas which provide information to aid in the diagnosis of cervical disease, namely HPV DNA (detection and genotyping) and HPV mRNA testing. A list of the HPV DNA detection technologies to be discussed in this review chapter can be seen in table 2.1, with HPV mRNA technologies listed in table 2.2. Unlike other biological infections, HPV cannot be cultured in vitro. As a result molecular HPV detection technologies were developed, with PCR based the most commonly used. Two approaches to PCR based detection are; Consensus PCR and type specific PCR. The consensus PCR approach allows simultaneous amplification of a particular gene region across multiple HPV types. There are 3 well described consensus PCR systems that form the basis of most commercially available HPV tests today. These include the GP5+/6+ +, MY09/11 and PGMY09/11 (a modified MY09/11).

The MY09/11 primers are degenerate primers (primers with similar sequences) made up of 24 oligonucleotide sequences that amplify over 30 different HPV types (Bernard *et al.*, 1994). The GP5+/6+ primer set was developed from an original set of general primers (GP5/6) through elongation at the 3' end to reduce background amplification and increase specificity and sensitivity (de Roda Husman *et al.*, 1995). The GP5+/6+ primers amplify a 150 bp region within the 450 bp MY09/11 product. The GP5+/6+ primers are only complementary to a few HPV genotypes, and to account for this, annealing is performed at lower temperatures to allow for less stringent binding (de Roda Husman *et al.*, 1995). The use of a nested PCR approach using (MY09/11 product, and GP5+/6+) can improve sensitivity of HPV detection (Strauss *et al.*, 2000, Husnjak *et al.*, 2000).

Table 2.1: HPV DNA detection technologies.

HPV assay	Specimen input	Nucleic acid extraction method	Assay type	HPV genotypes detected	Detection limit	Recent published data Sensitivity/Specificity CIN 2+
hc2 HR HPV test (Qiagen Ltd)	4 mL PreservCyt	Sample conversion kit (Qiagen) or QiaSymphony automated extraction (Qiagen)	Hybridisation assay using whole genome probes	Positive or negative result. HR-HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	5000 copies/mL	95.8%/39.6% (CIN 2+ n=402) (Ratnam *et al.*, 2010)
hc2 HPV 16, 18, 45 Probe set (Qiagen Ltd)				Detects individual types 16, 18, 45		n/a
Digene HPV genotyping RH test (Qiagen Ltd)	200uL PreservCyt	QiaSymphony and EZ1virus mini kitv2 or QIAamp MinElute Virus Spin Kit	Reverse hybridisation based on GP5+/6+ L1 gene amplicon hybridisation	Detects individual types. 16,18,26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	4-100,000 copies	n/a
HPV genotyping LiquiChip Test RUO (Qiagen Ltd)	200uL PreservCyt	QiaSymphony and EZ1virus mini kitv2 or QIAamp MinElute Virus Spin Kit	Xmap technology HPV genotype specific bead based hybridisation of Gp5+/6+ amplicon	Detects individual types. 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	1-10,000 copies	n/a
Cervista (Hologic and Third Wave technologies)	2 mL PreservCyt	Genfind DNA extraction kit (Hologic)	L1 Invader™ technology	Detects 3 HR-HPV groups HR-HPV (51,56 and 66), (18, 36, 45, 59 and 69), (HPV 16, 31, 33, 35, 52 and 58)	1250-7500 copies per reaction	92.8% /44.2% (CIN 2+ n=69) (Einstein *et al.*, 2010)
Full-spectrum HPV amplification and detection system (GenoID)	250 µL PreservCyt	Amplilute extraction kit (Roche)	Consensus L1 PCR and HR/LR ELISA	Detects 3 different groups HR-HPV group 16,18,31,33,35,39,45,51,52,56,58,59,66,68. LR-HPV 6,11,42,43,44/55. Non-classified group. 2,3,7,10,13,26,27,28,29,30,34,40,53,54,57, 61,67,70,72,73,74,81,82,83,84,85,89,90,91	2-97 copies per reaction	~95%/~66% (CIN 2+ n=16) (Jeney *et al.*, 2009)

Table 2.1: HPV DNA detection technologies continued.

HPV assay	Specimen input	Nucleic acid extraction method	Assay type	HPV genotypes detected	Detection limit	Recent published data Sensitivity/Specificity CIN 2+
GenoID real-time HPV assay (GenoID)	250 µL PreservCyt	Amplilute extraction kit (Roche)	Real-time molecular beacon PCR for L1 region	Dependant on kits and hardware, up to 15 HR-HPV and 5 LR-HPV types.	2 copies per reaction	~92%/~64% (CIN 2+ n=16) (Jeney *et al.*, 2009)
PapilloCheck (Greiner Ltd)	250 µL PreservCyt	Qiagen DNA mini kit (Qiagen) or Qiagen M48 Biorobot	E1 PCR to detect and slide hybridisation to genotype	Detects Individual types. HR-HPV 16,18,31,33,35,39,45,51,52, 53,56,58,59,66,68,70,73,82 LR-HPV 6,11,40,42,43,44	30-750 copies per reaction	96%/40% (CIN 2+ n=104) (Halfon *et al.*, 2009b)
Amplicor HPV test (Roche Biochemicals)	250 µL PreservCyt	Amplilute extraction kit (Roche)	Consensus L1 PCR and probe detection	Positive or negative result HR-HPV 16,18,31,33,35,39,45,51,52,56,58,59,68	100-240 copies per reaction	98.9%/21.7% (Total population n=949) (Szarewski *et al.*, 2008)
Linear Array HPV Genotyping Test (Roche Biochemicals)	250 µL PreservCyt	Amplilute extraction kit (Roche)	Specific L1 PCR and reverse line blot detection	Detects individual genotypes 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, CP6108	76-20000 copies/mL	98.2%/32.8% (Total population n=932) (Szarewski *et al.*, 2008)

Table 2.2: HPV mRNA technologies.

HPV assay	Specimen input	Nucleic acid extraction method	Assay type	HPV genotypes detected	Detection limit	Recent published data Sensitivity/Specificity CIN 2+
PreTect™ HPV-Proofer (Norchip) and the NucliSens EasyQ HPV (Biomerieux)	5 mL PreservCyt	Qiagen M48 Biorobot and total nucleic acid custom protocol with Magattract RNA cell mini kit Nuclisens EasyMag automated extraction	NASBA amplification with genotype identification for E6/E7 oncogene expression	Detects individual types 16,18,31,33,45	100 SiHa or CasKi cells (HPV 16) 5 HeLa cells (HPV18)	PreTect™ HPV-Proofer 71.4%/75.8% (CIN 2+ n=84) (Keegan *et al.*, 2009) NucliSens Easy Q 76%/63% (CIN 2+ n=37) (Halfon *et al.*, 2009a)
Aptima HPV Assay (Gen-probe)	1 mL PreservCyt	Aptima Target Capture System (Gen-probe)	Transcription mediated assay for E6/E7 oncogene expression	Detects individual types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	38-488 copies per reaction	56.2-*55.4%/98.8% (CIN 2+ n=753) (Dockter *et al.*, 2009)

*Automated detection

2.1. HPV DNA technologies

In this section, currently available HPV detection technologies that utilise DNA based strategies will be outlined. The type of technology, along with a brief description of the protocol, and relevant published data used in the individual assays will be discussed.

2.1.1. hc2 high-risk (Qiagen Ltd)

The hc2 system (Qiagen Ltd.) requires 4 mL of PreservCyt solution and uses RNA probes to hybridise to HPV DNA in solution. Following hybridisation, the DNA:RNA hybrids are captured onto the wells of a microplate coated in corresponding antibodies. The DNA:RNA hybrids are washed and a chemiluminesent substrate is added. The light emitted is measured using a Qiagen dedicated luminometer (Figure 2.1). The full hc2 protocol is outlined in Chapter 3, Section 3.5 of this thesis. The total time for the detection of 88 samples in a single run is approximately 6-7 hours. There are two different probe cocktails available for use with the hc2 system. The low risk kit is used to detect 5 LR-HPV types (6, 11, 42, 43, and 44) and the high risk kit is used for detection of 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The hc2 assay is currently being researched by Qiagen for full automation, from sample to results, with the equipment currently available to reduce the hands on time of the extraction steps.

The QIAsymphony system sample preparation/ assay setup (SP/AS) instrument has been developed to provide fully integrated automation of complete workflows, from sample preparation to assay setup. The system allows for the purification of DNA, RNA, and protein from a wide range of sample types including LBC cervical smears. Currently kits

are available with the QIAsymphony SP/AS for assay setup for many diagnostic tests and it is currently under development for the hc2 assay.

In 2003, the hc2 system became the first commercially available HPV detection kit to be approved by the FDA for use as an adjunct to cytology for ASCUS patients and in women over 30yr for detection of HR-HPV in cervical cytology samples (FDA, 2003, Wright, 2004). Since that time, the hc2 system has been used in many large scale studies to determine the role of HPV testing in primary screening and triage with cytology (ALTS, 2003a, ALTS, 2000, Castle *et al.*, 2004, Sargent *et al.*, 2008, Cuzick, 2003, Mayrand *et al.*, 2007). The assay is CE (Conformité Européene) marked. The sensitivity, specificity and limit of detection of the hc2 assay are shown in table 2.1. The assay cannot differentiate between different HPV types, and cross-reactivity with HPV types not included in the probe mix cocktail has been reported (Castle *et al.*, 2008b). As the hc2 assay is not based on target amplification (PCR), it has several advantages including reduced chance of contamination related to amplicon production, and no need for dedicated pre/post amplification facilities. The hc2 is well established as a HPV testing technology, and it has recently been suggest that the assay should be considered the gold standard of HPV testing performance (Meijer *et al.*, 2009).

A modification of the original hc2 test has been the development of a second generation hc2 genotyping test. The hc2 16, 18 and 45 assay (Qiagen Ltd), which specifically detects and genotypes samples for the 3 HR-HPV genotypes (HPV 16, 18, and HPV 45). Advantages of the assay over the original is that it is now cheaper, it allows for genotyping of the 3 most common HR-HPV types associated with CC and uses the same proven technology as the hc2 assay.

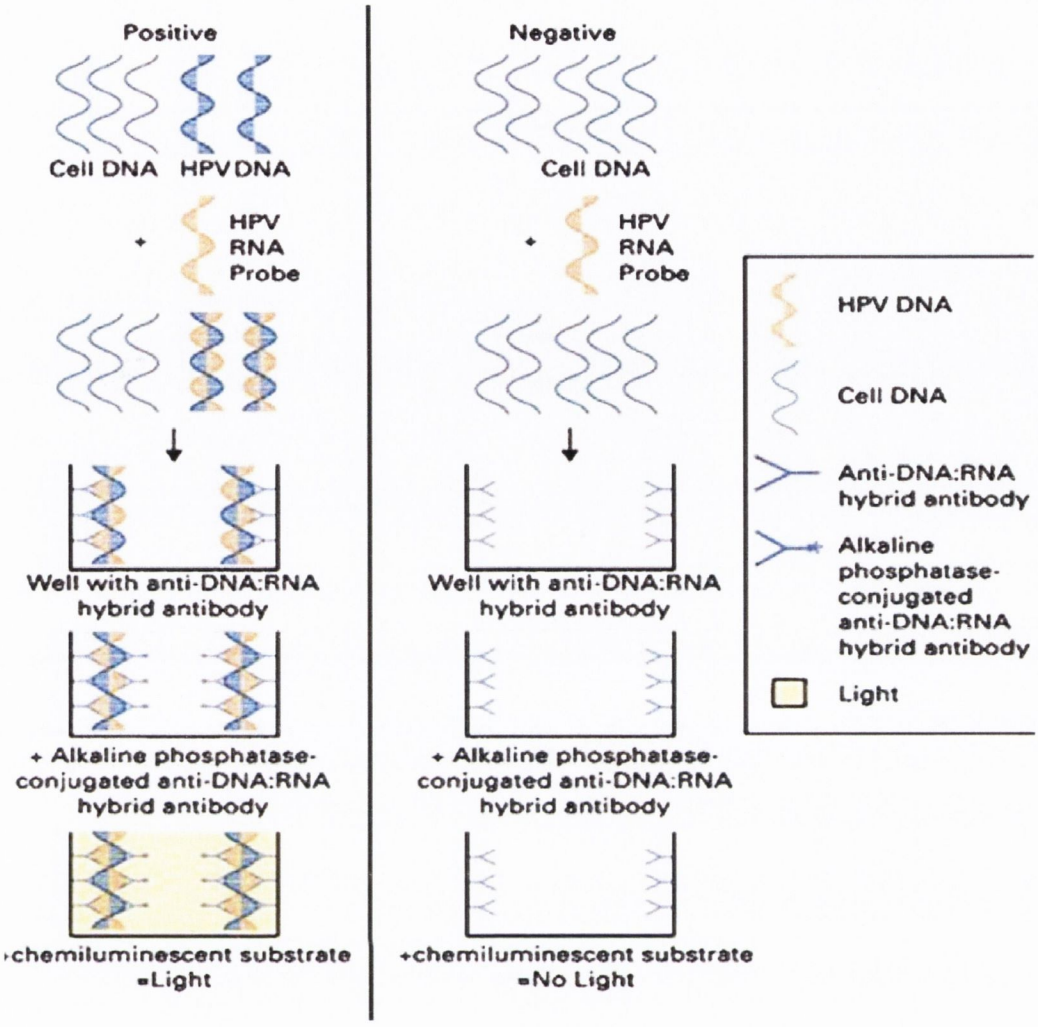


Figure 2.1: Schematic of the Hybrid Capture II assay chemistry. Target DNA combines with specific RNA probes creating RNA:DNA hybrids. The RNA:DNA hybrids are captured onto the sides of the wells which are coated in antibodies specific for RNA:DNA hybrids. Captured RNA:DNA hybrids are detected with multiple antibodies conjugated to alkaline phosphatase. This allows for signal amplification, and the bound alkaline phosphatase is detected with a chemiluminescent substrate. Upon cleavage by alkaline phosphatase, the substrate produces light that is measured on a luminometer in Relative Light Units (RLUs) (modified from http://www.merckmedicus.com, accessed 24-06-10).

2.1.2. Prototype Qiagen genotyping assays

There are a couple of genotyping assays currently undergoing clinical trials by Qiagen. The first is the Digene HPV Genotyping LQ Test which will provide rapid, reliable detection of 18 high-risk individual HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). The assay is currently semi-automated with the possibility of full automation in the future, and is based again on GP5+/6+ PCR. The assay uses the Qiagen LiquiChip Reader. DNA samples are aspirated, and added sequentially into the reader. Beads coated with capture probes specific for HPV DNA bind the target sequence. The bead:DNA complex binds to a reporter dye and fluorescent signals are recorded simultaneously and digitally processed to translate the signals into quantitative data for each reaction. There is currently no published data available.

The second assay is the Digene HPV Genotyping RH Test, an assay for the detection of 18 individual HPV types. The assay is similar to the Linear Array HPV Genotyping Test assay as it uses reverse hybridisation biotinylated amplicons that are hybridised with specific oligonucleotide probes, which are immobilized as parallel lines on membrane strips. HPV types are detected by visual inspection following a chromogenic reaction. Positive results show up as a purple line on the test strip. It has been reported that the assay has shown comparable results when compared to reverse line blot assays for the detection of HPV genotypes. The HPV Genotyping RH Test has reported good concordance with hc2 positive samples (86.6%) and negative women (97.9%). The assay is designed for lower volume of samples, compared to the Digene HPV Genotyping LQ Test. HPV Genotyping RH Test is now CE approved.

2.1.3. Cervista HR-HPV (Hologic and Third Wave Technologies)

Cervista HR-HPV technology has produced a HR-HPV detection assay, and a genotyping assay for the typing of HPV 16 and HPV 18. The Cervista HR-HPV assay requires 2ml of PreservCyt samples and uses Invader Probe technology (Third Wave, Ltd). Briefly 2 mL of PreservCyt specimen from 88 samples are added to a 96 well extraction plate. The approved extraction for use with the Cervista assay is the Genfind™ Extraction Procedure, a magnetic bead based silica extraction method. The cells are lysed, DNA is bound to magnetic beads, the beads undergo several washes to remove any contaminant and DNA is eluted from the magnets using 10 mM Tris buffer. The DNA is transferred to a standard 96 microplate and the Invader HPV reagents are added. Amplification using invader technology then occurs for 4 hr at 63°C. The Invader chemistry is composed of two simultaneous isothermal reactions; the first detects the specific DNA target sequence and generates a one-base overlapping structure if the desired sequence is present. This structure is created with the probe and the Invader oligo on the target. Cleavase enzymes specifically cleave the overlapping probes and release the 5' flaps plus one nucleotide. During the primary reaction, the probes cycle on and off the target, and more and more of the overlapping substrate is formed as cleavage occurs. The amount of the flaps released is relative to the amount of target DNA in the sample. The flaps cleaved in the primary reaction are then free to combine with a fluorescence resonance energy transfer (FRET) probe in a secondary and simultaneous occurring overlapping cleavage reaction. The cleaved FRET probes then generate the fluorescent signal (Figure 2.2) (Day *et al.*, 2009). Like the hc2 assay, the Cervista assay requires approximately 6-7 hours to provide results, however the hands on time is reduced as the Cervista amplification takes 4 hours. The Cervista system is a relatively new HPV technology, and received FDA approval in 2009. To date, Cervista assays and hc2 are the only FDA approved HPV tests. The use of

the Genfind™ extraction system allows for the extraction of 88 samples in a single run and the Cervista detection is performed on 32 samples simultaneously.

The detection assay has been developed from the HPV phylogenetic tree. The assay targets the A5/A6, A7 and A9 virus groups, which represent 14 HPV types [A5/A6 (HPV 51, HPV 56 and HPV 66), A7 (HPV 18, 36, 45, 59 and 69), and A9 (HPV 16, 31, 33, 35, 52 and 58). Thus the twelve columns of a microplate are separated into a group of four columns (32 wells) for each master mix. The Cervista assay is currently being developed by Hologic and full automation is expected in 2011. The Cervista HPV detection assay has a detection limit of 1250-7500 copies/reaction (for all 14 HPV types and the internal control) and the Cervista genotyping assay has a detection limit of 1250-2500 (HPV 16 and HPV 18), which are equivalent to that of the hc2. Unlike hc2, it has not been shown to cross-react with other HPV types (Day *et al.*, 2009). The performance of the system has been reported as comparable to that of the hc2 assay for the triage of women with ASCUS (Einstein *et al.*, 2010).

As signal is amplified as opposed to nucleic acid amplification found in PCR techniques, there is no requirement for specialised pre/post amplification hoods/areas, and the inclusion of a sample specific internal control ensures any negative results are not due to insufficient sample cellularity (Day *et al.*, 2009)

As mentioned (Chapter 1 Section 1.4), there has been a re-classification of HR-HPV types, with HPV 66 now considered a possible carcinogen, as such it has been noted that those HPV testing assays that include possible carcinogens may provide more false

positives if used in screening (Castle, 2009). However, it should be noted that HPV prevalence can vary from population to population, and it could be possible that there are isolated populations where possible carcinogens are important in the development of CC.

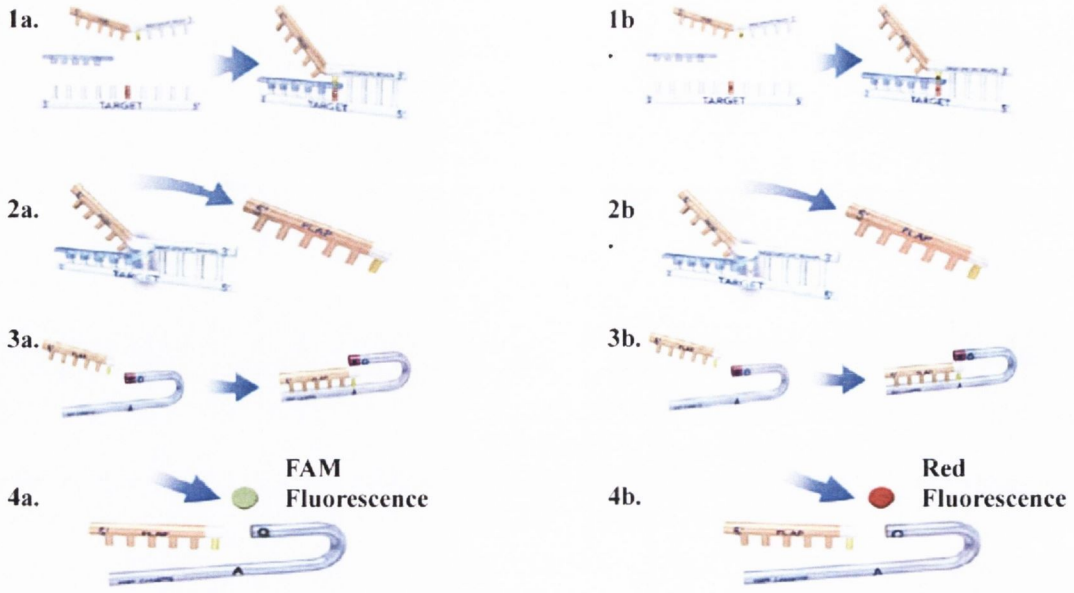


Figure 2.2: Cervista HPV DNA detection chemistry. **(1a.)** HPV oligos form invasive structure on the target HPV DNA (1b.) HIST2HBE oligos form invasive structure on the genomic DNA (control). **(2a.** and **2b.)** The Cleavase® enzyme recognizes the structure and cleaves probe oligos. **(3a.)** Flaps from the HPV probe oligos form invasive structure on the FAM FRET oligos. **(3b.)** Flaps from the HIST2HBE probe oligos form invasive structure on the Red FRET oligos. **(4a.** and **4b.)** The Cleavase® enzyme recognizes the structure and releases fluorophores from the FRET Oligos, creating the fluorescent signal. (adapted from Day *et al.*, 2009).

2.1.4. Amplicor HPV (Roche Biochemicals)

The Amplicor HPV test (Roche, Germany), a PCR based assay for the detection of HR-HPV DNA of 13 HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) Briefly, DNA is extracted from 250 µL of PreservCyt under denaturing conditions at elevated temperatures in the presence of proteinase K, as described in Chapter 3, Section 3.6.1. The Amplicor HPV test uses consensus PCR of the L1 region of the HPV genome, and of the β-globin gene (control) using biotinylated primers. The amplified DNA is chemically denatured to form single-stranded DNA. Amplicons are added to the wells of a micro well plate coated in capture probes for HR-HPV or the β-globin gene, to identify the viral or human DNA. Hybridisation to capture probes is carried out and HR-HPV DNA is detected following addition of streptavidin-horseradish peroxidase conjugate in a darkroom and a substrate solution containing hydrogen peroxide and 3,3',5,5'-TMB to allow the colorimetric reaction. The absorbance of the wells is detected at 450 nm, using an automated micro well plate reader and provides a positive or negative result for HR-HPV.

The Amplicor assay is carried out in a 96-micro well plate format, and has been validated for use on liquid based cytology media [(PreservCyt® (Hologic) or SurePath® (BD)]. Unlike the hc2 system, there is no "grey zone" as there is an amplification control. One of the major benefits of the Amplicor assay is the low volume of media required for the assay, and as such HPV diagnosis can be carried out after completion of liquid cytology screening, even in samples with extremely low cellularity that result in less residual sample. The Amplicor assay similar to the Cervista assay contains an internal control which ensures that negative results are not due to insufficient sampling. The assay has a limit of detection of 100-240 copies of HPV DNA per reaction. It has also been reported

that the Amplicor assay has similar sensitivity for cervical abnormalities compared to that of the hc2 assay (Monsonego, 2005). A recent study into several HPV testing techniques included the Amplicor assay and reported a high sensitivity (98.9%) and relatively low specificity (21.7%) when compared to other assays (Szarewski *et al.*, 2008).

2.1.5. PapilloCheck (Greiner Ltd)

The PapilloCheck® (Greiner Bio-one, Germany) assay is different from other HPV DNA detection and genotyping assays as it is based on the detection of a fragment of the E1 gene of 24 (HPV 6, 11, 40, 42, 43, 44, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82) different high and low-risk HPV-types. DNA is extracted from 250 µL of cervical smear specimen and then a fragment of the E1 gene is amplified by PCR. The PapilloCheck® assay includes an internal sample extraction control human gene Adenosine deaminase1 (ADAT1) for each sample. The amplified products are hybridised to specific DNA-probes fixed onto spots on a "low density DNA array-chip" and the bound DNA is fluorescently labelled. On each chip there is space to test up to 12 samples for the 24 different HPV types. The chips are washed to remove unbound DNA and the DNA-chip is automatically scanned and analysed. The software automatically produces a run report (Greiner Bio-one, Germany). Each sample has several controls (e.g. spot homogeneity of the DNA-array, sample preparation, DNA hybridisation and PCR) which allows for easy troubleshooting of test failures and helps to prevent false negative and false positive results. The PapilloCheck® assay workflow is shown in figure 2.3. The assay also has a detection limit 30-750 copies per reaction. There have been a few studies published on the PapilloCheck® assay since its recent release, suggesting that it produces comparable results for detecting HPV in cervical cytology samples as other established

HPV detection and genotyping assays (Dalstein *et al.*, 2009, Hesselink *et al.*, 2009, Halfon *et al.*, 2009b, Schopp *et al.*, 2010). The PapilloCheck® assay has also shown good sensitivity to detect HPV genotypes in LSIL and HSIL samples (Table 2.1) (Halfon *et al.*, 2009b, Jones *et al.*, 2009).

Figure 2.3: PapilloCheck® workflow. 1. Liquid based smear samples are taken. **2.** DNA is extracted (Qiagen or Roche extraction technology) from the smear samples. **3.** The DNA is amplified using a polymerase chain reaction (PCR) to amplify a part of the El gene of the 24 HPV types tested and also amplifying the internal human house keeping gene. **4.** Hybridisation and fluorescent labelling of the El PCR product is perform in a water vapour saturated atmosphere at room temperature. **5.** Three rapid and stringent washing steps are performed to remove unbound material. **6.** The DNA-chip is scanned using the CheckScanner™ software, a two-colour laser. **7.** The CheckReport™ Software automatically evaluates the controls and HPV specific signals and generates a report (taken from www.greinerbioone.com, accessed 12-08-10).

2.1.6. Full-spectrum HPV amplification and detection system (GenoID Ltd)

The Full-Spectrum HPV Detection and Genotyping Assays (GenoID Ltd) have been developed by GenoID® using an optimized primer set developed in 1991 (Yoshikawa *et al.*, 1991). It is claimed that "the new system outperforms in all features the original method and has a potential to be the most advanced HPV detection system available today" (GenoID, 2010). The assay uses a consensus PCR approach targeted at the L1 region and an ELISA detection for the genotyping of LR-HPV (HPV 6, 11, 42, 43 and 44/55), HR-HPV (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and non-classified HPV (HPV 2, 3, 7, 10, 13, 26, 27, 28, 29, 30, 34, 40, 53, 54, 57, 61, 67, 70, 72, 73, 74, 81, 82, 83, 84, 85, 89, 90, and 91) genotype groups. The HPV type specific ELISA genotypes for 14 HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

The assay is currently only configured to work on PreservCyt specimen (Cytyc Corporation). A 1.25 mL of sample is centrifuged to form a pellet and 250 µL of an internal control (tested in parallel) solution is added to prevent false negative results. DNA is extracted using Roche extraction technology (AmpliLute Liquid Media Extraction Kit or High-Pure PCR Template Preparation Kit, Roche Diagnostics). The PCR reaction is then carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems) with the provided master mix. The amplicons produced are detected in a solid phase hybridisation format for detection of the different HPV groups. The biotinylated PCR products are captured onto the surface of a microplate well coated with streptavidin. The plate is washed to remove the complementary chain from the captured product and hybridisation to specific fluorescein labelled probes is carried out. The bound probes are reacted with horseradish peroxidase (HRP) and Tetramethylbenzidine (TMB)

to produce a blue colour. For specific genotyping the PCR amplicon is used in the HPV type specific ELISA assay using the same methodology.

The assay is one of the most sensitive assays for detection of HPV with a limit of detection of 2-97 copies per reaction. There is little published data in relation to the Full Spectrum HPV Detection and Genotyping test (Jeney *et al.*, 2007). In the study the assay was compared to the hc2 assay for 81 samples, with 83.9% agreement between the assays. The discordant samples were sequenced to determine true positivity which resulted in an estimated sensitivity of 91.1% and estimated specificity of 100% for the assay for the detection of HPV (Jeney *et al.*, 2007). Preliminary results comparing the Full-Spectrum assay with several other HPV tests was presented in poster form at the 25th International Papillomavirus conference 2009, and the CIN 2+ sensitivity and specificity are estimated in table 2.1 (Jeney *et al.*, 2009). The assay has received CE approval and trials are currently underway to determine the sensitivity and specificity in determining differing grades of cytology and histology, in larger study populations.

2.1.7. GenoID Real-Time HPV Assay (GenoID Ltd)

The assay is designed for detection of a ~150 bp. region of the L1 gene of up to 15 HR-HPV and 5 LR-HPV types. The assay is only configured to work on PreservCyt (Cytyc Corporation), however it has been developed to work with several PCR systems, the Roche lightcycler® 2.0, Applied Biosystems 7900HT, Applied Biosystems StepOnePlus™ and the Corbett Life Science Rotor-Gene 6600. Depending on the system used different groups of HPV types are detected. The Roche Lightcycler® 2.0 detects 19 HPV types in 2 channels. The separate channels detect; (1) HR-HPV (HPV 16, 18, 26, 31,

33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), and (2) LR-HPV (HPV 6, 11, 42, 43, and 44) genotypes. The two Applied BioSystems PCR systems detect 14 HR-HPV types in 2 channels. The first channel detects HR-HPV types 16 and 18, and the second channel detects 12 other HR-HPV types (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). The Corbett Rotor-Gene 6600 system detects 14 HR-HPV genotypes in 3 different channels. The first channel detects HPV 16 and 18, the second channel detects HPV 31, 33, 45, 52 and 58 and the third channel detects the seven remaining HR-HPV types (HPV 35, 39, 51, 56, 59, 66 and 68. The variation in the kits provided allows for labs to decide on a particular system that suits their needs. The Roche Lightcycler® 2.0 allows for the detection of the LR-HPV types, where as the Applied BioSystems allows for the differentiation of HR-HPV types 16 and 18 from other HPV types, and the Corbett system allows for the differentiation of infections into 3 groups "semi-genotyping".

The system uses the same extraction and amplification procedure outlined above for the GenoID® Full-spectrum HPV amplification and detection system.

The assay is reported as very sensitive for the detection of HPV with a lower limit of detection of 2 copies per reaction. The assay has been validated against the Full-Spectrum HPV Detection and Genotyping Assays (Takacs *et al.*, 2008) and 89.4% concordance between the assays has been reported. The real time assay has estimated sensitivity of 95.45% specificity of 91.57% and when compared to the Full-Spectrum HPV Detection and Genotyping Assays for the detection of HPV (Takacs *et al.*, 2008). The sensitivity and specificity of the assay for the detection of CIN 2+ histology is shown in table 2.1 (Jeney *et al.*, 2009).

2.1.8. Linear Array HPV Genotyping Test (Roche Biochemicals)

Reverse hybridisation methods were adapted for genotyping of HPV infections, the most common of which is the line-probe assay (now commercially available as the Linear Array HPV Genotyping Test) (Li-Pa, Roche diagnostics) (Gravitt *et al.*, 1998). The assay detects 37 different 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 and CP6108) with internal β-globin controls. Briefly, DNA is extracted from 250uL of PreservCyt under denaturing conditions as described in Chapter 3, Section 3.6.1. The DNA is then amplified using biotinylated primers targeting a sequence of nucleotides within the L1 region of the HPV genome as described in Chapter 3, Section 3.6.2. Hybridisation of amplicons to probes which are bound to test strips is performed to detect the various genotypes fully outlined in Chapter 3, Section 3.6.3. The strips are then read visually by comparing the pattern of blue lines to the Linear Array HPV Genotyping Test reference guide and interpreted as described in Chapter 3, Section 3.6.4. Figure 2.4 shows a diagrammatic representation of the protocol (Gravitt *et al.*, 1998). The reverse hybridisation method allows for the binding of a mixture of PCR products to bind to multiple immobilised oligonucleotide probes simultaneously (Gravitt *et al.*, 1998, Kleter, 1999, Melchers, 1999). The assay has been considered for many years as the standard in HPV genotype detection. The Linear Array HPV Genotyping Test has a limit of detection of 76-20000 copies per mL depending on the HPV type. The Linear Array HPV Genotyping Test has a reported sensitivity of 98.2% and specificity of 32.8% in ASCUS patients with CIN 2+ histology (Szarewski *et al.*, 2008).

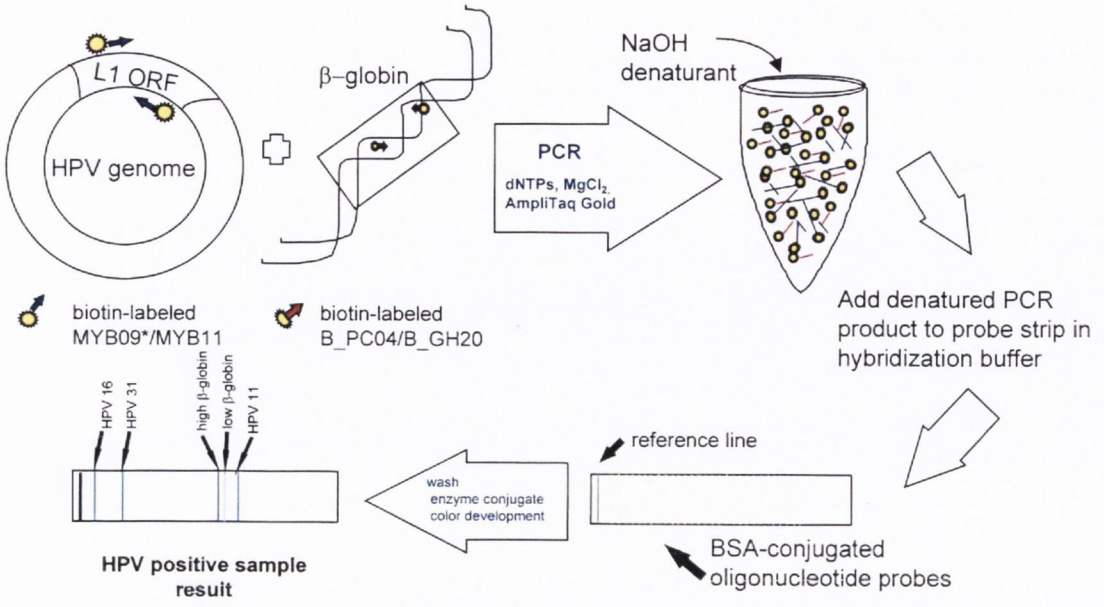


Figure 2.4: Linear Array HPV Genotyping Test procedure. The line blots assay reaction, Diagram from Roche Molecular diagnostics. DNA is extracted using the Amplicor extraction kit. The sample DNA is replicated using PCR protocol. Denatured PCR product is added to the detection strip in the hybridisation buffer. BSA-conjugated to the probes allows for detection (taken from Gravitt *et al.*, 1998).

2.2. mRNA testing technologies.

The detection of oncogenic HPV mRNA may be of greater value for detecting high-grade cervical disease compared to established HPV DNA testing assays as they focus on detection of HPV oncogene expression. This section will describe HPV mRNA testing technologies currently available.

2.2.1. PreTect™ HPV-Proofer and NucliSENS EasyQ HPV test.

The PreTect™ HPV-Proofer (Norchip AS) uses the Nucleic Acid Sequence Based Amplification (NASBA) to detect HPV mRNA in a background of DNA by an isothermal reaction. The test detects mRNA from the 5 most commonly found HR HPV types (HPV 16, 18, 31, 33 and 45) (Molden, 2007). The NucliSENS EasyQ HPV Test is identical to the PreTect™ HPV-Proofer assay, differing only in commercial trade name (Jeantet *et al.*, 2009).

Total Nucleic Acid (TNA) is extracted using the M48 Qiagen extraction technology (Chapter 3 Section 3.7). TNA is added to the reaction mix and the NASBA reaction is carried out at 41°C, where the target mRNA is amplified and the products detected by use of single stranded molecular beacon oligonucleotide probes (Figure 2.5).

It has been shown that HPV detection by PreTect™ HPV-Proofer is more specific and has a greater positive predictive value than the hybrid capture DNA test and therefore may be more specific in the triage of women for detecting high-grade cervical disease (Table 2.2) (Keegan *et al.*, 2009). A recent study was carried out on the NucliSENS EasyQ HPV test to determine if HPV DNA could act as a target for the molecular beacon

probes of the HPV NASBA assays (Boulet *et al.*, 2010). The authors used a plasmid based approach to test the robustness of the assay, and suggest that NucliSENS EasyQ HPV test may have the capacity to detect low copy DNA infection. However the authors state that the findings do not reflect the clinical performance of the assay (Boulet *et al.*, 2010).

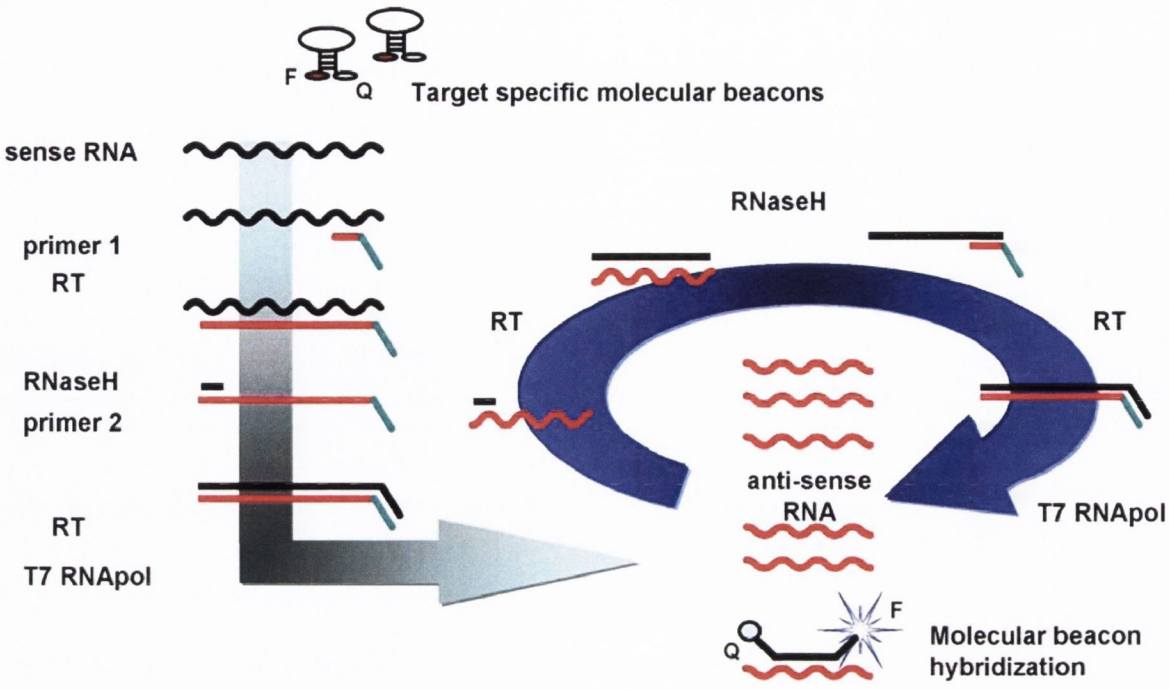


Figure 2.5: Nucleic acid based sequencing assay (NASBA) technology. Schematic presentation of NASBA including molecular beacon detection. The light arrow represents the initiation phase and the dark arrow (curved) represents the cyclic phase. The activities of reverse transcriptase (RT), RNase H, T7 DNA dependent RNA polymerase (T7 RNA pol) and the primer and molecular beacon probe binding activities are indicated. In the absence of a complementary sequence, the stem of the molecular beacon hairpin structure is closed and the quencher (Q) prevents the detection of the fluorescent signal emitted by the fluorophore (F). By hybridisation of the loop sequence of the beacon with a complementary sequence, the stem region is forced open and the fluorescent signal becomes detectable (taken from Molden *et al* 2007).

2.2.2. APTIMA® HPV Assay

The APTIMA HPV Assay is another mRNA based HPV detection test available. The assay incorporates an internal control to control for nucleic acid capture, amplification, and detection, as well as operator or instrument error.

The APTIMA HPV Assay is calibrated for use with PreservCyt cervical smear samples. For the test 1 mL of PreservCyt solution is used, a pellet is produced and cells are lysed to release the mRNA. The target mRNA is isolated in solution using capture oligomers (complementary to specific regions of the HPV mRNA). During hybridisation oligomers bind to specific regions of the HPV mRNA target molecule. The oligomer:mRNA complex is then captured on a magnetic particle bead by hybridisation of the deoxyadenosine region on the capture oligomer and the poly-deoxythymidine molecules. Magnets are then applied to allow for the supernatant to be aspirated. The particles are washed and the HPV mRNA is amplified using reverse transcriptase and T7 RNA polymerase.

The APTIMA HPV Assay performed well when compared to hc2 for the detection of HR-HPV with a sensitivity and specificity of >92% and 99% for the APTIMA HPV Assay and 93% and 82% for the hc2 test respectively. The sensitivity and specificity for the detection of CIN 2+ samples was 91% and >55% and 95% and 47% for APTIMA® HPV Assay and hc2 respectively. In CIN 3+ samples the sensitivity was 98% and 53% for the APTIMA HPV Assay and 99% and 44% for the hc2 (Castle *et al.*, 2007). Castle *et al.*, (2007) stated that the APTIMA HPV Assay is sensitive and very specific for detection of HR-HPV and shows similar clinical sensitivity and specificity as the hc2 assay. The sensitivity and specificity reported by Dockter *et al.*, (2009) also show comparable results

with the hc2 assay. More studies are needed to determine the clinical benefit of HPV mRNA detection by the APTIMA HPV Assay.

2.3. Summary

This chapter is designed to highlight the HPV detection technology "arms race" currently being fought out by biotechnology companies. As mentioned, detection of HPV DNA has been the dominant technology for many years. The leading assay for the last decade has been the hc2 assay. Until 2009, the assay was the only FDA approved HPV test with approval for use with cytology in ASCUS/women over 30 years. For this reason hc2 was chosen as the primary HPV detection method in this thesis. The Cervista HPV test, and the Cervista HPV 16 and HPV 18 genotyping assays have since received FDA approval.

As mentioned in Chapter 1, there has been a recent re-classification of HR-HPV genotypes and are now broken down into 4 groups (Table 1.1). Many of the current HPV detection technologies are designed to detect the HR-HPV genotypes as defined by the 2002 classification. It has been suggested that HPV detection assays that include HPV genotypes from the re-classified possible carcinogens may result in a higher number of false positives (Castle, 2009). Castle *et al.*, (2009) presented the Cervista system as an example of this, suggesting that the assay may result in more women testing positive for HR-HPV DNA (400/10000 people) with very little pick-up of clinically relevant infection (2 extra CIN 2+ in every 10000), as it includes the possible carcinogenic HPV 66 genotype. The hc2 assay is designed to detect the 13 genotypes that now make up the three most clinically relevant HPV genotypes (Most common carcinogenic HPV types 16, 18, 45, 31, 33, 35, 52 and 58, other carcinogenic HPV types 51, 56, 39and 59, and probable carcinogenic HPV type 68) and despite well established cross-reactivity with some of the possible carcinogenic group genotypes and thus possibly inflating the proportion of false negatives with little increase in true positive results, the hc2 assay is still considered the benchmark for HPV DNA detection assays (Castle, 2009). This is an

interesting comment, do the current batch of HPV tests include too many genotypes from groups considered probable or possible carcinogens? If so then maybe HPV assays that allow for the determination of specific genotypes is required. Genotyping of a large number of HPV types however can be costly with less clinical benefit relative to cost for each additional HPV genotype, along with the fact that the development of HPV detection assay is a long process of 10 or more years (Castle, 2009). As a result HPV assays being developed today are being designed to today's definitions of what constitutes a HR-HPV. In response to this some of the biotechnology companies are starting to switch to genotyping tests that include only the most clinically relevant HPV types. The HPV 16, 18 and 45 genotyping assay, the Cervista HPV 16 and HPV 18 genotyping assay and the PreTect™ HPV Proofer/NucliSens EasyQ HPV mRNA detection assays are all examples of this change of direction.

It has been suggested that it is unlikely that biotechnology companies will tailor their HPV tests to the HPV profile of specific populations (Castle, 2009), but with no apparent slow down of this technology arms race, it may be a possibility in the future. With many of the companies producing genotyping assay, a trend that appears to be the latest twist in the battle, it could be that certain populations have particularly relevant HR-HPV genotypes not considered one of the most prevalent carcinogenic types. Should such a population be found, it is conceivable that a tailored assay be developed/current assay modified by a technology company looking for a new advantage in the war.

Cuschieri *et al.,* (2004a) suggested that HPV prevalence can vary greatly between different populations, with this in mind should HPV testing be incorporated into the cervical screening algorithm for a population , HPV data for that specific population

would be required. This is also true of HPV type specific data. It has been suggested that if HPV testing is to become part of the CC screening algorithm data on the neoplastic progression of the less common HR-HPV genotypes be established (Cuschieri *et al.*, 2004a). Thus the prevalence of the less common HR-HPV needs to be determined.

In the four years since the beginning of the projects outlined in this thesis there has been a vast increase in the number of commercially available HPV detection assays. Notably the hc2 assay is no longer the only assay with the coveted FDA approval.

A few years ago the new technology was HPV mRNA testing. The testing of HPV mRNA could be an improvement over the available detection technologies in the determination of women who would progress/regress through the grades of cervical disease (Molden *et al.*, 2005c, Molden *et al.*, 2005b). Since then there have been ~30 publications relating to HPV mRNA testing. One publication looking at biomarkers of cervical disease included several HPV detection assays including the PreTect™ HPV-Proofer (Szarewski *et al.*, 2008). The main theme of the paper is that currently available assays do not have sufficient sensitivity and specificity for the detection of cervical disease. The aim of HPV testing technologies should be to identify HPV positive women who may develop/have developed cervical disease, as such a HPV detection assay with good sensitivity and specificity is required.

HPV testing technologies are being developed at an astonishing rate, and it is at this point that the future of HPV testing must be decided. The genotypes to be included in the next generation of HPV tests needs to be clarified (Castle, 2009). However these genotypes cannot be decided without data provided by HPV prevalence studies representative of the many different populations worldwide. As such the information provided in this thesis

will hope to provide the data relating to HPV DNA prevalence and genotype in the Irish population and HPV DNA and HPV mRNA prevalence in the HIV positive Irish population.

Chapter 3

Material and Methods

3.0 Materials and Methods

This chapter provides a complete description of the methodologies and techniques employed in this thesis. In some cases background information is given where deemed necessary. Specific techniques will be referred to in individual chapters however full description is limited to this chapter only.

3.1. Clinical specimens

3.1.1. Irish cervical screening population

PreservCyt™ smear samples from the general cervical screening population (n=3193) were collected for the study from three hospitals and from the CervicalCheck. The samples were collected through the cytology department in the recruiting centres, Coombe Women and Infants University Hospital, Dublin, Ireland, University College Hospital Galway, Galway, Ireland, and Antrim Area Hospital, Antrim, Northern Ireland. Two sampling events were carried out at the Coombe Women and Infants University Hospital, Dublin in 2006 (n=233) and 2007 (n=489). These samples were representative of the eastern part of the country due to the catchment area of the hospital. A single sampling event was carried out at University College Hospital Galway, Galway (n=504) in 2008, with the samples collected representative of the western areas of Ireland. Sampling in Antrim Area Hospital (n=981) was carried out in 2008-2009, with the samples collected being representative of the population of Northern Ireland. With the introduction of CervicalCheck (NCSS) samples had to be recruited directly from QUEST Diagnostics following cytological examination. Samples from areas of Ireland not covered in the initial testing based in Dublin and Galway were selected from the

CervicalCheck population for recruitment n=986. Figure 3.1 shows the number of samples per county by recruitment centre.

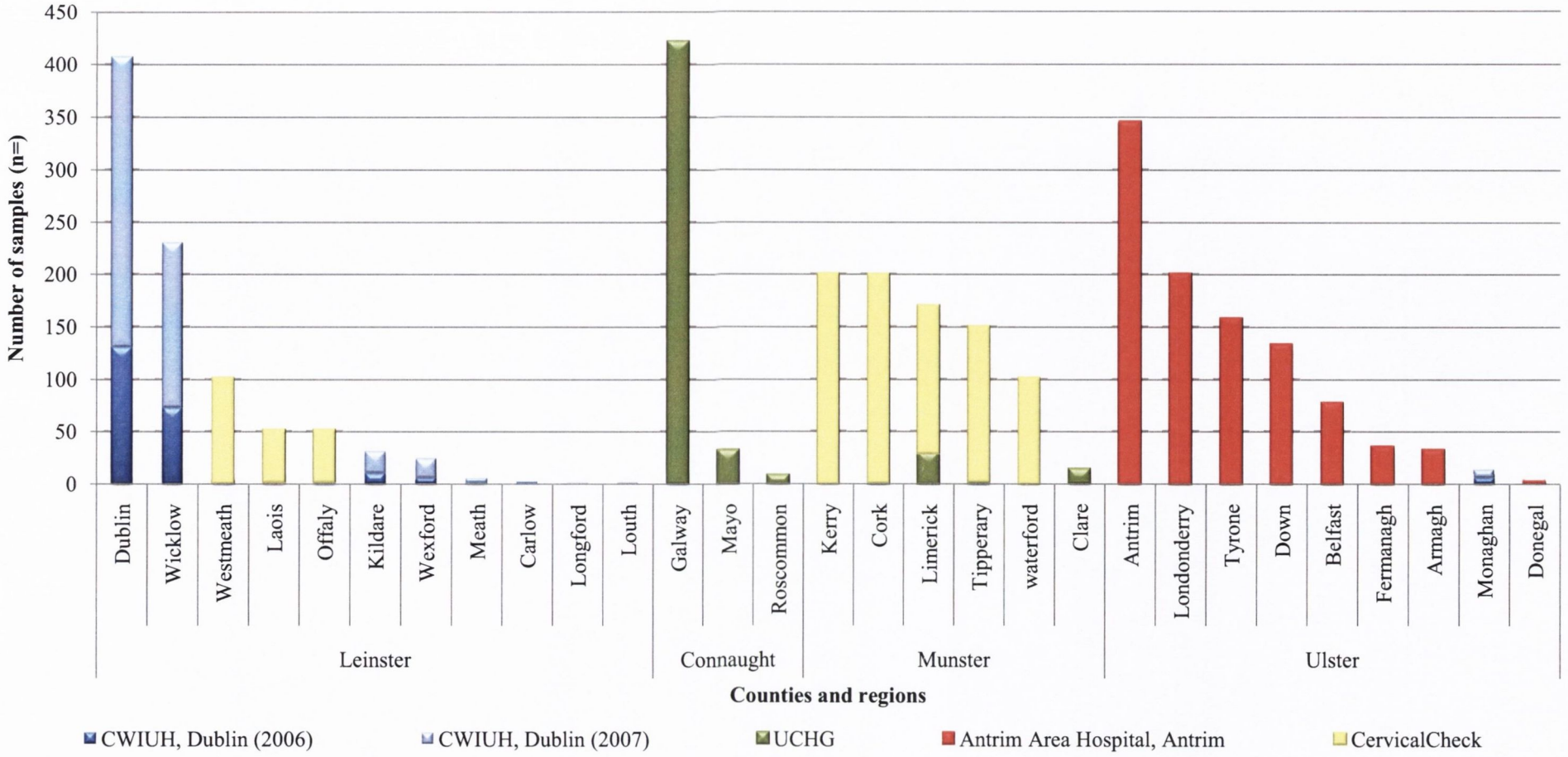


Figure 3.1: The distribution of samples recruited to the study by county and recruitment centre.
CWIUH= Coombe Women and Infants University Hospital, UCHG= University College Hospital, Galway.

Only county data was collected to identify the location of individual samples, thus, it was not possible to show any difference between rural and urban samples. Following recruitment to the study all samples were anonymised, assigned a unique study ID and no identifiable patient information was recorded. The PreservCyt™ smear specimens were processed and analysed according to BSCCP guidelines by the individual centres that provided the samples, with the exception of samples from the NCSS which were assessed using TBS. Those samples received from The Coombe Women and Infants University Hospital, Dublin and University College Hospital Galway, Galway were tested by the Cytology laboratories at both hospitals. The samples received from Antrim Area Hospital, Antrim were diagnosed by the cytology laboratory in the hospital as part of the screening programme of Northern Ireland. Finally those samples received from Quest diagnostics were diagnosed by Quest diagnostics as part of CervicalCheck (NCSS). In accordance with recommendations for cytology terminology, all cytological diagnoses were translated into the TBS for analysis (Herbert et al., 2007). The residual specimen was transported to the Molecular Pathology Research Laboratory, based at The Coombe Women and Infants University Hospital and University of Dublin, Trinity College, Dublin. HPV testing was performed on specimens with satisfactory or adequate material to generate a cytological diagnosis, HPV testing was carried out on samples recruited through The Coombe Women and Infants University Hospital, Dublin, Ireland with all cytological and HPV testing being carried out locally in the Hospital, University College Hospital Galway, Galway and QUEST Diagnostics in the Molecular Pathology Research Laboratory in The Coombe Women and Infants University Hospital, Dublin. HPV testing on those samples recruited through Antrim Area Hospital, Antrim, was carried out locally by hospital staff. Specimens that did not fulfil these criteria were omitted from the study. Ethical approval for this study was obtained from the Research Ethics Committees at the

Coombe Women and Infants University Hospital, Dublin, University College Hospital Galway and Antrim Area Hospital, Antrim.

3.1.2. HIV positive cohort of women

All female patients attending the HIV clinic at St James's Hospital, Dublin, were invited to participate in the study. PreservCyt™ smear samples were taken initially at baseline and regularly during follow-up (from ~6-18 months). PreservCyt™ clinical samples (n=321) were collected from each patient, with follow-up smears on 88 patients. The PreservCyt™ smear samples were processed and analysed according to BSCCP guidelines by the Department of Cytology, Central Pathology Laboratory, St. James' Hospital, Dublin, and translated into TBS for analysis. The residual specimens were transported to the Molecular Pathology Research Laboratory, Coombe Women and Infants University Hospital and University of Dublin, Trinity College, Dublin. Data was collected in the HIV clinic at St James's Hospital, Dublin. Ethical approval was obtained from The Joint Federated Hospital Research Ethics Committee at St. James's Hospital.

3.2. Database management

Distiller (SlidePath, Ireland) is a secure web based information management system for the sharing of files and data between project collaborators in the CERVIVA consortium. The distiller system was used to create and manage the database of all samples. The system allowed for the creation and extension of the database, with sample data being entered individually or by batch loading of data (clinical, demographic etc.) Separate databases were designed for each of the individual CERVIVA studies with sufficient

options to record all relevant data for each individual study. The system was chosen by the consortium as it uses SSL security technology, encrypted passwords, multi-level user access control and allows for secure database backup. The system also allows for easy data mining using constrained searches across multiple data parameters, with the ability to export results using common spreadsheet software and provide descriptive statistics on all search results (www.slidepath.com).

3.3. Clinical and demographic data

Clinical and demographic data was recorded on all samples in the study to determine associations between HPV infections and to allow the determination of risk factors associated with HPV.

3.3.1. Data on Irish population

For the population based HPV prevalence study, the following clinical and demographic data was recorded: age, geographical region, parity, smoking history, contraception, previous smear history, cytological diagnosis, and HPV status. Geographical regional parameters were restricted to Irish counties, with samples collected from 27 counties of Ireland, however as sampling was carried in batches to prevent bias some counties are better represented than others. A full breakdown of the different demographics listed above for this population is given in table 4.1.

3.3.2. Data on HIV positive subpopulations

For samples collected from the HIV positive cohort of women, information on age, ethnicity, parity, smoking history, contraception, possible risk factor for HIV infection, number of years since HIV positive diagnosis, HIV viral load, CD4 cell count, sexually transmitted disease screening, previous cytological diagnosis, and baseline cytology, cytology on follow-up smears, HPV DNA, and mRNA data was also collected. The HIV positive cohort of women attending the Department of Genito-Urinary Medicine and Infectious Disease clinic in St. James' Hospital are from varied backgrounds therefore ethnicity was recorded to determine trends in HPV in different ethnic groups. A full breakdown of the different demographics listed above for this population is given in table 5.1.

3.4. Statistical analysis

Power calculations were carried out for the Irish screening population to determine a prevalence of 17.5% (95% CI 16% - 19%) [based on previous studies; Ireland, 19.8% LR-HPV and HR-HPV (Keegan *et al.*, 2007), Scotland, 15.7% HR-HPV (Cuschieri *et al.*, 2004a)] with a 1.5% precision to estimate an overall required sample size of 2465. The age standardised HR-HPV prevalence was determined using World and European Standard populations (Ahmad *et al.*, 2000, Waterhouse, 2003). The age standardised rate was determined by first calculating the expected frequency of HPV positives. To do this age-specific rates in the tested population are calculated and these rates are multiplied by the standard population at each age. The overall age standardised rate is then calculated by dividing the sum of expected frequencies by the sum of the standard populations (http://meteor.aihw.gov.au). Pearson Chi-Square test was used to determine statistical

significance for the association between HPV and clinical/demographic factors. Mcnemars test was used to determine concordance of HPV tests. Multiple logistic regression was carried out to determine predictors of HPV infection taking into account the clinical and demographic factors (Age, years since HIV positive diagnosis, smoking history, parity, contraception, Ethnicity, CD4 cell count, cytology, and HPV DNA and mRNA positivity). The numbers included in this analysis was n=299, there were 22 missing values from the original sample of n=321. The analysis was powered to show an OR >=2.15 with 80% power and 5% level of significance. All statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) software version 16.0. Statistical analyses were carried out with the assistance of Dr. Kathleen Bennett, Senior Lecturer and Statistician in the Department of Pharmacology and Therapeutics.

3.5. HR-HPV DNA detection

The hc2 assay (Qiagen) was chosen for the detection of HPV DNA in the studies carried out as part of this thesis. The hc2 was, at the time, the only HPV DNA test with FDA approval. The samples used for hc2 testing were no more than 3 months old and had been stored between 2°C and room temperature. As described in Chapter 2, hc2 is a nucleic acid hybridisation assay that uses signal amplification for the detection of HPV DNA. The hc2 assay contains probes that allow detection of 13 HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

3.5.1. Sample conversion DNA extraction for hc2

A 10 mL conical Sarstedt tube was labelled with the appropriate specimen identification number. All specimens were aliquoted one at a time. PreservCyt™ smear samples were

vortexed vigorously until the cells appeared to be homogenously dispersed. An aliquot of 4 mL was taken and delivered to the bottom of the conical tubes to minimize cellular material adhering to the inside of the tubes. 0.4 mL of Sample Conversion Buffer was added to each tube and the tubes were mixed using a vortex mixer. The tubes were then transferred to a swing bucket rotor and centrifuged at 2900 ± 150 x g for 15 ± 2 minutes. The manufacturer recommends batching samples into batches of 32. Therefore in cases where a greater number of samples were denatured ($n \leq 47$) a blank control was also prepared to ensure that the increase in the number of samples being prepared did not result in contamination. During centrifugation the Specimen Transport Medium/Denaturation Reagent Mixture (STM/DNR) was prepared in a 2:1 ratio, according to table 3.1. To determine the total volume of STM/DNR required the STM and DNR volumes "per tube" were multiplied by the number of specimens being processed. The STM/DNR mixture was mixed thoroughly by vortexing. The conical tubes were carefully removed from the centrifuge one at a time, inspected to ensure that a pink/orange pellet was present, and placed into a rack. Samples without a visible pellet were not tested as per manufacturer's guidelines.

Table 3.1: STM and DNR volumes for preparation of STM/DNR Mixture.

PreservCyt™ Volume	STM volume per tube for final STM/DNA mixture	DNR volume per tube for final STM/DNR mixture	STM/DNR mixture to be added per tube
4 mL	120 µL	60 µL	150 µL

The cap was removed from a sample and set aside on a clean lint-free paper towel. The supernatant was then decanted into a waste bottle containing 1% Virkon (Anachem Ltd. UK). The inverted tube position was maintained and the tube was gently blotted on to absorbent lint-free paper towels until liquid no longer dripped from the tube. The tube was placed in the rack and 150 µL of STM/DNR mixture was added to each pellet. The

cap was replaced and the pellets were re-suspended by vortexing each tube for at least 30 seconds at the highest settings. In the case of pellets that were difficult to re-suspend additional vortexing for 10-30 seconds or until the pellet floats free from the bottom of the tube was carried out and the tube replaced into the rack. If following this, any specimens contained pellets, the specimen ID was noted and the next step of the protocol was followed as recommended. The above steps were repeated for all samples individually. A clean area of the lint-free paper towel was used for each sample. The hc2 calibrators and controls were removed from the fridge and allowed to equilibrate to room temperature. 500 µL of denaturation reagent was added to calibrators and positive control, and 1mL was added to the negative control. The calibrators and controls were vortexed thoroughly and added to the rack. When all samples and controls were processed the rack was placed in a water bath at 65 ± 2°C for 15 ± 2 minutes, with a water level sufficient to cover all the liquid in the tubes. The rack was then removed from the water bath and the samples were vortexed individually for 15-30 seconds. The rack was returned to the water bath at 65 ± 2°C to continue denaturation for a further 30 ± 3 minutes. Following denaturation samples were stored at 2-8°C overnight or at -20°C for a maximum of 3 months.

3.5.2. Hybridisation

The Microplate Heater I was set to 65 ± 2°C for at least 60 minutes prior to use to ensure that it had reached uniform temperature across the plate. DNA extracted from the samples and controls by alkali denaturation removed from storage (2-8°C or -20°C) and allowed to equilibrate to room temperature. The HR-HPV probe mix was prepared by mixing the appropriate quantities of HR-HPV probe and probe diluent. The HR-HPV probe mix contains specific RNA probes for the 13 HR-HPV types. 25 µL of HR-HPV probe mix is

required per test. As recommended, additional probe mix was prepared to allow for the volume of mix that could be lost through use of pipettes and on the sides of the vial. The manufacturers suggested guideline for probe mix was followed by addition of 160 µL of probes to 4 mL of probe diluent for a full run. Following the guidelines for preparation of 96 tests (88 samples, 3 positive and 3 negative calibrators a low and high risk HPV quality control) 160 µL of probe was added to 4 mL of probe diluent. Using the Digene Hybrid Capture System Version 2 Software the plate layout was designed as seen in table 3.2. The samples were removed from -20°C storage and allowed to thaw to room temperature. A 96 well hybridisation plate was labelled to ensure easy visual identification of its orientation. The controls, calibrators and samples were individually vortexed for at least 5 seconds and 75 µL was added to the bottom of corresponding wells in a 96 well hybridisation plate using a clean Extra-Long Pipette Tip for each transfer to avoid cross-contamination of calibrators and controls. The 75 µL aliquot was taken from the bottom of the control, calibrator or sample tube and consideration was given to the movement to ensure that the tip did not touch the sides to prevent a false positive result. Also while adding the aliquot to the hybridisation plate care was taken to prevent the tip from touching the sides of the plate and to limit the formation of air bubbles. As described in the layout exported from the hybrid capture software, the Negative Calibrator (NC) and High-Risk Calibrator (HRC) were tested in triplicate and the Quality Control 1 Low-Risk (QC1-LR) and Quality Control 2-High-Risk (QC2-HR) were tested once for each test plate as per manufacturer's instructions.

Table 3.2: Example layout for a run of 24 microplate wells.

Row	*Column*											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NC	Spec . 1	Spec . 9	Spec . 17	Spec . 25	Spec . 33	Spec . 41	Spec . 49	Spec . 57	Spec . 65	Spec . 73	Spec . 81
B	NC	Spec . 2	Spec . 10	Spec . 18	Spec . 26	Spec . 34	Spec . 42	Spec . 50	Spec . 58	Spec . 66	Spec . 74	Spec . 82
C	NC	Spec . 3	Spec . 11	Spec . 19	Spec . 27	Spec . 35	Spec . 43	Spec . 51	Spec . 59	Spec . 67	Spec . 75	Spec . 83
D	HRC	Spec . 4	Spec . 12	Spec . 20	Spec . 28	Spec . 36	Spec . 44	Spec . 52	Spec . 60	Spec . 68	Spec . 76	Spec . 84
E	HRC	Spec . 5	Spec . 13	Spec . 21	Spec . 29	Spec . 37	Spec . 45	Spec . 53	Spec . 61	Spec . 69	Spec . 77	Spec . 85
F	HRC	Spec . 6	Spec . 14	Spec . 22	Spec . 30	Spec . 38	Spec . 46	Spec . 54	Spec . 62	Spec . 70	Spec . 78	Spec . 86
G	QC 1-LR	Spec . 7	Spec . 15	Spec . 23	Spec . 31	Spec . 39	Spec . 47	Spec . 55	Spec . 63	Spec . 71	Spec . 79	Spec . 87
H	QC 2-HR	Spec . 8	Spec . 16	Spec . 24	Spec . 32	Spec . 40	Spec . 48	Spec . 56	Spec . 64	Spec . 72	Spec . 80	Spec . 88

Table shows the position of calibrators, controls and samples. Negative calibrator (NC), HR-HPV calibrator (HRC), quality control 1 LR-HPV (QC-1 LR), quality control 2 HR-HPV (QC2-HR) and specimens 1 to 88 (Spec. x)

Following the addition of the final sample, the plate was covered with a plate lid and incubated for 10 minutes at room temperature before the addition of the HPV probe mix. The prepared and thoroughly vortexed HR-HPV probe mix was aliquoted into a disposable reagent reservoir. Carefully, 25 µL of HR-HPV probe mix was added to each well that contained calibrators, quality controls or sample using an 8-channel pipette, with fresh tips for each row. The volume of probe was dispensed into the wells carefully to avoid the tips touching the sides of the wells or back splashing if dispensed to quickly. The hybridisation microplate was then covered with the plate lid and placed on the Hybrid Capture System Rotary Shaker I set at 1100 ± 100 revolutions per minute (rpm) for 3 ± 2 minutes. The plate was visually inspected to ensure all calibrators and controls changed from a purple colour to a yellow colour during shaking with the PreservCyt™ solution samples turning a pink colour. If the wells remained a purple colour following shaking an additional 25 µL of HPV probe mix was added and the plate was again placed on the Hybrid Capture System Rotary Shaker I set at 1100 ± 100 rpm for 3 ± 2 minutes. Following this step, all calibrators and controls turned a yellow colour and PreservCyt™ samples pink. The plate was then transferred to the preheated Microplate Heater I carefully to prevent contamination from splashing. The plate was incubated at 65 ± 2°C for 60 ± 5 minutes to hybridise the HR-HPV DNA in the calibrators, controls and samples to the specific RNA probes for the 13 HR-HPV types found in the HR-HPV probe mix to produce HPV RNA:DNA hybrids.

3.5.3. Hybrid Capture

The capture microplate was prepared by removing all but the required number of Capture Microplate wells from the plate frame provided in the hc2 Kit. The surfaces of the wells

of the Capture Microplate are coated in antibodies specific for the RNA:DNA hybrids produced during hybridisation. Each row of the capture plate was numbered to corresponding to the numbers on the hybridisation plate (1, 2, 3. . . . .) using a solvent resistant marker. The hybridisation microplate was carefully removed from the Microplate Heater I and the lid removed and placed on a clean surface. The entire contents of the calibrators, quality controls and samples were transferred from the Hybridisation plate to the bottom of the corresponding capture microplate well using an 8-channel pipettor. New pipette tips were used on the 8-channel pipettor for each column transferred. The microplate was covered with the plate lid and placed on the Rotary Shaker I at 1100 ± 100 rpm, at room temperature for 60 ± 5 minutes, allowing the RNA:DNA hybrids to bind to the antibodies on the surface of the microplate.

The wash buffer was prepared during this capture step in 1L batches at a time by mixing 33.3 mL of Wash Buffer Concentrate with 966.7 mL of distilled H_2O in the Automated Plate Washer I Wash Reservoir. The Automated Plate Washer I Rinse Reservoir was filled with distilled H_2O and Automated Plate Washer I Waste Reservoir of the Hybrid Capture System was emptied during the capture step. When the capture step was complete the Capture Microplate was removed from the Rotary Shaker I and the plate lid was removed and placed on a clean surface. The liquid was removed from the wells by fully inverting the plate over a sink and followed immediately with a sharp downward motion to remove the contents while being careful to avoid backsplash from the bottom of the sink. The plate was kept in an inverted position and blotted on to lint-free paper towels firmly 2-3 times. The plate was visually inspected to ensure that all liquid had been removed from the wells and that the top of the plate was dry.

3.5.4. Hybrid Detection

The appropriate volume of Detection Reagent 1 (alkaline phosphatase conjugated antibodies specific for the RNA:DNA hybrids in a buffered solution with 0.05% w/v sodium azide) aliquoted into a disposable reagent reservoir as per manufacturer's recommendations (Table 3.3). A 75 µL aliquot of Detection Reagent 1 was dispensed into each well of the Capture Microplate using the reverse pipetting technique to improve the consistency of the reagent delivery. If the tips touched the well or there was back splash the tips were replaced and the above procedure followed. The addition of reagents to the plate was always carried out from left to right. A visual inspection of the plate was carried out to ensure that all the wells have been filled with 75 µL of Detection Reagent 1 by observing the intensity of the pink colour in each well and ensuring that each well had similar colour intensity. The plate was covered with a plate lid and incubated at room temperature for 30-45 minutes.

Table 3.3: Detection reagent quantities.

Number of Tests/Strips	Volume of Detection Reagent 1 or 2
96/12	contents of bottle
72/9	7.0 mL
48/6	5.0 mL
24/3	3.0 mL
1 test	0.125 mL

3.5.5. Washing

The alkaline phosphatase conjugated antibodies were now bound to the RNA:DNA hybrids. The excess Detection Reagent 1 was then removed using the Hybrid Capture Automated Plate Washer I. The Automated Plate Washer I remained turned on 24 hours a day, 7 days a week to allow for routine rinsing of the system for cleaning. Therefore before washing, the volume of the Wash Reservoir was verified to be above 1 L, the Rinse Reservoir was full and the Waste Reservoir was empty, with the cap securely fastened. The rubber tubing was also checked to ensure there were no bends or kinks that may cause any obstruction to the transport of liquids or cause a problem with the Automated Plate Washer I pump.

The plate lid was removed and the plate was placed on the Automated Plate Washer I platform. A visual check was made to determine that the power was on and that the Automated Plate Washer I display read "Digene Wash Ready". If only a partial column of capture wells was being used, empty microplate wells were placed in the capture plate to complete the column prior to washing. The number of strips to be washed was entered my pressing the "Rows" key followed by "+" or "-"keys to adjust as necessary. The "Rows" key was pressed to return to the original screen. The "Start/Stop" was pressed to begin the wash step. During the washing step the Automated Plate Washer I performed six fill and aspirate cycles per well. When finished the Automated Plate Washer I display once again read "Digene Wash Ready". The microplate was removed from the platform and the plate inspected to ensure that there was no residual pink liquid in the microplate wells.

3.5.6. Signal Amplification

The appropriate volume of CDP-Star® with Emerald II (Detection Reagent 2) (a chemiluminescent substrate) was aliquoted into a disposable reagent reservoir as per manufacturer's recommendations (Table 3.3). A 75 µL aliquot of Detection Reagent 2 was dispensed into each well of the Capture Microplate using the reverse pipetting technique to improve the consistency of the reagent delivery. The plate was covered with a plate lid followed by several layers of lint free paper towels to avoid direct sunlight. The plate was incubated at room temperature for 15 minutes.

The microplate was read on the Digene Microplate Luminometer 2000 instrument after 15 minutes of incubation and no longer than 30 minutes after incubation. The chemiluminesent substrate is cleaved by the bound alkaline phosphatase and results in emitted light. The light emitted was measured in relative light units (RLUs) with a cut-off value determined by positive and negative calibrators. A negative cut-off point of ≤ 1.0 relative light units/ cut-off value (RLU/CO) was set as per manufacturer's instructions. Samples with RLU/CO of 1.0-2.5 were considered intermediate and those with an RLU/CO of >2.5 were considered positive. Samples with an intermediate result were denatured from a new aliquot of 4 mL of PreservCyt™ and tested again. Retested samples with RLU/CO of >1.0 were classified as positive and those with an RLU/CO of <1.0 were retested a final time from a new aliquot of PreservCyt™ as per manufacturers instructions. Following the third test, samples with RLU/CO of >1.0 were classified as positive and those with an RLU/CO of <1.0 were classified as negative as per manufacturers instructions. The software allows for the printing of detailed reports clearly determining HR-HPV DNA positivity. If a full microplate was not used the wells were removed from the microplate holder. The holder was rinsed thoroughly with distilled

water, allowed to dry and stored for use in future assays. The results were printed and entered into the distiller database system.

3.6. HPV genotyping

All samples positive for HR-HPV DNA by hc2 were genotyped using the Linear Array HPV Genotyping Test (Roche Diagnostics, Germany). 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.

3.6.1. DNA extraction

DNA is extracted from a 250 µL 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 (~77 µL) frozen at -20°C for up to 2 months

for use in subsequent extractions. 30 mL 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 10 mL Sarstedt centrifuge tube by adding the appropriate volume of dissolved CAR to appropriate volume of AL as shown in table 3.4. The solution was mixed by gently inverting the tube 10 times, as vortexing could have resulted in foaming of the solution.

Table 3.4: 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

2 mL screw cap Sarstedt tubes for each specimen and control to be extracted were labelled clearly using a solvent resistant marker. An 80 µL aliquot of ATL was added to each labelled 2 mL Sarstedt tube. Each specimen and control was vortexed individually for at least 10 seconds before 250 µL of the PreservCyt specimen was added to the appropriately labelled 2 mL tube. 20 µL of Proteinase K was added to each 2 mL 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. 250 µL 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 300 µL 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 16000 RCF for 3 minutes. The waste collection tube was discarded and the CLM was placed into a new waste collection tube. 750 µL 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 16000 RCF for 3 minutes. The waste collection tube was discarded and the CLM was placed into a new waste collection tube. 750 µL 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 16000 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 1.5 mL 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. 120 µL 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 eluate was visually identified at the bottom of the 1.5 mL

ETL. The 1.5 mL 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.

3.6.2. 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 125 µL of vortexed LINEAR ARRAY HPV Magnesium Solution (Mg^{2+}) (< 1% Magnesium chloride, Amaranth dye and 0.05% Sodium azide) to one vial (580 µL) 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 upstream

and downstream primers and 0.06% Sodium azide). Approximately two vials of HPV MMX mixed with 125 µL of MG2+ (1400 µL) 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 50 µL 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 50 µL 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 100 µL. The programme was started, with a completion time of approx 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.

3.6.3. 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 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 25 mL of SSPE to 97 mL of distilled water. The solution was mixed well and 3.125 mL 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 33.25 mL of SSPE to 630 mL of distilled water. The solution was mixed well and 3.325 mL 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 5 mL of Working Ambient Wash Buffer to a clean media bottle (120 mL 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 6.25 mL of CIT to 118.75 mL 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 4 mL aliquots were dispensed into each well of the 24-well tray containing a Strip. Each sample and control was vortexed immediately before 75 µL 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 5 mL of Working Ambient Wash Buffer for each strip being tested and the solution was thoroughly mixed. For a full run of 24 samples 360 µL of SA-HRP was added to 120 mL 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 3.5 mL transfer pipette (Sarstedt AG & Co., Germany) for each well. 4 mL 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 3.5 mL transfer pipette for each well. The Working Stringent Wash Buffer was removed from the 53°C ± 2°C water bath and immediately 4 mL 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 3.5 mL transfer pipette. 4 mL 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 3.5 mL transfer pipette.

4 mL 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. 4 mL 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 3.5 mL transfer pipette.

4 mL 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 4 mL of Substrate A to 1 mL 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 3.5 mL transfer pipette. 4 mL 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 3.5 mL transfer pipette. 4 mL 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.

3.6.4. 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 3.2). 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 3.5. The Linear Array HPV Genotyping 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 3.6

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.

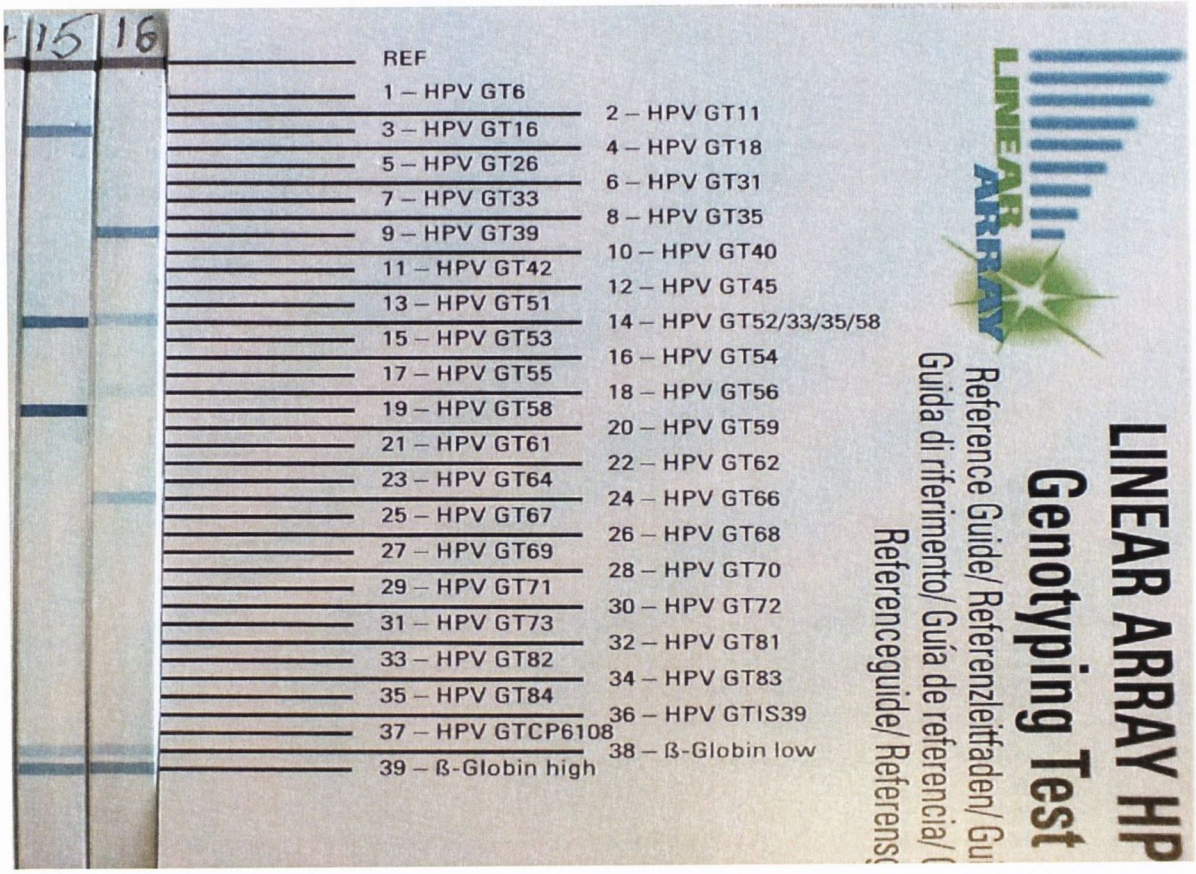


Figure 3.2: HPV Linear Array HPV Genotyping Test Strips and Reference Card. The Strips (left) are lined up so that the black strip at the top is lined up with the REF line on the genotyping card. The HPV genotypes found in the sample is then read from the reference card. Sample 15 above contains positive 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 3.5: 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 3.6: 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.

3.7. RNA Extraction

Pre-preparation of the samples was required before Total Nucleic Acid (TNA) extraction was carried out on the BioRobot® M48 Workstation using the Magattract M48 Nucleic Acid Extraction kit (Qiagen Ltd. Cat no: 958236). 400 µL of lysis buffer was prepared fresh for the preparation of samples for TNA extraction. Lysis buffer was prepared in sufficient volumes to allow for the preparation of the number of samples being processed.

The PreservCyt ™ samples were vortexed individually for 10 seconds before a 5 mL aliquot was transferred from into a 10 mL Sarstedt centrifuge tube using a sterile 3.5 mL transfer pipette. The samples were centrifuged at 1125±100g for 12 minutes, the supernatant was removed using a 3.5 mL transfer pipette, and washed in 1mL of 96-100% ethanol The samples were centrifuged at 1125±100g for 12 minutes to produce another pellet. The supernatant was removed using a 1mL filter tip to prevent disturbing the cell pellets. 400µL of lysis buffer (prepared by adding 10µL of β-Mercaptoethanol per 1mL of Tissue Lysis Buffer (RLT) (Qiagen Ltd. UK). was added to the cell pellets and the samples were homogenised by vortexing at max speed for 1 minute or pipetting up and down 15-20 times to lyse the cells. The samples were left for 10 minutes at room temperature and individually checked to ensure lysis. Following lysis the samples were stored at -70°C or used directly in the TNA extraction protocol. Lysate from cells was stored for several months at -80°C with the majority of samples were processed within a few days of lysis.

TNA extraction was performed using the Magattract M48 Nucleic Acid Extraction kit (Qiagen Ltd.) for the BioRobot® M48 workstation (M48) (Qiagen Ltd. UK). The TNA extraction was performed using a customised TNA extraction protocol designed by

Norchip for use with the HPV PreTect Assays,. Homogenised lysate was transferred to 1.5 mL sample tubes compatible with the sample rack of the BioRobot® M48 workstation. The M48 was turned on and the QIAsoft M Operating System was launched. The custom TNA protocol was selected from the drop-down menu the QIAsoft M software was followed as per manufacturer's instructions for the remaining steps required to set up the M48 custom NorChip protocol as outlined below.

1.5 mL tubes were selected in the QIAsoft M48 software as the preferred elution tubes. The number of samples being extracted was entered (in a multiple of 6); if the samples being tested were less than a multiple of 6 empty tubes and plastic ware was prepared as if the number of samples was a multiple of 6 was and water was used as blanks to replace the missing specimens. The sample volume was set at 400 µL and elution volume at 50 µL. The tip guard and waste shoot was checked to ensure that they were clean and that there is a waste bag attached. The on screen protocol was followed and the required number of M48 filter tips (Qiagen Ltd.) were loaded into the M48. Any tip racks that had been filled as a result of adding the extra tips were manually reset in the software to allow the robot to utilise the new tip racks during the extraction. As described on screen the reagents (RNase free H2O, RPE wash buffer (RPE), buffer MW from the Magattract M48 RNA Cell mini kit (Qiagen Ltd.) and 96% Ethanol (made up in RNase free H2O) were dispensed in a nucleic acid extraction hood into large reagent containers to the volumes required and the containers were placed in the M48 robot in the appropriate positions. The suspension of magnetic beads was vigorously mixed using a vortex at max speed for 5 minutes when opening a new container and 1 minute in all other cases. The required amount was dispensed using a 1000 µL pipette into a small reagent container (Qiagen Ltd) in the hood. The required volume of buffer ME (RNase free water) was dispensed

into another small reagent container and the reagent containers were placed in the correct positions inside the M48. The required number of sample preparation plates (Qiagen Ltd) were also loaded onto M48 workstation. The required number of 1.5 mL tubes with no caps (Sarstedt) were loaded into position on the heating block 1 section of the M48 and the required number of appropriately labelled 1.5 mL screw cap sample collection tubes (Sarstedt) were loaded into position on the heating block 2 section of the M48. The caps were removed from the screw cap tubes and placed on the clean surfaces to the sides of the heating blocks in the workstation.

The lysed samples were vortexed once defrosted and 400 µL of each lysed sample was added into a labelled 1.5 mL microfuge tube with no cap. The samples were then loaded into the sample racks in sequence from left to right, with the positions from 1-96 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 and 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 –70°C for HPV mRNA detection by the Norchip AS PreTect™ HPV-Proofer assay (NorChip AS, Norway). The unused suspension E was aspirated from the reagent container and dispensed into a 10 mL Sarstedt centrifuge tube and the tube was labelled with the appropriate expiration date and the tube was stored inside the Magattract M48 RNA Cell Mini kit for use in the next extraction. The remaining reagents and plastics were disposed of into cytotoxic waste along with the used tips in the waste bag. The M48 was cleaned

using 70% IMS and the waste bag was replaced. The Custom NorChip programme was exited and sterilization by ultraviolet light was carried out for 4 hours.

3.8. HPV mRNA testing by PreTect™ HPV-Proofer Assay

The PreTect™ HPV-Proofer is a qualitative assay for the detection of HPV E6/E7 oncogenic mRNA for high-risk HPV types 16, 18, 31, 33 and 45, as described in Chapter 2. The assay also detects mRNA from the human U1 small nuclear ribonucleoprotein specific protein A (U1A) to monitor sample mRNA integrity (control). The kit uses real-time Nucleic Acid Sequence Based Amplification (NASBA) technology for nucleic acid amplification and molecular beacon probe detection. NASBA is an enzyme driven amplification process that occurs under isothermal (41°C) conditions (detailed in Chapter 2 section 2.2.1). Oligonucleotide primers specific for the RNA target, determine the specificity of the reaction, with molecular beacon probes increase the specificity by signalling out detection of target during the amplification. This technology coupled with reading taken every 1.5 minutes on a fluorescence reader makes the PreTect™ HPV-Proofer a real-time assay. The PreTect™ HPV-Proofer involves two procedures; nucleic acid amplification and nucleic acid real-time detection with molecular beacon probes.

3.8.1. Nucleic acid amplification

The NASBA reaction begins with the hybridisation of the P1 primer to the target RNA. The P1 primer contains a 5'-terminal T7 RNA polymerase promoter sequence. The enzyme Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) elongates the primer along the length of the RNA template creating a cDNA copy forming a RNA:DNA hybrid. The enzyme RNaseH recognises the RNA:DNA hybrid as a substrate and hydrolyses the RNA portion of the RNA:DNA hybrid leaving a single-stranded DNA

molecule. This single-stranded DNA is now free for the second primer (P2) to anneal to. AMV-RT is again required, it elongates the P2 primer making a promoter region and subsequently a transcriptionally active double stranded DNA molecule. T7 RNA Polymerase can now recognise the functional promoter and produced multiple copies of RNA transcripts that are anti-sense to the original target RNA sequence. It is this antisense RNA that can now be bound by the molecular beacon probes or by the P2 primer. If bound by the P2 primer AMV-RT elongates the primer producing a cDNA copy forming a RNA:DNA hybrid. RNaseH acts to hydrolyse the RNA portion of the hybrid leaving a single-stranded DNA molecule. The P1 primer can now bind to the DNA and AMV-RT produces the T7 RNA promoter and another transcriptionally active double stranded DNA molecule. T7 RNA Polymerase can now transcribe the DNA to produce more RNA which is anti-sense to the original target. As the cycle continues more anti-sense RNA becomes available for the molecular beacons to bind.

3.8.2. Nucleic acid real-time detection with molecular beacon probes

Amplified RNA is detected in real-time by molecular beacon probes. The molecular beacons are single-stranded oligonucleotides with a stem-loop structure, with the loop portion containing the complementary sequence of the amplified anti-sense RNA produced by the NASBA reaction. The stem is unrelated to the target and has a double stranded structure. On one arm of the 5' end of the stem is labelled with a fluorescent dye and on the 3 ' end is labelled with a quencher molecule. When not bound to the RNA target the probe does not produce fluorescence as the energy is transferred to the quencher molecule. When hybridised to its complementary RNA target the molecular beacon undergoes a conformational change resulting in a physical separation of the fluorophore

and quencher and thus allows for the emission of fluorescence specific for the fluorophore. As the intensity of the fluorescence is related to the concentration of the amplicon, real-time measurements of fluorescence result in a typical exponential curve with an eventual plateau occurring.

3.8.3. PreTect™ HPV-Proofer assay protocol

Each PreTect™ HPV-Proofer kit supplies enough reagents to test 30 samples with 6 supplied controls on a 96 well plate. On delivery the PreTect™ HPV-Proofer kits were stored at -20°C. Prior to testing a kit was removed from the –20°C freezer along with TNA samples from storage at –80°C, samples were left to thaw on ice with the reagents from the PreTect™ HPV-Proofer kits thawing at room temperature. The PreTect™ Analyser and accompanying pc were turned on. The Proofer Plate Design software was started and the excel macros were enabled. 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.

171 µL of enzyme sphere diluent was added to 3 tubes, each tube containing 3 enzyme spheres. The solution was mixed by flicking, spun briefly to ensure spheres were submerged. The tube was left stand at Room Temperature (RT) in the hood for at least 20 minutes to dissolve. 240µl of reagent sphere diluent was added to 3 tubes, each tube containing 3 reagent spheres. The solution was immediately vortexed until the spheres were completely dissolved and the tube was centrifuged briefly for approximately 3

seconds. The reagent solution was aliquoted into the 3 empty colour coded tubes (ISC/HPV 16, HPV 18/31, and HPV 33/45). The rest of the reagent sphere containing reagent sphere diluent was frozen at -70°C for up to 2 weeks and was re-used for a following run if required. A 30µl aliquot of the U1A/HPV16 primer/molecular beacon was added to the empty (MT) U1A/HPV16 tube. 42µl of NASBA Water was added to the MT U1A/HPV16 tube, and 48µl of KCl, was added to the MT U1A/HPV16 tube. The solution was mixed by pipetting up and down several times. 30µl of the HPV 18/HPV31primer/molecular beacon was added to the MT HPV 18/HPV31 tube. 42µl of NASBA Water was added to the MT HPV 18/HPV31 tube, and 48µl of potassium chloride solution (KCl), was added to the MT HPV 18/HPV31 tube. The solution was mixed by pipetting up and down several times, and 30µl of the HPV 33/HPV45 primer/molecular beacon was added to the MT HPV 33/HPV45 tube. 42µl of NASBA Water was added to the MT HPV 33/HPV45 tube, and 48µl of KCl, was added to the MT HPV 33/HPV45 tube. The solution was mixed by pipetting up and down several times using a 200 µL pipette.

Before the addition of the master mix U1A/HPV 16 to the 96 well non skirted plate (ABgene Inc. USA) the solution was again mixed by pipetting up and down. 10µl of the prepared master mix U1A/HPV 16 was added to the 30 test wells (all of columns 1-3 and A-F of column 4) and in the positive and negative control wells (G4 and H4). HPV 18/HPV31 solution was again mixed by pipetting up and down and 10µl was added to the 30 test wells (all of columns 5-7 and A-F of column 8) and in the positive and negative control wells (G8 and H8). HPV 33/HPV45 solution was again mixed by pipetting up and down and 10µl was added to the 30 test wells (all of columns 9-11 and A-F of column 12)

and in the positive and negative control wells (G12 and H12). The distribution of master mix on the 96 well plate can be seen in (Figure 3.3).

The plate was removed from the PCR hood and the rest of the protocol was carried out on the bench in the pre-amplification room. The NucliSens EasyQ (Biomerieux S.A., France) thermocycler was turned on and a cycle programme of 2 minutes at 65 followed by 2 minutes at 41 C. The extracted TNA samples that were going to be added to column 1, 5 and 9 of the plate were vortexed, pulse centrifuged at max speed for 5 ± 2 seconds and placed a in column of 8 in plastic rack that holds 1.5 mL tubes. The lids were removed and placed on a clean surface. The samples were added to the plate in 5 µL aliquots as per figure 3.3 with corresponding positive controls added to well G4, G8 and G 12, and negative controls (NASBA Water) were added to wells H4, H8, and H12. The cap strips (ABgene Inc. USA) were then added to the plate and the plate was placed into the thermocycler. The preheated lid was closed and the programme was started. The thermocycler was used to incubate the plate 65 ± 1°C for 2 minutes, followed by incubation at 41 ± 0.5°C for 2 minutes. While the thermocycler programme was running the enzyme solution was mixed by pipetting up and down (solutions containing enzymes were never vortexed) and 5µl of the enzyme was added to lids of a new set of cap strips (ABgene Inc., USA). On completion of the thermacycler 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 3000 rcf at 20°-41° 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. When the run was completed the data was saved in

the same folder as the .ppd file, as a .pla and .glb file. The data was then exported to the same folder and the KC4 software was closed. The PDA excel programme was used to produce the report. The macros were enabled and the data from the current run loaded (specific text file and Bio-Tek text file). The results sheet was saved and printed for analysis.

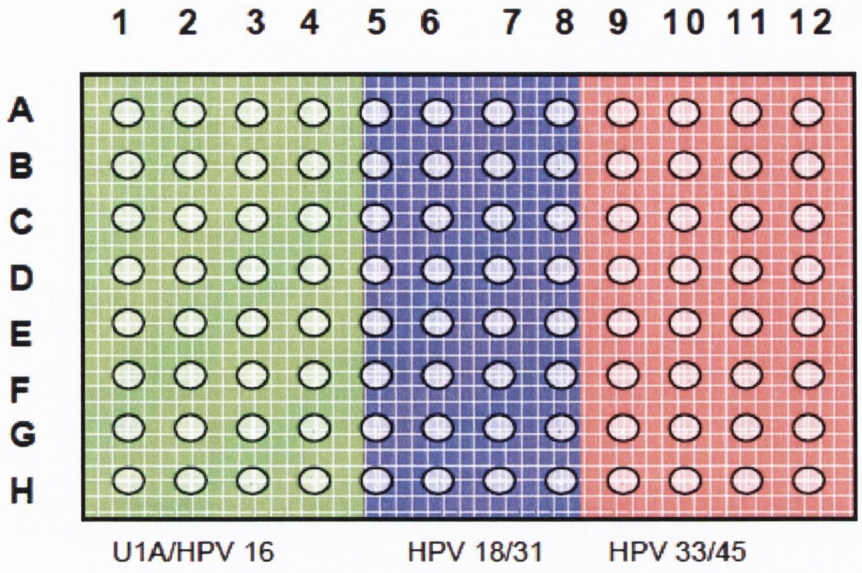


Figure 3.3: Plate with colours to show correct placement of samples. 10µL of the U1A/HPV 16 mastermix was added to columns 1-4, 10µL of the HPV 18/31 mastermix was added to columns 5-8, and 10µL of the HPV 33/45 mastermix was added to columns 9-12. The corresponding positive controls were added to well G4, G8 and G 12, and negative controls (NASBA Water) added to H4, H8 and H12.

3.8.4. PreTect™ HPV-Proofer analysis

For each PreTect™ HPV-Proofer run the U1A fluorescence was measured for each sample. The result was regarded as positive when the relative fluorescence signal increase during the amplification process was ≥1.7 and the curve is S-shaped (Figure 3.4). The results are regarded as negative when the relative fluorescence signal increase is less than 1.4 (<1.4). U1A, a human housekeeping gene, controlled and validated the complete procedure from sampling to amplification and detection. A negative result for U1A may have been due to, incorrect sampling, poor sample quality, degradation of sample, unsuccessful TNA isolation, incorrect dilution factors, or unsuccessful amplification/detection. 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 due to the low expression rate of the U1A housekeeping gene in human cells. In these cases a valid and positive HPV result was recorded as recommended by the manufacture. The advised validation decision diagram as recommend by the manufacture is outlined in table 3.7.

A positive run control was valid if the relative signal increase during the amplification process was ≥1.7 and the curve is S-shaped. If a positive control was negative (e.g. the relative fluorescence signal increase was <1.4 all results for the corresponding primer and probe set were invalid and all samples must were retested for that primer and probe set where possible. A negative control was regarded as positive when the relative fluorescence signal increase during the amplification process was ≥1.4. If a negative

control was positive all results for the corresponding primer and probe set were invalid and all samples were retested for that primer and probe set where possible.

Positive results were determined by their relative fluorescence compared to the stabilised background. The results were regarded as positive when the relative fluorescence signal increase during the amplification process was ≥1.7 and the corresponding curve is S-shaped. Non S-shaped curves (e.g. block waves) were not found in this study but if present the result would have been invalid and the samples would have been retested.

Indeterminate results are recorded when the signal increase during amplification was between 1.4 and 1.7. These results were considered borderline positive and the assay was repeated. The samples were retested, by re-extracting a larger sample volume (e.g. 10 ml sample input instead of the default 5 ml input when possible) and they were retested with the PreTect™ HPV-Proofer assay. Two borderline positive results from the same sample for any of the HPV type 16, 18, 31, 33, 45 were considered as a positive result.

A negative result was recorded when the relative signal increase during amplification was <1.4. Calculation of the relative fluorescence signal increase and qualitative interpretation of the results (positive, negative, undetermined) was automatically performed by the PreTect Analysis Software . The software quality controls all curves verifying that, the linear regression slope was positive, the fluorescence signal at the start of the reaction was lower than the mean of all fluorescence measurements, the minimum fluorescence signal was greater than 0, and that the control result criteria was met (outlined below). The curves were regarded as valid when all of the above criteria were met. Manual validation of curves pertaining to a positive result was still carried out as required to

ensure that the curve was S-shaped. Non S-shaped curves (e.g. block waves) were not found in this study but if present the result would have been invalid and the samples would have been retested.

A positive run control was valid if the relative signal increase during the amplification process was ≥1.7 and the curve is S-shaped. If a positive control was negative (e.g. the relative fluorescence signal increase was <1.4 all results for the corresponding primer and probe set were invalid and all samples must were retested for that primer and probe set where possible. A negative control was regarded as positive when the relative fluorescence signal increase during the amplification process was ≥1.4. If a negative control was positive all results for the corresponding primer and probe set were invalid and all samples were retested for that primer and probe set where possible.

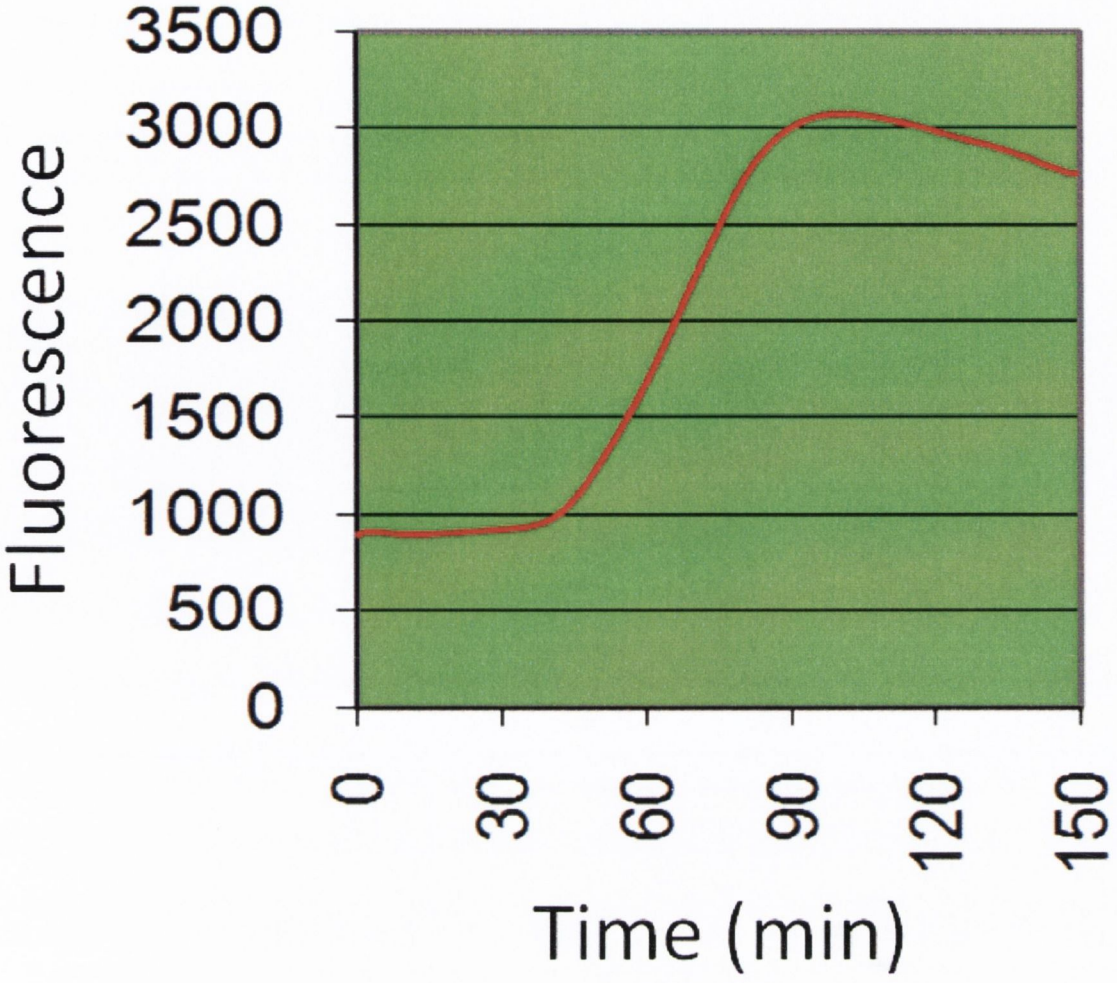


Figure 3.4: S-Shaped curve from the PreTect™ HPV Proofer.

Table 3.7: Interpretation of Pretect™ HPV-Proofer assay HPV and U1A results.

Sample HPVX result	U1A result	
	+	-
+	Valid	Valid
-	Valid	Invalid
Indeterminate	Indeterminate	Invalid

Chapter 4

Population based type specific HPV prevalence in the Irish cervical screening population.

4.0 Population based type specific HPV prevalence in the Irish cervical screening population

4.1. Introduction

CC is the third most prevalent cancer found in women worldwide (Ferlay *et al.*, 2010). In Ireland, CC is the eight most frequently diagnosed cancer in women, with 232 cases and 83 deaths in 2006 (NCRI, 2009). It is well established that persistent HPV infection is the causative agent in the development of CC (Walboomers *et al.*, 1999, Munoz, 2000). Over 120 types of HPV have been identified to date and these have been separated into 2 groups, LR-HPV and HR-HPV types (de Villiers *et al.*, 2004). The HR-HPV types are so called as they are associated with cervical disease, with HR-HPV types present in 99.7% of CC cases (Walboomers *et al.*, 1999). Infection with HR-HPV types is common with ~80% of women becoming infected at some stage during their lives (Jenkins 1996). However CC is quite rare, and with a large proportion of the population exposed to HPV during their lifetime, it has been suggested that some women may have a genetic predisposition for the development of cervical disease, and that genetic involvement may comprise a greater component of risk by comparison to other cancers (Magnusson, 2000, Martin *et al.*, 2006). HPV 16 and 18 are thought to account for 70% of all CC's (Smith *et al.*, 2007). Recently vaccines against the HR-HPV types 16 and 18 have been developed which are expected to reduce the number of women with cervical disease in the future (Roden, 2006). The population prevalence rates for HPV have been described in many populations with HPV prevalence ranging from 2% to greater than 35% worldwide (de Sanjose *et al.*, 2007). A recent study carried out in Ireland found that HPV prevalence in an urban cohort in Dublin, was found to be high at 19.8% with HR-HPV infections accounting for 74% of all HPV infections (Keegan *et al.*, 2007). A report detailing HPV

genotype distribution has also been carried out in a population of self referred Irish women with visible external genital warts. This study showed that the majority of women were infected with multiple HR-HPV types, with some women infected with up to 8 different HPV genotypes (Menton *et al.*, 2009).

It has been well established that HPV prevalence and distribution in a specific population is dependant on age, with HPV prevalence highest in younger age groups (Schiffman, 1992). This pattern remains a constant in HPV prevalence studies, but slight changes may be seen depending on the age at which the female population becomes sexually active and the overall patterns of sexual activity (Hibbitts *et al.*, 2006, Deacon *et al.*, 2000). Patterns of sexual activity are ever-changing in populations and especially in Ireland in recent years. The growth of the European Union has increased the number of foreign immigrants to Ireland and the effects of the Celtic Tiger on the Irish population have aided this process. It may be possible that these changes have been more rapid in some areas (e.g. urban) than others (e.g. rural). In the past two years, two major programs for the prevention of CC in Ireland were implemented, namely the CervicalCheck (Irish Cervical Screening Programme), and the HPV Vaccination Programme. The screening of women for signs of cervical dysplasia and CC as part of an organised programme is relatively new in Ireland and was only introduced as a nationwide screening programme in September 2008 (www.CervicalCheck.ie). Before the introduction of CervicalCheck, screening for CC was opportunistic, with smears taken from women via their local GP office, sexually transmitted disease clinics, "Well Woman" and family planning clinics and following childbirth. CervicalCheck provides free smear tests through primary care settings to 1.1 million eligible women aged 25-60 years (CervicalCheck.ie). All women who enter the programme will have a second test within 3 years of their entry, however

after this initial screening women aged 25 – 44 years will continue with screening tests every 3 years and those women aged 45 – 60 years will be tested every 5 years provided they have a negative smear. The Irish HSE has introduced a HPV Vaccination Programme, which commenced September 2010. All girls who are starting 1st and 2nd year in secondary schools will be offered the GARDASIL® HPV vaccine.

HPV prevalence surveys have been and are being carried out in many countries to investigate the factors associated with HPV infection, such as persistence, clearance, new infection, and the impact of vaccination. Our current study and other studies within the framework of the CERVIVA consortium will provide baseline figures for many of these parameters for the island of Ireland.

4.1.1. Aims

The aims of this project were:

1. To determine the prevalence of HPV in the Irish cervical screening population.

2. To determine the HPV genotype distribution in the Irish cervical screening population.

3. To set a baseline knowledge of HPV in Ireland for use in determining the impact of the proposed national HPV vaccination programme in the future.

4.2. Methods

4.2.1. Study population

PreservCyt™ smear specimens were obtained from a population of women (n=3,243) attending for routine cervical screening tests in Ireland. Specimens were collected through Cytology departments at the Coombe Women and Infants University Hospital, Dublin (n=737), during 2006 and 2007, University College Hospital Galway, (n=506) in 2008, Antrim Area Hospital, Northern Ireland, (n=1000), 2008 and 2009. After commencement of the national screening programme CervicalCheck in 2008, an additional 1,000 specimens were recruited through the CervicalCheck and The National Cancer Screening Services Board (NCSSB).

All specimens were anonymised, assigned a unique study ID and HPV testing was carried out while blinded to the cytology results. Only specimens with satisfactory results for cytology and HPV testing were included in the analysis (Hybrid capture positive cases that were unsatisfactory when tested with the Linear Array HPV Genotyping Test were excluded). With this in mind the final number available for statistical analysis was 3193.

Cytological diagnosis was carried out in the individual centres listed above. The samples collected through the Coombe Women and Infants University Hospital, Dublin, and University College Hospital, Galway were diagnosed using *BSCCP* guidelines for determining cytological grades. Those samples collected from Antrim Area Hospital, Antrim, and through CervicalCheck were diagnosed using TBS for Cervical Cytology. All cytology was translated into TBS for analysis (Herbert *et al.*, 2007).

County data was collected for all the samples used in the study (n=3193). The county data was used to separate Ireland into 4 provinces (North; Ulster, South; Munster, East; Leinster, and West; Connaught). The counties representative of the west (n=460) were Galway, Leitrim, Mayo, Roscommon, and Sligo. The counties representative of the south (n=834) were Clare, Cork, Kerry, Limerick, and Waterford. The counties representative of the east (n=904) were Carlow, Dublin, Kildare, Kilkenny, Laois, Longford, Louth, Meath, Offaly, Westmeath, Wexford, and Wicklow. The counties representative of the north (n=995) of Ireland were Antrim, Armagh, Cavan, Fermanagh, Londonderry, Donegal, Down, Monaghan, and Tyrone. This distribution of counties was chosen as it was in line with the traditional North (Ulster) South (Munster), East (Leinster), and West (Connaught) boundaries of Ireland. With the determination of regions based on counties 995/3193 (31.1%) samples were from the north of Ireland, 834/3193 (26.1%) samples were from the south of Ireland, 904/3193 (28.3%) samples were from the east of Ireland, and 460/3193 (14.4%) samples were from the west of Ireland.

At two centres, Coombe Women and Infants' University Hospital, Dublin, and University College Hospital, Galway, sampling was carried out in 2 defined time periods and a single time period respectively. For the other two centres sampling was carried out of several periods of consecutive sampling.

Clinical data on smoking, parity, geographical region, age, date samples were taken, cytology, previous smear history, and hormone/oral contraceptive use, was used for analysis where recorded on the cytology request form, however only data that provided statistically significant results was presented in this thesis.

4.2.2. HPV Molecular Testing

4.2.2.1. Hybrid Capture II Assay

HPV DNA testing was carried out using the hc2 assay (Qiagen Ltd.), as described in Chapter 3, Section 3.5.

4.2.2.2. Amplicor HPV DNA test

The Amplicor HPV test (Roche Biochemicals, Germany), a PCR based assay for the detection of HR-HPV DNA of 13 HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was used instead of the hc2 assay for HPV detection in samples from Antrim Area Hospital. This HPV testing was performed in Antrim Area Hospital, Antrim. Briefly, DNA was extracted from 250uL of PreservCyt under denaturing conditions at elevated temperatures in the presence of proteinase K. Amplification of target DNA was carried out using consensus PCR of the L1 region of the HPV genome, and of the β-globin gene (control) using biotinylated primers. The amplified DNA was chemically denatured to form single-stranded DNA. The amplicons were added to the wells on a micro well plate coated in capture probes for HR-HPV or the β-globin gene, to identify the viral or human DNA. Hybridisation to the capture probes was carried out at 37°C for 1 h. HR-HPV DNA was identified following the addition of streptavidin-horseradish peroxidase conjugate in a darkroom and a substrate solution containing hydrogen peroxide and 3,3',5,5'-TMB to allow the colorimetric reaction, carried out in a darkroom and stopped by the addition of a weak acid. The absorbance of the wells was detected at 450 nm, immediately using an automated micro well plate reader.

4.2.2.3. HPV genotyping using the Linear Array HPV 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 Chapter 3 Section 3.6.

4.2.3. Statistical analyses

The age standardised HPV prevalence was determined for World and European Standard populations (Ahmad *et al.*, 2000, Waterhouse, 2003), by dividing the sum of expected frequencies (Age-Specific Rates x Standard populations) by the sum of the standard populations (http://meteor.aihw.gov.au). Pearson Chi-Square test was used to determine statistical significance for the association between HPV and clinical/demographic factors using. Statistical Package for the Social Sciences (SPSS) software version 16.0. Statistical analyses were carried out with the assistance of Dr. Kathleen Bennett, Senior Lecturer and Statistician in the Department of Pharmacology and Therapeutics.

4.3. Results

4.3.1. Characteristics of the study population

The age range of the study population was from 17-89 years of age. The average age of women in the study who presented for routine smears in Ireland between the years 2006-2009 was 39.1 years old. The median age of the study population was 38 years. The average for each region was 39 years (North), 39.3 years (South), 37.6 years (East), and 41.8 years (West). The median age for each region was 38 years (North and South), 36 years (East), and 40 years (West). The distribution of the number of women in different age categories can be seen in figure 4.1. A breakdown of the population by age and demographics is shown in table 4.1 Overall 8.1% (259/3193) of patients screened were in the <25 years group and 3.9% (123/3193) were in the >60 years age group. The age groups between 25-60 years were well represented; 16.8% (537/3193) were aged between 25-29 years, 15.5% (494/3193) were aged between 30-34 years, 15.1% (485/3193) were aged between 35-39 years, 12.5% (398/3193) were aged between 40-44 years, 10.6% (339/3193) were aged between 45-49 years, 10.1% (321/3193) were aged between 50-54 years, and 7.4% (237/3193) in 55-60 years age group (Figure 4.1).

In the study population, 88% (2811/3193) were aged 25-60 years, representing those women who are eligible to participate in CervicalCheck. The average age of this cohort was 39.5 years old and the median age of the cohort was 38 years. Within this selected cohort (25-60) the age distribution was as follows; 25-29 years [19.1% (537/2811)], 30-34 years [17.6% (494/2811)], 35-39 years [17.2% (485/2811)], 40-44 years [14.2% (398/2811)], 45-49 years [12.1% (339/2811)], 50-54 years [11.4% (321/2811)] and 55-60 years [8.4% (237/2811)] (Figure 4.1).

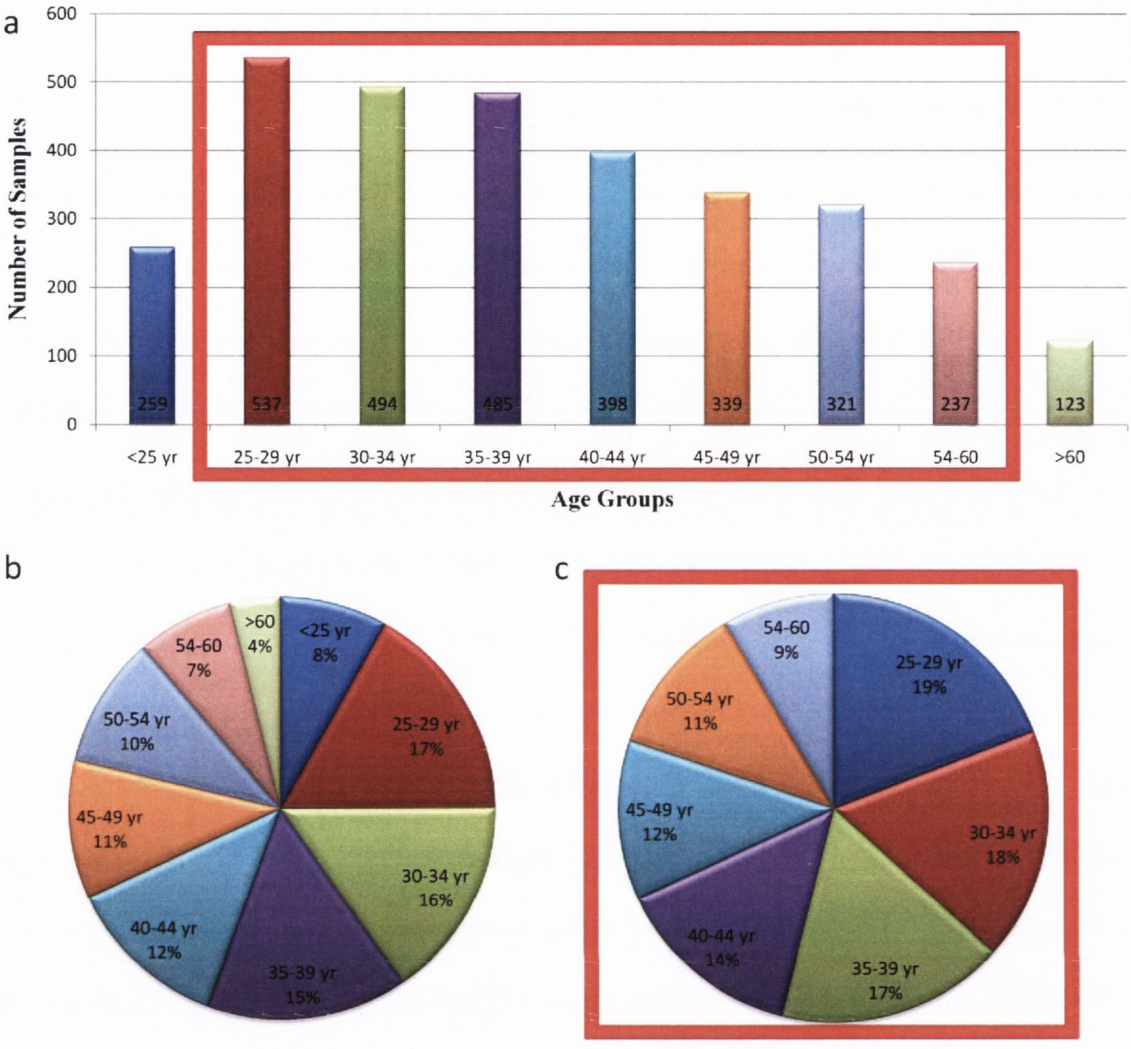


Figure 4.1: Distribution of the population by age. (a) Distribution of the entire study population (17-89 yr) by age; **(b)** Relative representation (%) of each age group in the entire study population; **(c)** Relative representation (%) of each age group in the 25-60 cohort. yr: years

Table 4.1 Breakdown of population by age group in relation to demographic data.

		Age groups								
		<25 years	25-29 years	30-34 years	35-39 years	40-44 years	45-49 years	50-54 years	54-60 years	>60 years
Geographic data	North	132	142	125	146	114	112	100	74	50
	South	21	149	161	117	127	106	81	56	16
	East	86	188	146	143	92	77	79	65	28
	West	20	58	62	79	65	44	61	42	29
	Total	259	537	494	485	398	339	321	237	123
Parity	Nulliparous	44	113	69	35	20	14	8	13	2
	Parous	11	83	148	159	173	134	113	89	36
	Not recorded	204	341	277	291	205	191	200	135	85
	Total	259	537	494	485	398	339	321	237	123
Smoking History	Smoking	4	4	5	1	2	4	8	4	0
	Non-smoking	3	5	8	4	3	2	0	3	1
	Not recorded	252	528	481	480	393	333	313	230	122
	Total	259	537	494	485	398	339	321	237	123
Contraception	No oral contraception	0	7	9	9	11	7	6	10	1
	Oral contraception	36	63	36	20	8	4	4	2	1
	Not recorded	223	467	449	456	379	328	311	225	121
	Total	259	537	494	485	398	339	321	237	123

4.3.2. Cytological Findings

The majority [88.5% (2826/3193)] of the study population recruited had a normal cytological diagnosis with 11.5% (367/3193) of the study population having an abnormal cytological diagnosis. Table 4.2 shows the breakdown of abnormalities in the study population, across different regions. The majority of abnormalities were in the low grade categories with 5.8% (185/3193) ASCUS and 3.9% (125/3193) LSIL. In the remaining abnormal cytology grades 0.2% (7/3193) were AGUS, 1.5% (47/3193) were HSIL and 0.1% (3/3193) were CIS, on cytology (Table 4.2).

Analysis of the regional data demonstrated different trends in cytological abnormalities across the 4 provinces; North (9%), South (14.9%), East (11.8%), and West (10%) (Table 4.2). The data revealed a significantly higher rate of abnormalities in the South when compared to the data from the North (χ^2=14.89, p<0.001). However, the rates of abnormalities across all other regions were not statistically different when compared or compared to the population as a whole.

The abnormality rate was significantly higher in women <30 years of age [17.5% (155/796)] compared with women ≥30 years [8.8% (212/2397)] (χ^2=66.350 p<0.001). The majority of abnormalities [60.2% (221/367)] occurred in the younger age groups (<25years, 25-29years, and 30-34yr) which accounted for 40.4% (1290/3193) of the population (Table 4.3).

In the 25-60 year cohort we found that 10.7% (300/2811) patients were abnormal (ASCUS or greater) as determined by cytology. In the 25-60 year cohort; 5.5%

(155/2811) samples positive by cytology for ASCUS, 0.3% (7/2811) positive for AGUS, 3.5% (98/2811) positive for LSIL, 1.4% (38/2811) positive for HSIL and 0.1% (2/2811) for CIS (Table 4.3).

Table 4.2: Breakdown of cytology by grade and region.

	Negative	[a]ASCUS	[b]AGUS	[c]LSIL	[d]HSIL	[e]CIS	Total Abnormal	Total
North	91.0% (905/995)	4.9% (49/995)	0.1% (1/995)	2.9% (29/995)	1.1% (11/995)	0.0% (0/995)	9.0% (90/995) (CI 95% 7.3%-10.8%)	995
South	85.1% (710/834)	8.8% (73/834)	0.2% (2/834)	3.7% (31/834)	2.0% (17/834)	0.1% (1/834)	14.9% (124/834) (CI 95% 12.5%-17.3%)	834
East	88.2% (797/904)	4.9% (44/904)	0.4% (4/904)	5.5% (50/904)	1.0% (9/904)	0.0% (0/904)	11.8% (107/904) (CI 95% 9.7%-13.9%)	904
West	90.0% (414/460)	4.1% (19/460)	0.0% (0/460)	3.3% (15/460)	2.2% (10/460)	0.4% (2/460)	10.0% (46/460) (CI 95% 7.3%-12.7%)	460
Total	88.5% (2826/3193)	5.8% (185/3193)	0.2% (7/3193)	3.9% (125/3193)	1.5% (47/3193)	0.1% (3/3193)	11.5% (367/3193) (CI 95% 10.4%-12.6%)	3193

[a]Atypical Squamous Cells of Undetermined Significance, [b]Glandular Atypical Squamous Cells of Undetermined Significance, [c]Low-grade Squamous Intraepithelial Lesion, [d]High-grade Squamous Intraepithelial Lesion, [e]Carcinoma In-Situ.

Table 4.3: Cytological diagnosis across different age cohorts.

	Negative	ASCUS[a]	AGUS[b]	LSIL[c]	HSIL[d]	CIS[e]	Total Abnormal	Total population
<25 yr	75.2% (195/259)	10.8% (28/259)	0.0% (0/259)	10.4% (27/259)	3.5% (9/259)	0.0% (0/259)	24.7% (64/259) (CI 95% 19.5%-30.0%)	259
25-29 yr	83.1% (446/537)	8.4% (45/537)	0.0% (0/537)	6.3% (34/537)	2.2% (12/537)	0.0% (0/537)	16.9% (91/537) (CI 95% 13.8%-20.1%)	537
30-34 yr	86.6% (428/494)	6.5% (32/494)	0.0% (0/494)	5.1% (25/494)	1.8% (9/494)	0.0% (0/494)	13.4% (66/494) (CI 95% 10.4%-16.4%)	494
35-39 yr	90.5% (439/485)	5.4% (26/485)	0.8% (4/485)	2.5% (12/485)	0.8% (4/485)	0.0% (0/485)	9.5% (46/485) (CI 95% 6.9%-12.1%)	485
40-44 yr	91.0% (362/398)	4.8% (19/398)	0.5% (2/398)	2.7% (11/398)	0.8% (3/398)	0.3% (1/398)	9.0% (36/398) (CI 95% 6.2%-11.9%)	398
45-49 yr	91.4% (310/339)	4.7% (16/339)	0.3% (1/339)	2.4% (8/339)	1.2% (4/339)	0.0% (0/339)	8.6% (29/339) (CI 95% 5.6%-11.5%)	339
50-54 yr	93.8% (301/321)	3.4% (11/321)	0.0% (0/321)	1.9% (6/321)	0.9% (3/321)	0.0% (0/321)	6.2% (20/321) (CI 95% 3.6%-8.9%)	321
55-60 yr	95.0% (225/237)	2.5% (6/237)	0.0% (0/237)	0.8% (2/237)	1.3% (3/237)	0.4% (1/237)	5.1% (12/237) (CI 95% 2.3%-7.9%)	237
60+	97.6% (120/123)	1.6% (2/123)	0.0% (0/123)	0.0% (0/123)	0.0% (0/123)	0.8% (1/123)	2.4% (3/123) (CI 95% -0.3%-5.2%)	123
Total	88.5% (2826/3193)	5.8% (185/3193)	0.2% (7/3193)	3.9% (125/3193)	1.5% (47/3193)	0.1% (3/3193)	11.5% (367/3193) (CI 95% 10.4%-12.6%)	3193

[a]Atypical Squamous Cells of Undetermined Significance, [b]Glandular Atypical Squamous Cells of Undetermined Significance, [c]Low-grade squamous intraepithelial lesion, [d]High-grade squamous intraepithelial lesion, [e]Carcinoma In-Situ.

4.3.3. HPV prevalence in the Irish population

A HR-HPV crude prevalence rate of 19.2% (614/3193) was determined in the study population. When adjusted to specifically focus on women in the 25-60 year age cohort the crude HR-HPV prevalence rate was 17.3% (487/2811) (Table 4.4). The European age standardised rates for the study population and the 25-60 year cohort were 19.7% and 15.7%, respectively. The World age standardised rates for the study population and the 25-60 year cohort were 22% and 16.8% respectively (Table 4.4). The age range of women testing positive for HR-HPV DNA was 17-73 years old. The average age of the HR-HPV DNA positive women was 33.1 years and a median age of 30 years. The average age of the 25-60 year cohort of women who tested HR-HPV DNA positive women was 35 years and a median age of 32 years.

HR-HPV DNA prevalence was highest in the younger age groups, at 44.4% (115/259), and 34.1% (183/537), in the <25 years, and 25-29 years age groups respectively. HR-HPV DNA prevalence decreased with increasing age from 21.7% (107/494) in the 30-34 years age group to 13.4% (65/485), 12.1% (48/398), 10.0% (34/339) and 8.7% (28/321) in the 35-39 years, 40-44 years , 45-49 years 50-54 years age groups respectively (Figure 4.2). Cumulatively 48.5% (298/614) of HR-HPV positive women were <30 years, which represented 24.9% (796/3193) of the study population. In the 25-60 year cohort, 34.1% (183/537) of HPV positive women were <30 years, representing a large proportion of the HR-HPV positives, and accounting for 19.1% (537/2811) of the overall 25-60 year cohort.

Table 4.4: Prevalence of HR-HPV by hc2, in the Irish female population presenting for routine cervical screening (n=3193), and in women aged 25-60 years (n=2811).

	Total Study Population	Total 25-60 yr
Total	3193	2811
HR-HPV DNA positive (hc2)	614	487
HR-HPV DNA Negative (hc2)	2579	2324
HR-HPV DNA Prevalence (hc2)	19.2%	17.3%
CI 95% ±	17.9%-20.6%	15.9%-18.7%
European Age Standardised Rate	19.7%	15.7%
World Age Standardised Rate	22.0%	16.8%

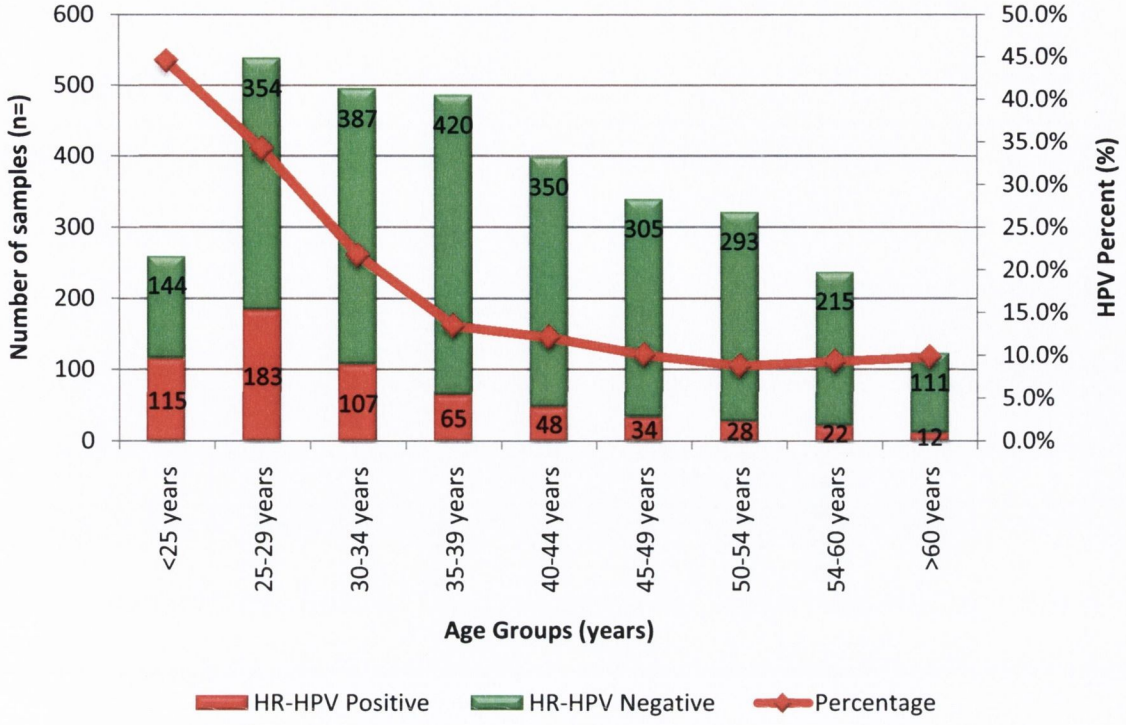


Figure 4.2: Prevalence of HR-HPV DNA across different age groups. HR-HPV positive samples are shown in red, HR-HPV negative samples are shown in green and the overall HR-HPV prevalence is shown on the secondary axis, and represented by the red line.

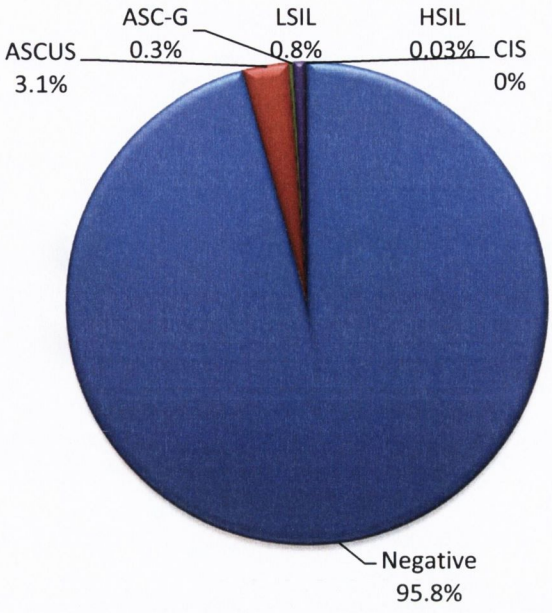
4.3.4. Relationship between HPV DNA status and cytology

Overall the HR-HPV prevalence rate found in this study was 19.2%. In total 70.3% (258/367) cases with abnormal cytology were positive for HR-HPV (χ^2>600, p<0.001). HR-HPV was also detected in 98% (49/50) of the cases with moderate to severe cytological abnormalities (HSIL and CIS).

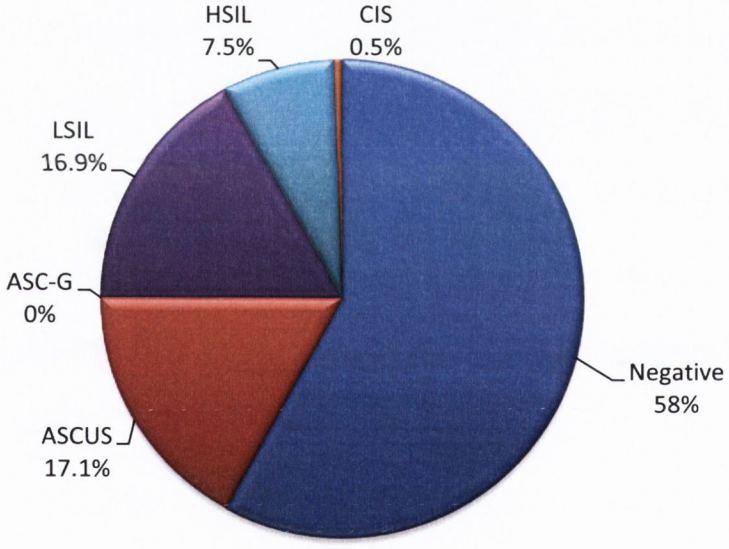
In the HR-HPV DNA negative cases (n=2579), the majority [95.8% (2470/2579)] were classified as normal for cytological diagnosis. A total of 4.2% (109/2579) of HR-HPV negative cases had an abnormal cytological diagnosis; 3.1% (80/2579) were ASCUS, 0.3% (7/2579) were AGUS, 0.8% (21/2579) were LSIL, 0.03% (1/2579) were HSIL and 0% (0/2579) were CIS (Figure 4.3 (a)). The majority of HR-HPV positive cases [58% (356/614)] were normal on cytological examination. While, 42% (258/614) of HR-HPV positive cases had an abnormal cytological diagnosis; 17.1% (105/614) were ASCUS, 16.9% (104/614) were LSIL, 7.5%) (46/614) were HSIL and 0.5% (3/614) were CIS (Figure 4.3 (b)).

The breakdown by cytological grade of the HR-HPV DNA results is shown in table 4.5. The HR-HPV prevalence rate in women <30 years was significantly higher than women ≥30 years (χ^2>200, p<0.001) (Table 4.5). Significantly higher HR-HPV prevalence rates were also found in younger (<30 years) cytologically normal and ASCUS women when compared to older women in those cytological grades [(χ^2>140 p<0.001) and (χ^2=10.295, p=0.001) respectively] (Table 4.5). There was no significant difference between the other cytological diagnostic groups and HR-HPV DNA prevalence (Table 4.5).

(a)



(b) HR-HPV Negative



HR-HPV positive

Figure 4.3: Cytological breakdowns in HR-HPV DNA positive and negative samples. HR-HPV negative **(a)**, and HR-HPV positive **(b)** samples by cytological diagnosis. ASCUS=Atypical squamous cells of undetermined significance, AGUS=Glandular Atypical squamous cells of undetermined significance, LSIL=Low-grade squamous intraepithelial lesion 1, HSIL=High-grade squamous intraepithelial lesion 2 and 3 combined, CIS=Carcinoma in-situ.

Table 4.5: HR-HPV status across different cytological disease categories in women <30 years, ≥30 years and total study population.

	Negative	[a]ASCUS	[b]AGUS	[c]LSIL	[d]HSIL	[e]CIS	Total Abnormal	Total
HR-HPV **Positive (hc2) (<30 years)**	26.5% (170/641) 95% CI 23.7-29.4%	71.2% (52/73) 95% CI 62.5-79.9%	0% (0/0)	90.2% (55/61) 95% CI 83.9-96.4%	100.0% (21/21)	0% (0/0)	82.6% (128/155) 95% CI 77.6-87.6%	37.4% (298/796) 95% CI 34.6-40.3%
HR-HPV **Positive (hc2) (≥30 yr)**	8.5% (186/2185) 95% CI 7.5-9.5%	47.3% (53/112) 95% CI 39.6-55.1%	0% (0/7)	76.6% (49/64) 95% CI 67.9-85.3%	96.2% (25/26) 95% CI 89.9-102.4%	100% (3/3)	61.3%(130/212) 95% CI 55.8-66.8%	13.2% (316/2397) 95% CI 12-14.3%
HR-HPV **Positive (hc2) (Total)**	12.6% (356/2826) 95% CI 11.6-13.6%	56.8% (105/185) 95% CI 50.8-62.7%	0% (0/7)	83.2% (104/125) 95% CI 77.7-88.7%	97.9% (46/47) 95% CI 94.4-101.3%	100% (3/3)	70.3% (258/367) 95% CI 66.4-74.2%	19.2% (614/3193) 95% CI 18.1-20.4%

[a]Atypical Squamous Cells of Undetermined Significance, [b]Glandular Atypical Squamous Cells of Undetermined Significance, [c]Low-grade squamous intraepithelial lesion, [d]High-grade squamous intraepithelial lesion, [e]Carcinoma In-Situ.

4.3.5. HR-HPV prevalence by geographical region

HR-HPV DNA prevalence in the population and by region is shown in table 4.6. The HR-HPV crude prevalence rate of 21.2% (211/995) found in samples representative of the North of Ireland, was the highest of all regions tested, followed by; South [19.4% (162/834)], East [19.3% (174/904)] and West [14.6% (67/460)] (Table 4.6). Similarly in the 25-60 year cohort the HPV prevalence was highest in the North [18.1% (147/813)] (Table 4.6). HR-HPV infection was significantly lower in the West compared to the other geographical regions [North (χ^2>8.976, p<0.01).), South (χ^2>4.806, p<0.05) and East (χ^2>4.595, p<0.05].

Table 4.6: HR-HPV DNA regional data for the study population.

	North	South	East	West	Total
HR-HPV **Positive** (hc2)	21.2% (211/995) 95% CI 19.1-23.3%	19.4% (162/834) 95% CI 17.2-21.7%	19.2% (174/904) 95% CI 17.1-21.4%	14.6% (67/460) 95% CI 11.9-17.3%	19.2% (614/3193) 95% CI 18.1-20.4%
[a]**EASR**	22.0%	19.3%	17.0%	21.6%	19.4%
[b]**WASR**	21.4%	21.8%	19.5%	24.4%	20.6%
25-60yr					
HR-HPV **Positive** (hc2)	18.1% (147/813) 95% CI 15.9-20.3%	19.1% (152/797) 95% CI 16.8-21.4%	17.6% (139/790) 95% CI 15.4-19.8%	12.4% (51/411) 95% CI 9.7-15.1%	18.2% (513/2811) 95% CI 17.1-19.4%
[a]**EASR**	21.5%	16.5%	14.1%	12.4%	17.2%
[b]**WASR**	21.3%	17.9%	15.4%	12.9%	17.9%

[a]European Age Standardised Rate, [b]World Age Standardised Rate.

4.3.6. HPV status in relation to parity

In total 39.6% (1264/3193) of patients had data relating to parity, with 25.2% (318/1264) of women nulliparous and 74.8% (946/1264) with 1 or more children. The average age of nulliparous women was 32.3 years with the average age in parous women of 42.3 years. The HR-HPV prevalence rate in nulliparous women [30.8% (98/318)] was significantly higher than the prevalence in parous women 12.4% (117/946) ($\chi 2$=57.398, p<0.001) (Table 4.7). As age is a significant covariate in relation to HPV regression analysis was used to determine the true effects of parity on HPV status. Following analysis, parity in relation to HPV status remained statistically significant after adjustment for age (Wald=11.724, p<0.001).

Table 4.7: HR-HPV status in relation to parity.

	Nulliparous	**Parous**	**Total**
HR-HPV **Positive** (hc2)	30.8% (98/318) 95% CI 25.7-35.9%	12.4% (117/946) 95% CI 10.3-14.5%	17.0% (215/1264) 95% CI 14.9-19.1%

4.3.7. HPV genotype prevalence in the Irish cervical screening population

HPV genotyping was performed on those specimens that tested positive for HR-HPV DNA by hc2 or Amplicor test (hc2 n=404, Amplicor n=210, Total n=614). HPV 16 was the most prevalent HPV genotype detected, in 29.0% (178/614), followed by HPV 31,HPV 52 [12.2% (75/614)/(75-123/614)], HPV 18 [11.6% (71/614)], HPV 51 [11.4% (70/614)] and HPV 39,HPV 66 [9.1% (56/614)] (Figure 4.4). A total 35 genotypes were detected in this study (Figure 4.4). HPV 64 and HPV 69 were not detected in the study (Figure 4.4). HPV 52 prevalence could not be defined in this study due to the limitations of the LA. Overall, 10.4% (42/404) of the samples hc2 HR-HPV positive samples were determined by linear array to be positive only for HPV types not present in the hc2 probe cocktail, with 1.9% (4/210) of the Amplicor HR-HPV positive samples also testing positive for HPV types not present in the Amplicor assay. Also 8.1% (50/614) of the samples that tested positive for HR-DNA were negative for HPV genotypes when tested using the LA.

We found a multiple HPV infection rate of between 56.5% and 58.5% (347-359/614). This range is attributed to a select number of 12 samples which were positive for the HPV 52 and HPV 33, 35 or 58 band by Linear Array HPV Genotyping Test (see Figure 3.2). The rate of multiple infection was higher in women <30 years [68.4% (201/294)] compared to women ≥30 years [41.3% (146/308)] ($\chi 2=27.077$ $p<0.001$). We also found that multiple infections were more prevalent in women with negative cytological diagnosis [51.6% (180/349)] compared to women with abnormal cytological diagnosis [66.4% (170/256)] ($\chi 2=13.320$ $p<0.001$), however, no difference could be determined between women with negative cytology and women with HSIL only as there where too few numbers in this cohort. Also, when examined, there was no difference found in the

HPV multiple infection rates between the different regions. Overall (not including infections with HPV 52 n=347), 38% (132/347) of samples with multiple infections where positive for two different HPV genotypes, 28.8% (100/347) were positive for three HPV genotypes, 16.7% (58/347) were positive for four HPV genotypes, with 16.4% (57/347) women testing positive for five or more HPV genotypes. LR-HPV types were found in 45.3% (278/614) of HR-HPV positive samples. Interestingly a number of cases that tested HR-HPV positive by hc2/Amplicor were only positive for LR-HPV types by Linear Array HPV Genotyping Test [5.7% (35-37/614)].

In the 25-60 year cohort of women tested HPV 16 [26.9% (131/487)] was the most prevalent HPV genotype detected, followed by; HPV 31, HPV 52 [12.1% (59/487)/(59-100/487) HPV 18 [10.7% (52/487)], HPV 51 [10.5% (51/487)], and HPV 66 [8.4% (41/487)] (Figure 4.5). Overall 8.6% (42/487) of the samples that tested positive for HPV DNA were negative for HPV genotypes by LA. We found a multiple HPV infection rate of between 51.7% and 54% (252-263/487). Again this range was attributable to the limitations of the Linear Array HPV Genotyping Test assay (Figure 3.2). LR-HPV types were found in 41.3% (201/487) of HR-HPV positive samples.

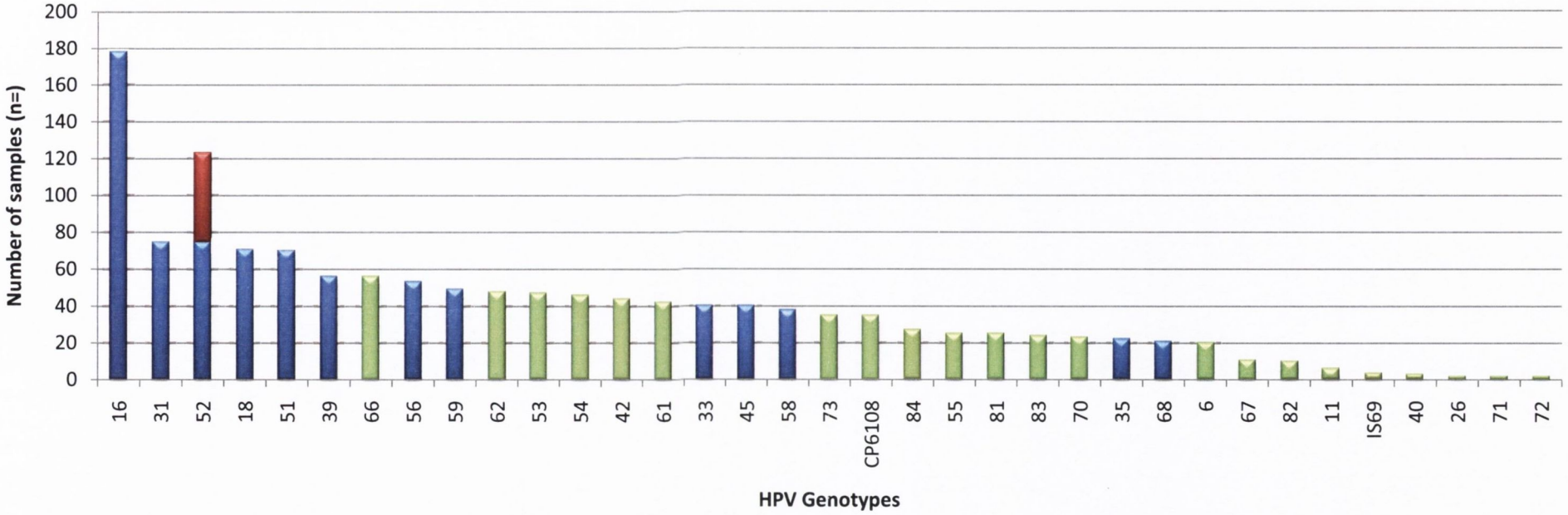


Figure 4.4: Distribution of specific HPV genotypes in the total study population. Specific HPV genotypes are ranked in order of prevalence in the positive samples. HPV types not detected by the hc2 assay are shown in green. HPV 52 was found in 12.2%-20% (75-123/614), but exact HPV 52 prevalence could not be defined in this study due to the limitations of the LA.

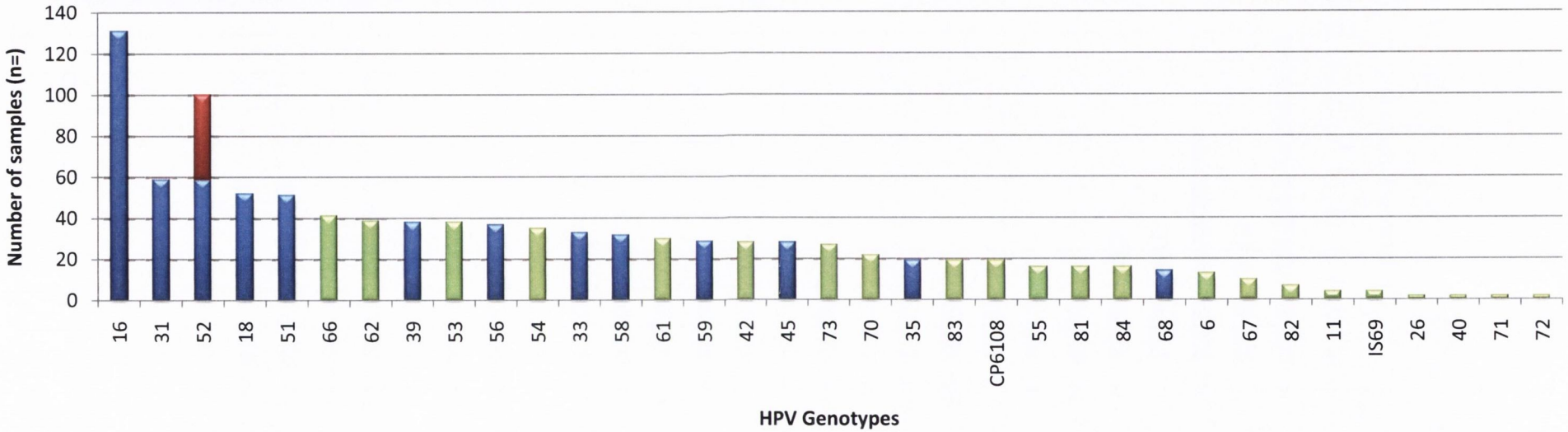


Figure 4.5: Distribution of specific HPV genotypes in the 25-60 years cohort. Specific HPV genotypes are ranked in order of prevalence in the positive samples. HPV types not detected by the hc2 assay are shown in green. HPV 52 was found 8.0%-19.6% (41-100/511), but exact HPV 52 prevalence could not be defined in this study due to the limitations of the LA.

4.3.7.1. HPV genotype distribution in abnormal cytology samples

Of the 258 cases of abnormal cytology that tested positive for HR-HPV by hc2 or Amplicor, 36% (93/258) cases were positive for HPV 16 when genotyped. The other dominant HPV types in samples with abnormal cytology were in decreasing order of prevalence as follows: HPV 31 [14.3% (37/258)], HPV 18 [13.6% (35/258)], HPV 66 [13.2% (34/258)] and HPV 52 [12.4-21.3% (32-55/258)].

HPV 16 was the most common genotype detected in high grade disease [55.1% (27/49)], followed by; HPV 31 [24.5% (12/49)] HPV 33, HPV 39 [10.2% (5/49)], HPV 52 [10.2%-20.4% (5-10/49)] and HPV 51, HPV 66 [8.2% (4/49)]. In HPV 16 positive cases, single type infection occurred in 40.7% (11/27) and with LR-HPV in 22.2% (6/27) of samples. Single type infections occurred in 50% (6/12) of HPV 31, 20% (1/5) of HPV 33 and HPV 39 and 25% (1/4) HPV 51 infections. HPV 66 occurred with another HR-HPV type in 50% (2/4) (HPV 16 and HPV 33 respectively), and in the other 2 samples HPV 66 was the only HR-HPV type.

HPV 18 was only present in 6.1% (3/49) HSIL and CIS samples positive for HPV, with 1 sample occurring with HPV 16 and the other 2 samples occurring with LR-HPV types only.

4.3.7.2. HPV genotype distribution per region

The genotype pattern for each geographical region shows the same nine genotypes (HPV 16, HPV 31, HPV 52, HPV 18, HPV 51, HPV 39, HPV 66, HPV 56 and HPV 59) occurring in the top ten (Table 4.8). All nine genotypes frequented the Eastern population,

with 8/9 occurring in the Western and Northern populations, and 6/9 detected in the top ten of the Southern populations.

HPV 16, HPV 18 and HPV 31 were consistently in the top 5 genotypes found in each region. HPV 16 is the most prevalent genotype found in all regions. HPV 16 was found in 28.4% (60/211) women from the North, 27.7% (45/162) from the South, 23.9% (42/176) from the East and 43.3% (29/67) from the West (Table 4.8). HPV 18 was persistently in the top 4 genotypes in each region, 28.4% (60/211) of women in the North, 14.2% (23/162) women in the South, 9.7 % (17/176) from the East and 34.3% (23/67) from the West (Table 4.8). HPV 31 was persistently in the top 5 genotypes in each region; 11.8% (25/211) North, 14.8% (24/162) South, 15.9% (28/176) East and 11.9% (8/67) West (Table 4.8).

Table 4.8: Distribution of the top 10 HPV genotypes found in each region.

HPV type	North*	HPV type	South*	HPV type	East*	HPV type	West*
16	29.4%	**16**	27.8%	**16**	24.1%	**16**	43.3%
51	13.7%	**31**	14.8%	**18**	13.2%	**66**	16.4%
52	12.3%-13.7%	**52**	13.6%-25.9%	**51**	12.1%	**52**	16.4%-19.4%
31	11.8%	**18**	10.5%	**39**	10.9%	**39**	13.4%
18	10.9%	**54**	9.3%	**31**	10.3%	**18**	11.9%
56	10.0%	**51**	8.6%	**52**	9.2%-22.4%	**31**	11.9%
59	10.0%	**66**	8.6%	**53**	9.2%	**42**	9.0%
61	10.0%	**53**	8.0%	**66**	9.2%	**51**	9.0%
39	9.0%	**62**	8.0%	**56**	8.6%	**56**	9.0%
33	8.5%	**70**	8.0%	**59**	8.6%	**33**	7.5%

Grey The top 9 genotypes in the entire population
Yellow Possibly under-represented due to pre-screening with hc2/Amplicor assays.
Green HPV 66 is shown in green as it is one of the 9 genotypes found in the total population and is also possibly under-represented due to pre-screening with the hc2/Amplicor assays.
*Percentages are greater than 100% due to multiple infections.

4.4. Discussion

This study describes for the first time, the HPV population prevalence and genotype distribution in the Irish cervical screening population and includes data to describe regional differences in HPV prevalence and genotype distribution. Also the data in this chapter relates to the sampling of an entire country, with samples from each of the provinces. The sample size (n=3193) was similar to equivalent studies carried out in areas of similar population sizes (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 2006). The aims of this project were, to determine the HPV prevalence and genotype distribution found in the Irish cervical screening population and to use that data to provide baseline knowledge of HPV in Ireland for use in determining the impact of the national HPV vaccination programme in the future.

Overall the determined HR-HPV prevalence rate was 19.2%, with HPV 16 being the most prevalent HPV genotype detected. Other dominant genotypes included; HPV 31, HPV 52, HPV 18, HPV 51 and HPV 39/HPV 66. The overall HR-HPV prevalence rate found in this population was high at 19.2%, considering the low incidence of CC in Ireland (10.40 deaths/100 000 per year from 1994-2007) (NCRI, 2009). This prevalence rate is higher than the rates seen in UK studies (15.4%, 7.6%, 15.5%, 7.1%, and 10.9%) where established screening programs exist (Cuschieri *et al.*, 2004a, Cuzick *et al.*, 2003, Sargent *et al.*, 2008, Peto *et al.*, 2004). Our data is also higher than previously reported data on an Irish cohort (Total HPV prevalence of 19.7%, 74% HR-HPV infections) (Keegan *et al.*, 2007). Cuschieri *et al*, 2004 described HR-HPV prevalence rate of 15.4% in a Scottish population of women attending for screening with a low incidence of CC (4.6 deaths /10000 per year) (Cuschieri *et al.*, 2004a). The author commented that such high HR-

HPV prevalence is in line with countries with relatively higher risks of CC such as Colombia (14.9%) and Paraguay (20%).

Sargent *et al*, (2008) found a HR-HPV prevalence of 15.5% in the ARTISTIC (A Randomised Trial in Screening to Improve Cytology) study. Peto *et al*, (2004) found using PCR testing (MY09/11) found a relatively lower HPV prevalence rate of 7.1% compared to our study and studies from the UK. The variation in HPV prevalence in various studies may be due in some part to the differences in how individual studies are designed, i.e. age range, test used etc. (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 2006, Clifford *et al.*, 2005). This highlights the variability in HPV prevalence rates in different populations (Cuschieri *et al.*, 2004a). Due to this variability, epidemiological evaluation on a population by population basis has been recommended (Cuschieri *et al.*, 2004a). Our data demonstrates this variability at a geographical level.

In our study the HR-HPV prevalence rate was significantly higher in younger age groups (<30 years). This is likely due to increased numbers of newly acquired sexual partners among younger women (Hibbitts *et al.*, 2006, Deacon *et al.*, 2000), coupled with possible HPV immunity, which may be acquired over time in older women.

A second peak of HPV prevalence has been reported in women older than 35 years (Cuzick *et al.*, 1999, Castle *et al.*, 2005). The second peak of HPV prevalence is not fully understood to date, with possible explanations being, acquisition of new HPV types, and possible differences in sexual history in older populations or increased persistence due to age-related immune senescence (Castle *et al.*, 2005). However, more research is required on the natural history of HPV in older women. HPV DNA testing has been used as a

primary screening tool in women ≥30 years of age only, due to the high HPV DNA prevalence in younger, resulting in a lower false positive rate in women ≥30 years (Herrington, 2001). This study would support this approach, as 26.5% of the cytology negative women <30 years of age were positive for HPV, while only 8.5% of cytology negative women ≥30 years were HPV positive (Table 4.5).

In total, 88.5% of cases in this study were normal by cytology, the abnormal cytology rate was 11.5%, as described in table 4.2. Similar rates have been seen in several studies carried out in both Ireland and the UK (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 2006, Keegan *et al.*, 2007). Keegan *et al*, (2007), described an abnormal cytology rate of 11% in women presenting for opportunistic screening, consisting of 4% ASCUS, 5% mild dyskaryosis, and 2% high grade dyskaryosis, in a cohort of opportunistically screened women from Dublin, Ireland. Similar rates were seen in Wales and Scotland where approximately 7% and 10% of opportunistically screened women had abnormal cytology respectively (Hibbitts *et al.*, 2006, Cuschieri *et al.*, 2004a).

We demonstrated that HPV prevalence increases with increasing grade of cytology (Table 4.5); a trend found in many prevalence studies worldwide, and would be expected as HR-HPV is the main cause of CC worldwide (Walboomers *et al.*, 1999). In our study we found regional variation in abnormality rates between the North and South regions. The difference was significant, and after further review, could not be explained by differences in age. One possible reason for the difference could be due to the fact that those women screened in the North live in Northern Ireland, part of the UK, where there is a well established CC screening program. We described an abnormal cytology rate which is relatively higher in women less than 30 years (17.5%) when compared to those over 30

years (8.8%). This is in line with published data (Kitchener *et al.*, 2006). Interestingly, the regions with the highest abnormal cytology (14.9%, South, 11.8% in the East), mirror the trends in Ireland for relative risks of developing CIS and CC (Figure 1.1). In the western population, HSIL abnormalities represented 26.1% of all abnormalities. This is high but not statistically higher than the population as a whole. Other data from the National Cancer Registry, Ireland (NCRI) indicates that there is a relatively higher risk of developing CC in parts of the West of Ireland (Figure 1.1) (NCRI, 2009). Unfortunately our sampling in the Connaught region did not include any samples from these counties. If we had managed to include samples from these counties or increased the sampling size this may well have produced a result.

One of the risk factors for the development of HPV infection is parity. Evidence suggests that parous women have a lower risk of association with HPV infection. (Kjaer *et al.*, 1997, Ronco *et al.*, 2005, Deacon *et al.*, 2000, Munoz *et al.*, 2002). Our data concurs with this; nulliparous 30.8%, parous 12.4%.

It is well established that HPV 16 and HPV 18 are among the highest risk and highest prevalent HPV types in screening populations and in high grade disease (De Vuyst *et al.*, 2009, Smith *et al.*, 2007). Thus the introduction of HPV vaccines against these HPV types will reduce the incidence of HPV 16/HPV 18 related infections. However we cannot exclude the impact that the vaccines could have on other HPV types. In keeping with published data, we report HPV 16 as the most prevalence HPV type in Ireland followed by HPV 31/HPV 52, HPV 18, HPV 51 and HPV 39/HPV 66 (Figure 4.4) [(HPV 16, HPV 18, HPV 66, HPV 33, HPV 53, HPV 31, and HPV 58) (Keegan *et al.*, 2007)] . Interestingly the HR-HPV types HPV 16, HPV 18, HPV 31, and HPV 66 were among the

top 10 types in both studies, with HPV 66 is possibly underestimated in this study as it is not included in the HR-HPV probe cocktail used in the hc2 assay. When compared to other HPV prevalence studies in Europe, the genotypes found in our study tend to be well represented in other populations. Cuschieri *et al*, (2004) found HPV 16, followed by HPV 18, HPV 51, HPV 31, HPV 52, and HPV 45 were the most prevalent genotypes from residual cytology specimens from a routine cervical screening population with the top 4 genotypes the same as found in the present study (Cuschieri *et al.*, 2004a). This trend was also seen with the exception of HPV 52 in the ARTISTIC trial (Sargent *et al.*, 2008). Hibbitts *et al.*, (2006), found a slightly different HPV distribution in a Welsh screening population. They found that again, HPV 16 is the most dominant type; however this was followed by HPV 35, HPV 66, HPV 59, HPV 56, HPV 58, HPV 18, and HPV 3. It should be considered therefore that not only does HPV DNA prevalence vary from population to population but also HPV genotype distribution can vary, not just between different countries but also between different populations within countries.

In this study, a high rate of multiple infection was noted (56.5-58.5%) when compared to published data from Scotland [(43.3%) (Cuschieri *et al.*, 2004a)]. Cuschieri *et al.,* (2004a) noted, that the multiple infection rate found in their population was considered high, regardless of cytological status of the samples, however, it is understood that there is a lack of consensus regarding the implications of multiple HPV infections (Cuschieri *et al.*, 2004a). Taking this into account our populations high rate of multiple infection warrants future study, especially in relation to multiple HPV infections that occur with the HPV types found in current commercially available vaccines. We did note a higher prevalence of multiple HPV infection in the younger age groups similar to other studies (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 20006, Sargent *et al.*, 2008). This is also likely related to the

increased numbers of newly acquired sexual partners and possible HPV immunity that may be responsible for the higher rates of HPV infections in younger age groups overall. It was also noted that multiple HPV infection was significantly higher in women with abnormal cytology when compared to women with negative cytology which is in line with published data (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 20006, Sargent *et al.*, 2008). Finally, we found that a large number of samples within our study had multiple HPV infections with five or more HPV genotypes (n=57). Menton *et al.*, (2009), found a similar set of women presenting to sexually transmitted disease clinics had multiple HPV infections with five or more HPV genotypes. As a result, there may be a pool of women who may aid in genotype replacement in the epidemiology of HPV infection following the widespread use of HPV vaccines.

In general the same HPV genotypes that were common across the different regions of the study (Table 4.8). HPV 16 was the most prevalent HPV genotype found in each of the four regions of Ireland represented in the study, however, the relative prevalence of HPV 16 in each region was different, with the relative prevalence of HPV 16 highest in the West of Ireland (Table 4.8). It should be noted however that the average age for the Western region was higher that that of the other regions which may have had an affect on the results. Interestingly HPV 66 did not occur in a high percentage of HR-HPV positive samples from Northern Ireland. This is to be expected as the Amplicor assay used for HR-HPV screening in the North region has no reported cross-reactivity with HPV 66, unlike the hc2 assay which has reported cross-reactivity with several HPV genotypes not included in the probe mix (Castle *et al.*, 2008b). As such HPV 66 may be significantly under-represented in this region. However, the fact that HPV 66 was detected in a high percentage of samples tested using the hc2 assay, would suggest that the assay did show

cross-reactivity with HPV 66 in this study. The number of samples that tested positive for HR-HPV DNA that where positive only for HPV genotypes not included in the hc2 assay is most likely due to the demonstrated cross reactivity of the hc2 test with other HR-HPV and LR-HPV types (Sargent *et al.*, 2008).

Obviously the variation in HPV type distribution not only within our population [North, South, East, and West, (Table 4.8)] but also in different populations of the UK, could lead to small pockets of rare HR-HPV genotypes following the onset of large scale vaccination programmes.

As eluded to above, HPV 66 is an emerging genotype in the Irish population. It was the 6th most prominent type detected. Very few studies have reported HPV 66 with such high prevalence in a screening cohort (Keegan *et al.*, 2007, Hibbitts *et al.*, 2006). The higher rates of HPV 66 infection in these studies may be attributed to the consensus PCR approach used rather than hc2. A Dutch study carried out on repeat cytology and colposcopy samples, using sequencing and original linear array technology found that HPV 66 was the 2nd most prevalent HR-HPV type in their population (Melchers, 1999). HPV 66 appears to be associated with abnormal cytology more common in ASCUS than in the other grades of cytology (Keegan *et al.*, 2007, Melchers, 1999). In the current study HPV 66 was the 4th most prevalent HPV genotype found in samples positive for HPV DNA by hc2, with abnormal cytology, and was found in 4/44 samples with HSIL cytology.

As mentioned (Chapter 1, section 1.4) HPV 66 was recently re-classified from probably carcinogenic to possible carcinogenic as it is "found so rarely that its percentage of

detection is less than the relative percentage of detection among the general population" (Schiffman *et al.*, 2009). This classification may not be appropriate in the Irish screening population as HPV 66 was present in 1.8% of our study population and 9.2% of HPV positive specimens. It was also the only HR-HPV type found in 4% of HSIL samples. As HPV 66 re-classification is based on its prevalence in CC from published data, which do not include studies from Ireland, it warrants further study.

The HPV genotypes found in abnormal cytology samples and the HSIL samples in this study are similar to those found in the meta-analysis of eighteen European HPV studies, where HPV 16 was the prominent genotype detected in high grade disease (De Vuyst *et al.*, 2009). Our data concurs with published data showing an increase in the prevalence of HPV 16 as the grade of cytology becomes more severe, a trend not seen in HPV 18 (De Vuyst *et al.*, 2009) and our data would support this. We found HPV 18 in 14.5% of ASCUS, 16.5% LSIL and 6.1% in HSIL HPV positive samples. It would be interesting to possibly determine if this trend is seen in HPV 16 and HPV 18 co-infections only, with HPV 16 so prevalent in high-grade, it should be tested to determine if the genotypes are competing viral load wise as the grade increases.

As HPV genotyping was carried out on those samples positive for HR-HPV DNA by the hc2 assay, the prevalence of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 can be considered as representative of the Irish screening population. The other 24 genotypes tested in the Linear Array HPV Genotyping Test assay (6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS69, and CP6108) therefore may underrepresented in this study as a result of the pre-selecting with the hc2 assay. Overall 8.1% of the hc2 positive samples had no HPV when tested by genotyping; this is

a smaller percentage than that found in the ARTISTIC trial where 20.5% of samples positive by hc2 did not register any HPV types when tested on the then prototype Linear Array HPV Genotyping Test assay (Sargent *et al.*, 2008). In our study we used the now commercially available Linear Array HPV Genotyping Test assay (not the prototype), it would be expected therefore that the number of hc2 positive samples without genotypes on the Linear Array HPV Genotyping Test would be lower (Sargent *et al.*, 2008). It should also be noted that in the ARTISTIC study a RLU of 1.0 was used compared to our study where we repeated samples with RLU of 1.0-2.5 as per manufactures updated guidelines, which were directly introduced to reduce this phenomenon following calls to increase the cut-off (Hesselink *et al.*, 2006). The majority of these cases could be related to low RLU with 22/33 samples had an RLU <10, also found in another recent study (Castle *et al.*, 2008a).

The data provided in this chapter defines a base level of HPV prevalence and specific genotypes in relation to age, cytological diagnosis, and parity in the Irish population. This study is the only study which describes the population based types specific HPV prevalence in this Irish cervical screening population. The data presented will provide baseline data on HPV prevalence prior to the introduction of HPV vaccination program. The data will also allow for informed decisions regarding any future development in relation to HPV molecular testing, ultimately helping Irish women receive the best possible CC care in the future.

Chapter 5

The prevalence and persistence of Human Papillomavirus in Human Immunodeficiency Virus (HIV) positive women

5.0 The prevalence and persistence of HPV in HIV positive women

5.1. Introduction

HPV is the primary aetiological agent in the development of CC and the most common STI, with ~80% of women infected during their life. HIV positive men and women are at increased risk of HPV associated diseases and cancer (Boshoff, 2002, Palefsky, 2006b). HIV positive women are shown to be at a five to seven fold risk of CC compared with the general population (Palefsky, 2006a). Indeed there is an increased prevalence of cervical associated HPV infections in HIV positive women (Strickler *et al.*, 2005, Palefsky, 2006b), and this prevalence increases as the immune status decreases. The main determinant of persistent HPV infection in HIV, is primarily related to CD4 levels and the patients immune status (Palefsky and Holly, 2003, Stern, 2005).

Worldwide, HPV prevalence rates are higher in HIV positive populations. Additionally HPV prevalence rates in HIV positive populations of Africa and South/Central America are higher when compared to Europe and North America (Clifford *et al.*, 2006). HPV genotype distribution in HIV positive women seems to differ from those published in general populations. A meta-analysis of several HPV studies showed that the HR-HPV types 16, 58, 18, 52, 31, 33, and 45 were the most common types found in HIV positive populations (Clifford *et al.*, 2006). The authors noted that HPV 16 prevalence increased with increasing severity of cervical disease, similar to trends seen in HIV negative populations, however, overall HPV 16 was underrepresented in HIV positive women with HSIL relative to HIV negative women (Clifford *et al.*, 2006).

With the introduction of anti-retroviral treatment HIV positive women now have an increased life expectancy. The role of anti-retroviral therapy in relation to the incidence of

CC and HPV infection is not known, however data suggests that anti-retroviral therapy may have an impact on HPV infections in HIV patients (Palefsky, 2007, Minkoff *et al.*, 2010, Shrestha *et al.*, 2010). To date, the relationship between HPV, HIV, anti-retroviral therapy and the development of cervical disease is not fully understood. This is largely due to the small number of individual cases of CC in HIV positive women in developed countries with access to anti-retroviral drugs and new and innovative approaches are needed to study these roles (Bratcher and Sahasrabuddhe, 2010).

While HPV DNA testing has been adopted as an approach to triage and manage cervical abnormalities, it is generally not recommended for use in HIV due to the high prevalence of HPV infection in these patients. It is well recognised that HPV contributes to cervical malignancy through the combined action of two viral oncoproteins, E6 and E7. Indeed many studies have reported on the potential benefits of detecting HPV mRNA to improve specificity of HPV detection for detecting high grade disease (Molden *et al.*, 2005c, Szarewski *et al.*, 2008, Keegan *et al.*, 2009, Ratnam *et al.*, 2010).

5.1.1. Hypothesis

We hypothesise that HPV DNA and HPV mRNA prevalence and persistence in HIV positive populations will be higher than in general populations. In addition we believe that anti-retroviral therapy will reduce the incidence and persistence of HPV DNA and mRNA through its effects on immunosupression.

5.1.2. Aims

- To determine HPV DNA prevalence in HIV positive women residing in Ireland.
- To determine HPV mRNA prevalence in HIV positive women residing in Ireland.
- To define the persistence and progression of HPV infection in HIV positive women.
- To determine the predictors of HPV DNA and mRNA infection in HIV positive women.

5.2. Methods

5.2.1. Study population

All female patients attending the HIV clinic at St James's Hospital, Dublin, were invited to participate in the study. PreservCyt™ smear samples were collected through the Department of Cytology at, St. James's Hospital from 2007 until the start of 2009, and through the Coombe Women and Infants University Hospital, Dublin, during 2009. Samples were taken from women initially at baseline (n=321) and at ~6-18 months follow-up (n=88). Routine management of HIV positive women is carried out through annual screening. Patients with normal cytology continue to be screened annually. Patients with abnormalities are followed by repeat smear at 6 months or referred for colposcopy. Thus 88 patients were recruited with both baseline and follow-up HPV data. The PreservCyt™ smear samples were processed and analysed according to *BSCCP* guidelines by the Department of Cytology, Central Pathology Laboratory, St. James' Hospital, Dublin, and the Department of Cytology, Coombe Women and Infants University Hospital, Dublin respectively. Following diagnosis specimens were transported to Trinity College Molecular Pathology Research Laboratory, based in the Coombe Women and Infants University Hospital, Dublin. Upon arrival samples were anonymised and assigned a study ID for HPV testing.

All clinically relevant data (age, ethnicity, smoking, contraception, parity, viral load, CD4 cell count, date of HIV diagnosis etc.) was collected in the HIV clinic at St James's Hospital, Dublin for entry on to the secured database system Distiller (Chapter 3, Section 3.2) located in the Coombe Women and Infants University Hospital, Dublin. Data relating to anti-retroviral therapy use was not considered as clinically relevant. This is due to the

fact that not all women who were prescribed anti-retroviral drugs would be committed to taking the drugs as prescribed, as such HIV viral load was considered as an indicator that an individual was receiving affective anti-retroviral therapy. Ethical approval was obtained from the Joint Federated Hospital Research Ethics Committee at St. James's Hospital.

5.2.2. HPV DNA testing

HPV DNA testing was carried out using the hc2 assay (Qiagen Ltd.), as described in Chapter 3, Section 3.5.

5.2.3. HPV mRNA testing

The PreTect™ HPV-Proofer uses NASBA technology to detect HPV E6/E7 mRNA transcripts from five HPV types (HPV16, HPV 18, HPV 31, HPV 33 and HPV 45). The PreTect™ HPV-Proofer assay was carried out on all samples, as described in Chapter 3 Section 3.8.

5.2.4. Statistical analyses

Pearson Chi-Square test was used to determine statistical significance for the association between HPV DNA/mRNA and clinical/demographic factors. McNemars test was carried out to determine concordance of HPV tests and multiple logistic regression was carried out to determine predictors of HPV infection. All statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) software version 16.0. Statistical analyses were carried out with the assistance of Dr. Kathleen Bennett, Senior Lecturer

and Statistician in the Department of Pharmacology and Therapeutics, Trinity College Dublin.

5.3. Results

5.3.1. Characteristics of the study population

At baseline, the age range of the study population was from 17-71 years, the average age was 34.6 years and the median age of the study population was 34 years. The distribution of the number of women in different age categories can be seen in figure 5.1. A breakdown of the population by age and demographic/clinical data is shown in table 5.1.

Overall 9.7% (31/321) samples were <25 years old, 17.4% (56/321) were aged between 25-29 years, 25.9% (83/321) were aged between 30-34 years, 21.2% (68/321) were aged between 35-39 years, 15% (48/321) were aged between 40-44 years, and 10.9% (35/321) were aged >45 years (Figure 5.1).

The majority [71.3% (229/321)] of the study population had normal cervical cytology, while 28.7% (92/321) had an abnormal cytological diagnosis (Table 5.2). The relative representation of each cytological grade is shown in table 5.2. Low-grade abnormalities were predominant in the study with 39.1% (36/92) of cases ASCUS, 45.7% (42/92) of cases diagnosed with LSIL. In total 15.2% (14/92) were HSIL, by cytology.

Table 5.1: Breakdown of clinical and demographic data by age group.

		Age groups						
		<25 years	25-29 years	30-34 years	35-39 years	40-44 years	> 45 years	Total
Ethnicity	European	11	22	24	19	29	18	123
	African	19	32	56	45	17	13	182
	Asian	0	1	1	0	1	0	3
	South American	0	1	0	0	0	0	1
	North American	0	0	0	0	0	1	1
	Not recorded	1	0	2	4	1	3	11
	Total	31	56	83	68	48	35	321
Years since HIV positive diagnosis	<5 year	22	34	34	29	14	12	145
	5-9 years	9	19	37	27	18	8	118
	10+ years	0	2	7	8	15	12	44
	Not recorded	0	1	5	4	1	3	14
	Total	31	56	83	68	48	35	321
Immunosuppression	CD4 >200x10^6/L	30	49	72	53	41	30	275
	CD4 <200x10^6/L	1	7	11	14	6	5	44
	Not recorded	0	0	0	1	1	0	2
	Total	31	56	83	68	48	35	321
Viral suppression	HIV Viral load <50 copies/mL	10	25	44	36	33	15	163
	HIV Viral load >50 copies/mL	21	31	39	31	14	18	154
	Not recorded	0	0	0	1	1	2	4
	Total	31	56	83	68	48	35	321
Parity	Nulliparous	15	12	10	6	6	1	50
	Parous	15	44	71	58	41	31	260
	Not recorded	1	0	2	4	1	3	11
	Total	31	56	83	68	48	35	321
Smoking history	Smokers	10	17	28	19	21	15	110
	Current non-smokers	19	36	52	44	26	17	194
	Not recorded	2	3	3	5	1	3	17
	Total	31	56	83	68	48	35	321
Contraception	Hormonal	1	4	2	5	0	1	13
	[a]Barrier	11	28	44	25	24	6	138
	Barrier and hormonal	3	4	2	2	0	0	11
	[b]Physical contraception	2	4	3	3	3	0	15
	Not sexually active	12	11	26	23	20	23	115
	None	1	2	3	3	0	1	10
	Not recorded	1	3	3	7	1	4	19
	Total	31	56	83	68	48	35	321

[a]Barrier methods include condoms and dental dams, [b]Physical contraception methods include tubal ligation and intrauterine devices.

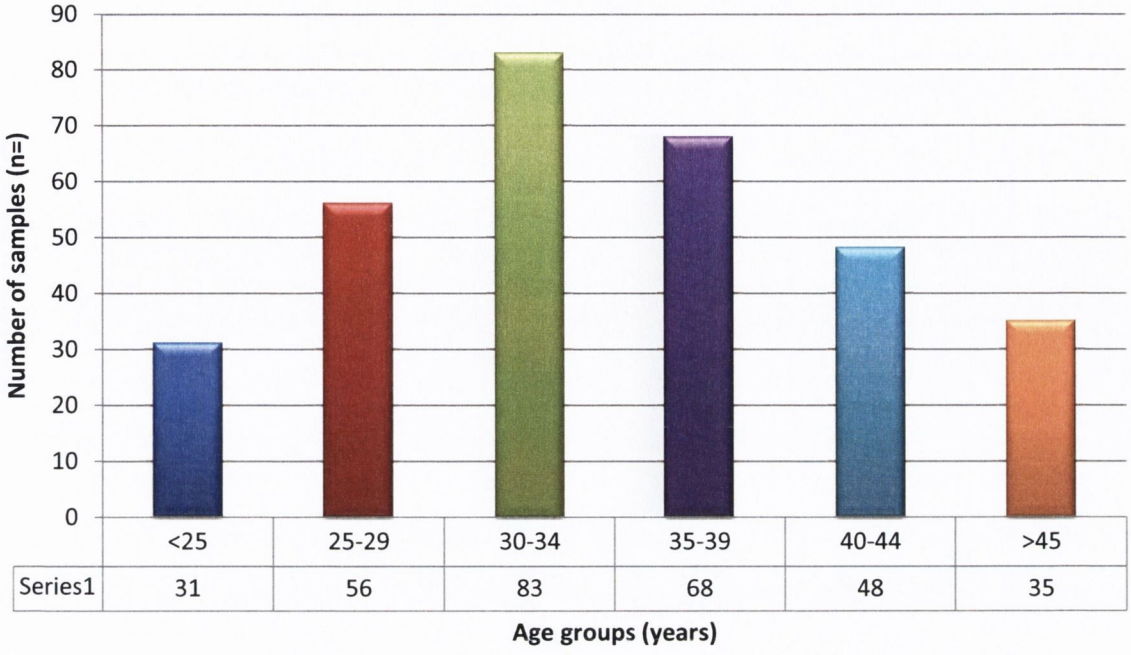


Figure 5.1: Distribution of the study population by age. The number of taken from patients in the different age groups of the population. The 30-34 year age group was the best represented age group in the study. Each age group had at least 30+ samples. Total population n=321.

Table 5.2: Breakdown of cytology by grade.

	Negative	[a]ASCUS	[b]LSIL	[c]HSIL	Total Abnormal	Total
Total	71.3% 229/321)	11.2% (36/321)	13.1% (42/321)	4.4% (14/321)	28.7% (92/321) (CI 95% 23.7%-33.6%)	321

[a]Atypical Squamous Cells of Undetermined Significance, [b]Low-grade squamous intraepithelial lesion, [c]High-grade squamous intraepithelial lesion.

5.3.2. HPV prevalence in HIV positive women

A HR-HPV DNA crude prevalence rate of 51.1% (164/321) was found in the study population. A HPV mRNA crude prevalence rate of 21.8% (70/321) was found in the study population (Table 5.3). The average age of HPV DNA positive women was 33.9 years and a median age of 33 years. The average age of the HPV mRNA positive women was 34.9 years and a median age of 34 years.

The crude HPV DNA prevalence rate was high across the different age groups with 64.5% (20/31) in the <25 years age group, 55.4% (31/56) in the 25-29 years age group, 50.6% (42/83) in the 30-34 years age group, 41.2% (28/68) in the 35-39 years age group, 37.5% (18/48) in the 40-44 years age group and 51.4% (18/35) in >45 years age group (Figure 5.2). There was no significant difference in the HPV DNA prevalence between women <30 years [58.6% (51/87)] and women ≥30 years [48.3% (113/234)].

The crude HPV mRNA prevalence was also high in all age groups, with 22.6% (7/31) in the <25 years age group, 26.8% (15/56) in the 25-29 years age group, 21.7% (18/83) in the 30-34 years age group, 19.1% (13/68) in the 35-39 years age group, 12.5% (6/48) in the 40-44 years age group and 31.4% (11/35) in >45 years age group (Figure 5.2). There was no significant difference between HPV mRNA prevalence between women <30 years [25.3% (22/87)] and women ≥30 years [20.5% (48/234)].

In total, 46.7% (150/321) of women were negative for HPV DNA and mRNA while 19.6% (63/321) of women were positive for HPV DNA and mRNA. A further 31.5% (101/321) were positive for HPV DNA and negative for HPV mRNA while 2.2% (7/321) were negative for HPV DNA and positive for HPV mRNA.

In total 82.6% (76/92) of cases with abnormal cytology were positive for HPV DNA with 41.3% (38/92) of cases mRNA positive. All [100% (14/14)] HSIL cases were positive for HPV DNA, while only 78.6% (11/14) were positive for mRNA. The breakdown of HPV DNA and HPV mRNA positivity by cytological grade is shown in table 5.3. There was no difference in HPV DNA and mRNA prevalence rates in cytologically normal and abnormal (ASCUS or worse) women <30 and ≥30 years However, in women <30 years and women ≥30 years, all [100% (6/6) and 100% (7/7) respectively] of HSIL samples tested positive for HPV DNA, with only 50% (3/6) and 100% (7/7) of HSIL samples tested positive for HPV mRNA in women <30 years and women ≥30 years respectively.

Logistic regression analysis revealed cytological status was one of the predictors of HPV DNA positivity along with smoking history and years since HIV positive diagnosis (Table 5.4). Cytological status was also the only predictor of HPV mRNA positivity (Table 5.5).

Table 5.3: HPV DNA and HPV mRNA positivity across the different cytological disease categories.

	Negative	[a]ASCUS	[b]LSIL	[c]HSIL	Total abnormal	Total positivity
HPV DNA positive	38.4% (88/229)	69.4% (25/36)	88.1% (37/42)	100.0% (14/14)	82.6% (76/92)	51.1% (164/321)
HPV mRNA positive	14.0% (32/229)	25.0% (9/36)	42.9% (18/42)	78.6% (11/14)	41.3% (38/92)	21.8% (70/321)

[a]Atypical squamous cells of undetermined significance, [b]Low-grade squamous intraepithelial lesion, [c]High-grade squamous intraepithelial lesion.

Table 5.4: Logistic regression analysis for factors associated with HPV DNA positivity.

	Odds ratio
Negative	1.0
ASCUS	5.03 (CI 95% 2.02-12.48)
LSIL	9.64 (CI 95% 3.44-27.03)
HSIL	2.01 x10^9
<5 years since HIV positive diagnosis	1.0
5-9 years since HIV positive diagnosis	0.45 (CI 95% 0.25-0.78)
10-19 years since HIV positive diagnosis	0.41 (CI 95% 0.17-0.97)
20+ years since HIV positive diagnosis	0.09 (CI 95% 0.02-0.44)
Current non-smoker	1.0
Current smoker	1.76 (CI 95% 0.99-3.12)

Table 5.5: Logistic regression analysis for factors associated with HPV mRNA positivity.

HPV mRNA logistic regression analysis	
Negative	1.0
ASCUS	2.1 (CI 95% 0.88-4.76)
LSIL	4.61 (CI 95% (2.25-9.45)
HSIL	22.57 (CI 95% 5.96-85.36)
<5 years since HIV positive diagnosis	1.0
5-9 years since HIV positive diagnosis	0.96 (CI 95% 0.49-1.8)
10-19 years since HIV positive diagnosis	0.6 (CI 95% 0.20-1.8)
20+ years since HIV positive diagnosis	0.6 (CI 95% 0.11-3.2)
Current non-smoker	1.0
Current smoker	1.5 (CI 95% 0.8-2.8)

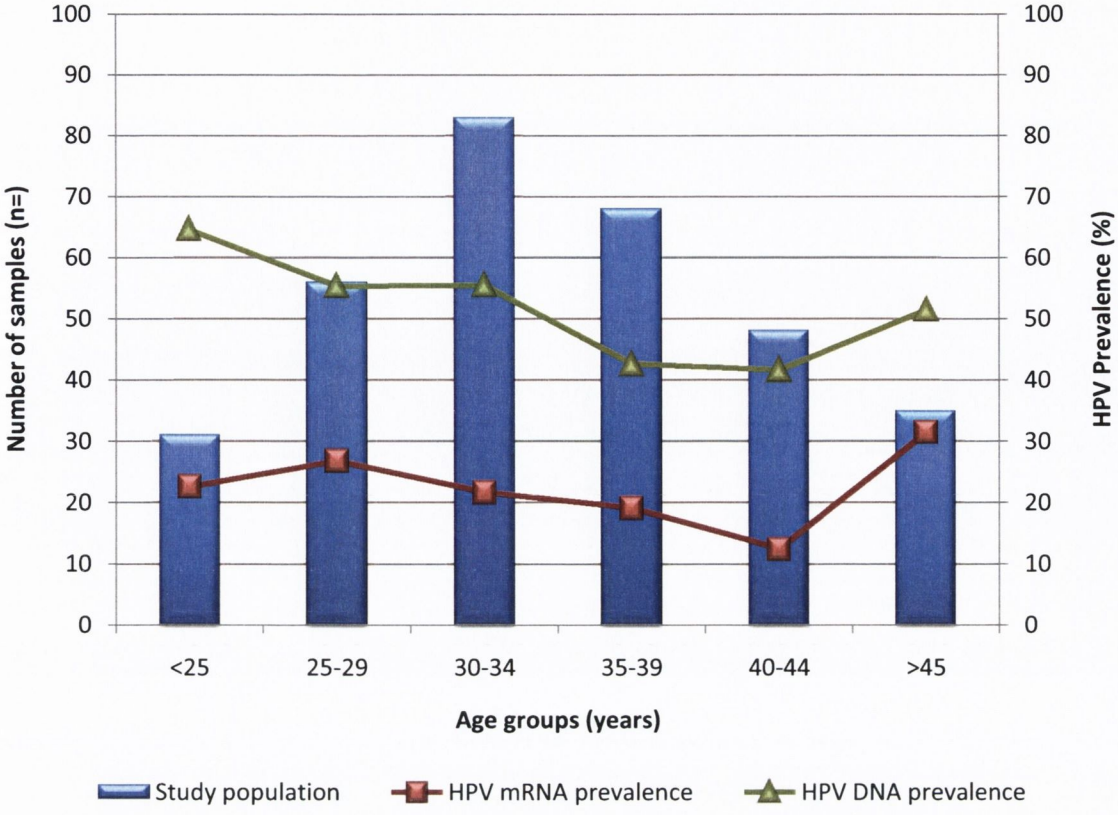


Figure 5.2: HPV DNA and HPV mRNA prevalence by age. The HPV DNA and HPV mRNA prevalence data is represented on the secondary vertical axis. The DNA and mRNA prevalence is high in each age group, with a similar pattern of prevalence seen from women greater than 25 years.

5.3.3. HPV in relation to demographic and clinical data

5.3.3.1. HPV status and CD4 cell count

CD4 cell counts were available for all patients with the exception of 2: 13.8% (44/319) of patients were immunosupressed with low CD4 counts (<200x10^6/L) and 86.2% (275/319) of patients had high CD4 counts (>200x10^6/L). At baseline, HPV DNA prevalence was significantly higher in women with low CD4 counts [72.7% (32/44)] when compared to women with high CD4 counts [48% (132/275)] (χ^2=9.284, p<0.005,) (Table 5.6). This trend was also observed for HPV mRNA data with 40.9% (18/44) of women with low CD4 counts positive for HPV mRNA compared to 18.9% (52/275) of women with high CD4 counts (χ^2=10.718 p=0.001). It was also noted that 52.3% (23/44) of women with low CD4 counts had abnormal cytology, significantly higher than the abnormality rate found in women with high CD4 counts [25.1% (69/275)] (χ^2=13.656, p<0.001).

It was also noted that there was a difference between HPV DNA prevalence in cytologically normal women with low CD4 counts [61.9% (13/21)] compared to cytologically normal women with high CD4 counts [36.4% (75/206)] (χ^2=5.219, p<0.05); a difference was also noted between HPV mRNA in both cohorts respectively [28.6% (6/21) and 12.6% (26/206)] (χ^2=4.004, p<0.05) (Table 5.6). All women with low CD4 counts and HSIL were positive for HPV DNA and mRNA, while only 66.6% (6/9) of women with high CD4 counts and HSIL were positive for HPV mRNA (Table 5.6).

Table 5.6: HPV DNA, mRNA and cytology data in relation to CD4 cell counts.

	Negative	[a]HSIL	Total Abnormal	Total
CD4 >200x10^6/L				
HPV DNA +	36.4% (75/206)	100.0% (9/9)	82.6% (57/69)	48.0% (132/275)
HPV mRNA +	12.6% (26/206)	66.7% (6/9)	37.7% (26/69)	18.9% (52/275)
CD4 <200x10^6/L				
HPV DNA +	61.9% (13/21)	100.0% (5/5)	82.6% (19/23)	72.7% (32/44)
HPV mRNA +	28.6% (6/21)	100.0% (5/5)	52.2% (12/23)	40.9% (18/44)

[a]High-grade squamous intraepithelial lesion.

5.3.3.2. HPV status and HIV viral load

HIV viral load data was available for all but 4 patients in this study; 51.4% (163/317) were virally suppressed [HIV viral load <50 copies/mL (women receiving anti-retroviral therapy) and 48.6% (154/317) were not virally suppressed [HIV viral load >50copies/mL (women not receiving anti-retroviral therapy)]. HPV DNA prevalence in virally suppressed women was significantly lower [45.4% (74/163)] compared to women who were not virally suppressed [57.8% (89/154)] (χ^2=4.869, p<0.05) (Table 5.7). No significant difference was detected between mRNA rates in virally suppressed patients [19.6% (32/163)] when compared to patients who were not virally suppressed [24% (37/154)] (Table 5.7).

When we analysed cytologically normal women in both cohorts, we found that HPV DNA was significantly (χ^2=4.662, p<0.05) lower in virally suppressed women [32.2% (39/121)] compared to non suppressed women [46.2% 49/106)]; however, no difference was apparent in HPV mRNA prevalence in the virally suppressed cohort [10.7% (13/121)] and the cohort of women who were not virally suppressed [17.9% (19/106)] (Table 5.7).

In virally suppressed women, 100% (8/8) of women with HSIL were positive for HPV DNA with 87.5% (7/8) positive for HPV mRNA. HPV DNA detection in women who were not virally suppressed was 100% (6/6), while the HPV mRNA prevalence was 66.7% (4/6) in non suppressed patients (Table 5.7).

Table 5.7: HPV DNA, mRNA and cytology in relation to HIV viral load.

	Negative	[a]HSIL	Total Abnormal	Total
Viral load <50 copies/mL				
HPV DNA +	32.2% (39/121)	100.0% (8/8)	83.3% (35/42)	45.4% (74/163)
HPV mRNA +	10.7% (13/121)	87.5% (7/8)	45.2% (19/42)	19.6% (32/163)
Viral load >50 copies/mL				
HPV DNA +	46.2% (49/106)	100.0% (6/6)	83.3% (40/48)	57.8% (89/154)
HPV mRNA +	17.9% (19/106)	66.7% (4/6)	37.5% (18/48)	24.0% (37/154)

[a]High-grade squamous intraepithelial lesion.

5.3.3.3. HPV status in relation to ethnicity, smoking, contraception and parity

Data relating to ethnicity was available for 310 women; 39.7% (123/310) were European, 58.7% (182/310) were African, 1% (3/310) were Asian, 0.3% (1/310) were South American and 0.3% (1/310) were North American. There was no significant difference between the different ethnic groups and HPV status. A breakdown of HPV status in relation to ethnicity is shown in table 5.8.

In the study, data relating to the number of years since HIV positive diagnosis was available for 307 women. The HPV DNA and mRNA prevalence in different diagnostic categories is shown in table 5.9. HPV DNA prevalence was significantly higher in women who were recently diagnosed HIV positive [64.2% (93/145), (<5 years)] than those diagnosed HIV positive [39.5% (64/162) more than 5 years (χ^2=18.579, p<.001). From logistic regression analysis of all the clinical and demographic data, the number of years since HIV diagnoses was a predictor of HPV DNA positivity, but not mRNA positivity (Table 5.4 and Table 5.5).

Data relating to smoking habits were available in 94.7% (304/321) of patients. 36.2% (110/304) were smokers and 63.8% (194/304) were current non-smokers (Table 5.10). We found that 61.8% (68/110) and 28.2% (31/110) of smokers were HPV DNA and mRNA positive respectively. In current non-smokers 46.4% (90/194) and 18% (35/194) of patients were HPV DNA and mRNA positive respectively (Table 5.10). When compared, HPV DNA and mRNA prevalence in currently non-smoking cohort was significantly lower than the smoking cohort (χ^2=6.693, p=0.001) (χ^2=4.247, p<0.05). Smoking history was also a predictor of HPV DNA positivity (Table 5.4), but not mRNA positivity (Table 5.5), following logistic regression analysis.

Data on parity was available for 96.6% (310/321) of patients; 16.2% (50/310) were nulliparous, and 83.9% (260/310) were parous. There was a significant difference between HPV DNA prevalence in parous [72% (36/50)] and nulliparous [49.6% (129/260)] women (χ^2=8.440, p<0.05). No significant difference was seen in HPV mRNA prevalence in parous [20.8% (54/260)] and nulliparous 26% (13/50) women. Data relating to contraception use was available for 302 patients however there was no significant difference between HPV status and contraception use.

Table 5.8: HPV status in relation to ethnicity.

	Europe	Africa	Asia	South America	North America
HPV DNA +	53.7% (66/123)	51.1% (93/182)	66.7% (2/3)	0.0% (0/1)	0.0% (0/1)
HPV mRNA +	25.2% (31/123)	19.2% (35/182)	33.3% (1/3)	0.0% (0/1)	0.0% (0/1)

Table 5.9: HPV status in relation to time since diagnosed HIV positive

	Years Since Diagnosed HIV positive		
	<5yr	**5-9yr**	**10+yr**
HPV DNA +	64.1% (93/145)	41.9% (49/117)	33.3% (15/45)
HPV mRNA +	24.8% (36/145)	20.5% (24/117)	15.6% (7/45)

Table 5.10: HPV data in relation to smoking history.

	Smokers	Non-smokers	Total
HPV DNA +	61.8% (68/110)	46.4% (90/194)	52.0% (158/304)
HPV mRNA +	28.2% (31/110)	18% (35/194)	21.7% (66/304)

5.3.4. HPV mRNA type distribution in HIV positive patients

The PreTect™ HPV-Proofer also provided information on E6/E7 mRNA expression of 5 HR-HPV types. HPV 45 [45.7% (32/70)] was the most prevalent mRNA type detected followed by HPV 33 [24.3% (17/70)], HPV 16 [22.9% (16/70)], HPV 18 [21.4% (15/70)] and HPV 31 [12.9% (9/70)]. We found multiple HPV mRNA infection in 24.3% (17/70) of the mRNA positive samples. Infection with two HPV mRNA types was found in 88% (15/17) of these cases with two cases of multiple infection with three different HPV mRNA type found (HPV 16, 18, 33 and HPV 16, 18, 45). Of the samples with two mRNA HPV types, co-infection with HPV 33 and HPV 45 was found in 33.3% (5/15) of the samples, with co-infection of HPV 16 and HPV 45, HPV 18 and HPV 33, HPV 31 and HPV 33 found in 13.3% (2/15) of samples with two HPV types. The other types of co-infection found in this population were infection with HPV 16 and HPV 33, HPV 16 and HPV 31, HPV 16 and HPV 18, HPV 18 and HPV 45, all found in 6.7% (1/15) HPV mRNA multiple infections.

The breakdown of HPV mRNA types for Europe was as follows; HPV 16 was found in 10/31 HPV mRNA positive samples, HPV 18 in 5/31, HPV 31 in 5/31, HPV 33 in 9/31 and HPV 45 in 13/31. The breakdown of HPV mRNA types for Africa was as follows; HPV 16 was found in 4/35 HPV mRNA positive samples, HPV 18 in 9/35, HPV 35 in 4/35, HPV 33 in 7/35 and HPV 45 in (18/35). Overall, 32.3% (10/31) of the HPV mRNA positive samples from Europe were positive for more than one HPV mRNA type, while 20% (7/35) of women from Africa had multiple infections. The most common multiple infection found in the European cohort was between HPV 33 and HPV 45 (n=4/10), compared to HPV 31 and HPV 33 co-infection found in the African cohort (n=2/7). HPV infection with three HPV mRNA types was found once in both cohorts, with HPV 16,

HPV 18 and HPV 33 infection found in 1/10 patients from the European cohort, and infection with HPV 16, HPV 18 and HPV 45 found in 1/7 patients from the African cohort.

The majority of HPV 45 positive samples were normal by cytology [62.5% (20/32)] while the other 4 types detected were primarily abnormal cytology (χ^2=11.246, p=0.001).

5.3.5. HPV DNA and mRNA persistence in HIV positive patients

Follow-up smear specimens at ~6-18 months after enrolment were available for 89 patients. At baseline, the age range of this cohort was from 17-67 years of age, the average age was 33.7 years and the median age of the study population was 32 years. The distribution of the number of women in different age categories can be seen in figure 5.3. In total, 98.9% (88/89) of the follow-up cohort of patients had a valid HPV DNA test at baseline and at ~6-18 month follow-up. In total, 77 patients had a valid HPV DNA and mRNA results at both baseline and ~6-18 month follow-up. HPV DNA positivity rate at baseline was 42% (37/88) with the prevalence at follow-up decreasing to 34.1% (30/88). The HPV mRNA positivity rate at baseline was 16.9% (13/78) with the prevalence at follow-up of 23.4% (18/78). No statistical difference was observed in HPV DNA or mRNA prevalence at baseline compared to follow-up (McNemar p=0.118 and p=0.227 respectively). It was noted however that in a subset of patients (n=20) HPV status changed between baseline enrolment and follow-up (Table 5.11).

There where also a small number of changes in HPV mRNA type noted, with five patients testing negative at follow-up for mRNA types that they where positive for at

baseline. One of these patients went from being HPV 33 positive at baseline to HPV 45 positive at follow-up; three patients tested HPV mRNA negative after testing positive for HPV 16, HPV 18 and HPV 33, 45 respectively. The final patient in this cohort was initially co-infected with HPV 18 and HPV 33 and tested negative for HPV 33 at follow-up. Another ten patients tested HPV positive for a HPV infection that was not detected at baseline. In these cases each individual only gained a single additional HPV type (HPV 16 n=2, HPV 18 n=3, HPV 31 n=1, HPV 33 n=1 and HPV 45 n=3).

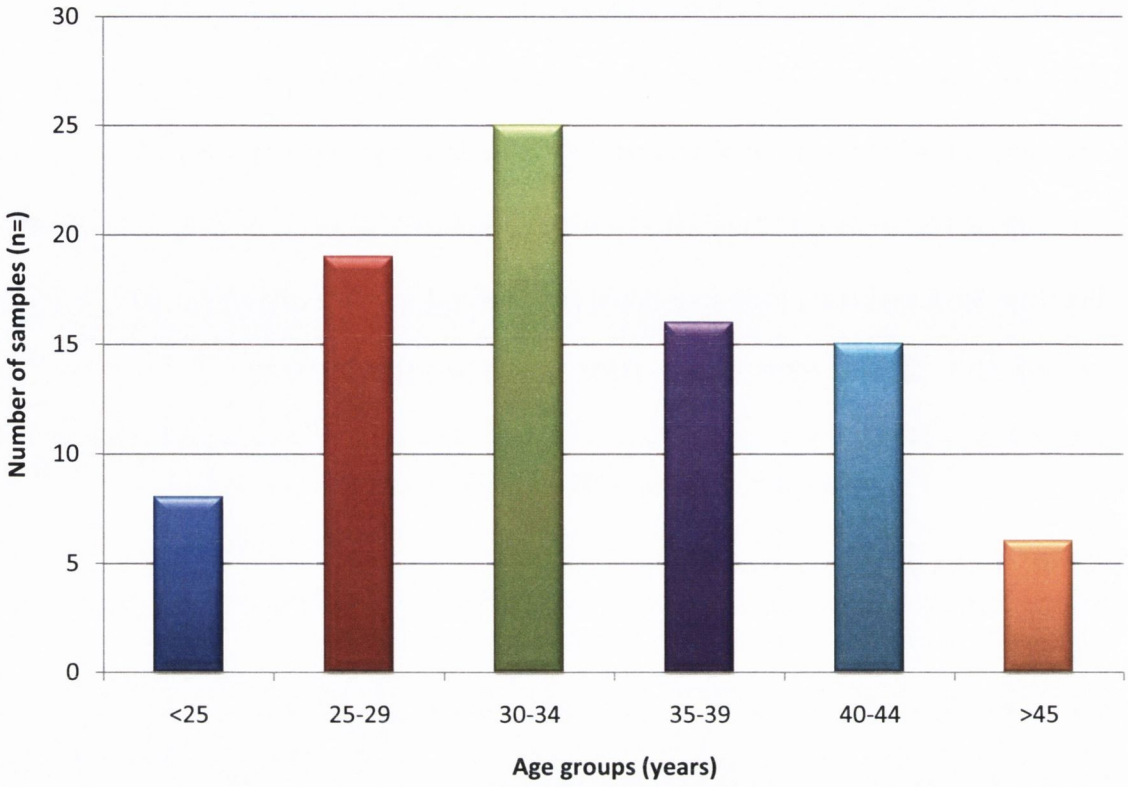


Figure 5.3: Age Distribution of patients with follow-up data. As with the total population tested the 30-34 year age group it the largest. The distribution pattern is similar to the pattern of distribution seen in the overall study population.

Table 5.11: Different scenarios for HPV positivity at baseline and at follow-up.

	HPV DNA status at baseline	HPV mRNA status at baseline	HPV DNA status after follow-up	HPV mRNA status after follow-up	Number of patients
HPV status positive (+) or negative (-)	-	-	-	-	37
	+	-	+	-	11
	+	+	+	+	9
	+	-	-	-	7
	-	+	-	-	2
	+	+	-	-	1
	+	+	-	+	1
	-	-	+	-	1
	-	-	-	+	1
	+	-	+	+	4
	-	-	+	+	3

Light green Women who were HPV DNA or mRNA positive at baseline, who tested HPV negative at follow-up.

Green Women tested positive for HPV DNA and mRNA at baseline who tested negative at follow-up.

Yellow Women who tested positive for HPV DNA and mRNA at baseline who were HPV DNA negative at follow-up.

Light orange Women who tested positive for a previously undetected HPV DNA infection.

Orange Women who tested positive for a previously undetected HPV mRNA infection.

Red Women who tested positive for a previously undetected HPV DNA and mRNA infections.

5.4. Discussion

This study describes for the first time the HPV DNA and mRNA prevalence in a cohort of HIV positive women living in Ireland. In this study we looked at the risk factors for HPV infections in HIV positive women.

We hypothesised that HPV DNA and HPV mRNA prevalence and persistence in HIV positive populations will be higher than in general populations. We have shown HPV DNA and mRNA prevalence of rates 51.1% and 21.8% respectively, in this population of HIV positive women residing in Ireland. This HR-HPV DNA prevalence rate is significantly higher than HPV DNA prevalence in women presenting for general screening in Ireland [19.2% (HR-HPV) Chapter 4 Section 4.3.3, 19.7% (total HPV) (Keegan *et al.*, 2007)]. A higher HPV DNA prevalence rate in HIV positive populations in comparison to HIV negative populations has been previously reported (Levi *et al.*, 2002, Luchters *et al.*, 2010, Tornesello *et al.*, 2008). Levi *et al* (2002) reported a HR-HPV prevalence rate of 57.7%, in a population of HIV positive women from Brazil, which is higher than that reported for HIV negative populations (12.3%) (Girianelli *et al.*, 2010). In a study carried out on HIV positive and HIV negative women in Italy, HPV DNA prevalence and abnormal cytology rate was significantly higher in the HIV cohort (39.3% and 29.5% respectively), compared to the HIV negative cohort of women (13.9% and 7% respectively) (Tornesello *et al.*, 2008). In relation to HPV DNA prevalence in our cohort of women, there was a significantly higher prevalence in women less than 30 years compared to women over 30 years. This is in line with published data relating to patterns of HPV DNA positivity in general populations in women <30 years (Chapter 4, Section 4.3.3) (Cuschieri *et al.*, 2004a, Keegan *et al.*, 2007).

There is currently no published data relating to the prevalence of HPV mRNA prevalence in HIV positive women. The HPV mRNA rate of 21.8% in our HIV positive women in this population is lower than the HPV DNA prevalence rate, however it is higher than that reported in general populations from Norway (Molden *et al.*, 2005b, Molden *et al.*, 2006). The reported HPV mRNA prevalence in these studies were 14.5% in women <30 years and 3% in women ≥30 years. This pattern of higher HR-HPV DNA prevalence rates in comparison to mRNA prevalence rates has been reported previously (Molden *et al.*, 2005b, Molden *et al.*, 2006). In addition type specific HPV DNA and mRNA prevalence rates are no different when specifically analysed for the 5 HR-HPV types included in the PreTect™ HPV-Proofer assay (Molden *et al.*, 2005b). The utility of HPV mRNA testing for predicting CIN has been well documented with data suggesting HPV mRNA is a more specific and less sensitive that HPV DNA testing and cytology (Molden *et al.*, 2005c, Keegan *et al.*, 2009, Trope *et al.*, 2009, Ratnam *et al.*, 2010).

Our second hypothesis is that treatment with anti-retroviral therapy will reduce the incidence of HPV DNA and mRNA through its effects on immunosupression. In our study, we found that virally suppressed women (women receiving anti-retroviral therapy) had a significantly lower HPV DNA prevalence than women who were not virally suppressed, however no difference was found in HPV mRNA prevalence rates between the two cohorts (Table 5.7). It has been shown that effective anti-retroviral treatment/use is associated with decreased HPV infection and SIL (Minkoff *et al.*, 2010). Studies on the impact of anti-retroviral therapy on HPV infection and related diseases are limited and inconsistent. Some studies demonstrate reduced progression of cervical lesions, while others have shown little or no effect (Bratcher and Sahasrabuddhe, 2010, Minkoff *et al.*, 2010, Shrestha *et al.*, 2010). Most studies looking at the effect of anti-retroviral therapy

on HPV related diseases have focused on cervical pre-cancer and cancer. This is one of the first studies which will examine HPV clearance and persistence in HIV positive women in the context of anti-retroviral therapy. Anti-retroviral therapy induces recovery of CD4 counts and suppression of viral HIV counts and therefore may be associated with clearance of HPV infection as a side event.

Within the study we investigated the persistence of HPV DNA and mRNA between baseline assessment and the ~6-18 months follow-up sampling event. No difference was observed in HPV DNA or mRNA status between the first HPV test and the follow-up test. We did find, however, that a number of patients (n=20) in our study changed HPV status from baseline to follow-up (Table 5.11), with changes in specific HPV types also noted in a small number of cases. It would be expected that some women over an ~6-18 month period would spontaneously shed HPV DNA infection, while others may become infected. However, HPV mRNA positivity is thought to be more predictive of persistent HPV infection (Cuschieri *et al.*, 2004b). Thus, the number of patients who shed mRNA infection should be low. In our study three patients who tested positive for HPV mRNA at baseline were negative at follow-up. In the patients, the positive baseline result was produced following an initial indeterminate result, thus the HPV mRNA infection may have been close to the limit of detection of the assay. Another reason for the changes in status may be due to sampling occurring at two different periods with differing levels of cellularity from one smear to the next. Finally it has been shown that ~35% of HIV positive women who test HPV DNA positive can have a negative HPV DNA test within 3 years (Kitchener *et al.*, 2007), thus the same might be true of HPV mRNA infections. In those cases where a change in specific HPV mRNA type was seen, there where too few numbers to draw any conclusions as to possible prevalence or persistence of specific HPV

mRNA types detected by the assay. For these reasons further study into the prevalence and persistence of HPV mRNA infections is required.

With the introduction of anti-retroviral drugs, the incidence of many virus related cancers has significantly decreased, however this trend has not been noticed in HPV related cancers (Bratcher and Sahasrabuddhe, 2010). However as life expectancy has increased due to viral treatment the risk of CC persists (Sirera *et al.*, 2007). In our study we found that HPV DNA prevalence in virally suppressed women (women receiving anti-retroviral therapy) was significantly lower (45.4%) than women who were not receiving anti-retroviral therapy (57.8%) (Table 5.7). However, we did not see a significant difference when we compared HPV mRNA data in these cohorts of patients. The effects of anti-retroviral therapy on HPV infection are still not defined and this is the first study to look at the effects on HPV mRNA infection. A possible reason for this could be due to sample size and the small numbers of HPV mRNA positives in the study (n=70). Also the HPV mRNA positive status may represent persistent infection in women who have recently begun anti-retroviral therapy.

The main risk factor for HPV infection is immunosupression as it has been shown that low CD4 counts have a direct relationship to HPV infection, recurrent HPV infection and cervical disease (Palefsky, 2006b). When HIV positive women become increasingly immunocompromised the risk for persistent infection increases. We found a significantly lower HPV DNA and mRNA prevalence in women with high CD4 counts (>200 x 10^6/L) (48% and 18.9% respectively) along with a lower rate of cervical abnormality (25.1%) compared to women with low CD4 counts (<200 x 10^6/L) (72.7%, 40.9% and 52.3% respectively) ($p<0.005$, $p\leq0.001$, and $p<0.001$ respectively) suggesting a direct

correlation between immunosupression and HPV infection and cervical abnormalities (Table 5.6).

In women with normal cytological diagnosis we found the same trend with lower rates of HPV DNA and mRNA in women with high CD4 cell counts. As we begin to understand the relationship between HPV, HIV and CC it may be possible to use HPV detection assays in the triage of HIV positive women for the management of cervical disease. It would be interesting to follow the women from this study with normal cytological diagnosis as we have access to HPV mRNA data, to determine the effect of CD4 cell counts on future disease progression. It may turn out that those women who are HIV positive, but have high CD4 cell counts and are negative for HPV DNA and mRNA could benefit from a similar screening period as women in the general population. We found that HSIL women with low CD4 counts were positive for both HPV DNA and mRNA, whereas HSIL women with high CD4 counts were positive for DNA but only 66.6% (6/9) were positive for HPV mRNA. It could be possible that these cases of HSIL that are negative for HPV mRNA, may represent only transient infection and disease. In HIV infection immunosupression is associated with higher rates of progression from LSIL to HSIL, however, it does not appear to be associated with CC (Palefsky, 2007).

In HIV positive patients, HPV DNA prevalence is higher than in HIV negative patients, and as mentioned earlier this can lead to increased false negative results when based on HPV DNA for clinical diagnosis. HIV positive patients also suffer increased low-grade cervical disease, but high-grade disease remains infrequent (Massad *et al.*, 2008). It has been suggested that there may be a genetic predisposition required for the development of high grade cervical disease (Martin *et al.*, 2006). It may be possible therefore when taking

these factors into consideration to produce an algorithm for the management of HIV positive patients using the PreTect™ HPV-Proofer as an adjunct for cytology in the triage of cervical disease. It was interesting to note that the HPV mRNA prevalence in each of the age groups in this population followed the pattern of HPV DNA prevalence, and it may be possible that this trend alone could make HPV mRNA testing a valuable tool in the management of HIV positive women (Figure 5.2).

In this population, we found a high rate of cervical abnormalities (28.7%) when compared to the general population of women living in Ireland [11.1% from Keegan *et al* (2007) and 11.5% from Chapter 4, Section 4.3.2]. It was noted however, that the rate of SIL in our study was similar to published data [Table 5.2 (17.4% LSIL and HSIL) (Levi *et al.*, 2002). In this study we had a limited number of HSIL cases (n=14), representing a HSIL prevalence of ~4%. Again, this result is explained by the fact that low grade abnormalities are relatively more common in HIV positive women, with HSIL cases infrequent (Massad *et al.*, 2008).

As expected, cytological diagnosis was determined as a predictor of HPV DNA infection,. However, smoking and years since HIV positive diagnosis were also predictors of HPV DNA infection. It has been established that current though not former smoking is associated with increased HPV infection, and our data would agree with this in our HIV cohort (Vaccarella *et al.*, 2008).

HIV positive patients are known to suffer increased infection with HPV types normally less prevalent in immunocompetent populations. The dominant HR-HPV types 16, 58, 18, 52, 31, 33, and 45 are the most prevalent types found in HIV positive populations'

worldwide (Clifford *et al.*, 2006). It is also known that HIV women suffer increased prevalence of HPV types other than HPV 16 (Clifford *et al.*, 2006). In our study, we determined the HPV mRNA prevalence of five of the seven most common HPV DNA types found in HIV positive populations. The most prominent oncogenic HPV mRNA was HPV 45 followed by HPV 33, 16, 18 and 31. We also described a possible association between HPV 45 mRNA and normal cytology. However it is the belief of this author that HPV 45 is currently highly prevalent in this cohort of women, and the low number of HPV mRNA positive samples in the study as a whole is not large enough to make definitive statements regarding oncogenicity of individual HPV mRNA types. As there has been no other studies published to date regarding HPV mRNA types in HIV positive patients, further studies into the prevalence of HPV mRNA types in HIV positive patients are required, especially in relation to the possible use of HPV vaccines in HIV positive cohorts. The HPV mRNA type distribution found in this study is different to that reported in mRNA studies carried out a general population in Norway, where HPV 16 was the most prevalent HPV mRNA type found, followed by; HPV 31, 33, 18 and finally HPV 45, This again highlights the need for further HPV mRNA type testing in HIV positive patients to determine specific HPV mRNA trends in HIV positive patients..

Recently, Boulet *et al*, (2010) have examined the PreTect™ HPV-Proofer methodology and suggest that the assay may have a capacity to detect low copy DNA infection. However, as the authors point out they have used a plasmid system to test the robustness of the NASBA protocol for the detection of mRNA. The data presented does not account for (a) T7 promoter site on the plasmids and (b) RNA free DNA preparations. The authors also state that the findings do not reflect the clinical performance of the assay (Boulet *et al.*, 2010).

The data provided in this chapter provides a look at the current prevalence of HPV DNA in the HIV positive population of Ireland. This study is the only study which describes the levels of HPV mRNA in HIV positive women and the association of HPV DNA and mRNA with possible risk factors found in HIV populations. Further studies are required to determine the uses of HPV testing in HIV positive patients. However, current HPV tests may not be good enough to be included in algorithms for the management of HIV positive women for cervical disease.

Chapter 6

Discussion

6.0 Discussion

CC is a major source of morbidity and mortality for women in Ireland and worldwide (~80 deaths in Ireland and 275,000 deaths worldwide yearly (NCRI, 2009, Ferlay *et al.*, 2010). The natural progression of cervical disease is from well-defined pre-cancerous abnormalities ASCUS to LSIL to HSIL to invasive carcinoma. In general, cervical abnormalities are present in ~10% of women. In relation to HIV positive women, the incidence of cervical pre-cancerous lesions and CC is relatively higher. CC is relatively rare by comparison (to cervical abnormalities) in both general populations and HIV positive populations. The screening by cytology for these pre-cancerous lesions has reduced the incidence of CC by allowing early treatment of cervical disease.

The main causative agent in the development of CC is previous/persistent infection by HR-HPV (Walboomers *et al.*, 1999, Munoz, 2000). Thus the majority of CC cases could theoretically be prevented by the detection/prevention/eradication of HPV infection. There are three approaches to combating HPV infection.

The first approach, which has been used for a number of years, is to screen different populations for the presence of HPV infection, and depending on the result a course of observation/treatment can be determined. Some of these approaches have been discussed at length in Chapter 2, Section 2.3 of this thesis. Briefly HPV DNA has been suggested for use in the management of borderline cytology abnormal women, in women ≥30 years and as a test of cure following treatment in a colposcopy setting (ALTS, 2003b, Cuzick, 2003, Zielinski, 2003, Wright, 2004, Cox and Cuzick, 2006, Kitchener *et al.*, 2006). HPV mRNA testing has also been suggested as a more specific test for identification of persistent disease (Cuschieri *et al.*, 2004b), and for use in the management of women with

low grade cervical abnormalities and women <30 years (Molden *et al.*, 2006, Ratnam *et al.*, 2010).

The second approach in preventing/decreasing the incidence of CC through the management of HPV infections is through prophylactic vaccination. As discussed in Chapter 1 Section 1.5, there are currently two prophylactic HPV vaccines which have been licensed for use in CC prevention: GARDASIL® (HR-HPV types HPV 16 and HPV 18 and LR-HPV types HPV 6 and HPV 11) and Cervarix (HPV16 and HPV 18). The introduction of a National Vaccination programme against HPV, and the possible effects of vaccination on the HPV prevalence and genotype distribution in a country have also been discussed in Chapter 4 section 4.1 and 4.4 respectively. The next avenue for HPV prophylactic vaccination is the development of effective vaccines against remaining oncogenic HPV types.

The third approach is to develop a therapeutic agent/vaccine to cure HPV infected individuals, to prevent persistent HPV infection and associated cervical disease. In regards to the management of HPV infection for the prevention of CC, the goal of research into HPV should lead to a cure of HPV infection. Currently therapeutic vaccines have been developed however, none have proved sufficient efficacy to be considered as a cure of HPV infection, or for possible inclusion in immunisation programmes (Roden and Wu, 2006). Therefore, future research into the development of effective therapeutic vaccinations is required.

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Together the approaches could be combined to help prevent deaths from CC in the future. At this point however, a combination of cytology, HPV detection and HPV vaccination will be required until such a point that effective therapeutic agents have been developed. Before HR-HPV types can be detected or vaccinated against, data regarding the prevalence of HR-HPV along with HPV genotype distribution is needed in a given population, along with rates of persistent infection. In relation to HIV positive populations, data regarding the possible effects of immunosupression and therapies specific for HIV infection in relation to HPV infection must be determined.

In this thesis the primary aim was to assess HPV prevalence and genotype distribution in two populations of women living in Ireland; first, the cervical screening population and the second a specific population of HIV positive women living in Ireland. The main hypothesis was that HPV prevalence in the general screening population would be similar to other studies from the UK and Ireland, even though Ireland did not have an established cervical screening programme. We also suggested that HPV DNA and mRNA prevalence and persistence will be higher in a HIV positive population of women when compared to general screening population. Within this thesis we have validated these hypothesis using two approaches. Firstly we examined the population type specific prevalence of HPV in a cohort of the general screening population (n=3193) and secondly we assessed HPV DNA and mRNA persistence in HIV positive women (n=321) at baseline and at ~6-18 month follow-up.

The differing profile of HPV prevalence and genotype distribution in general populations and in HIV positive populations has been discussed in Chapter 1 (Section 1.4.4), Chapter 2 (Section 2.3) and Chapter 4 (Section 4.4). Briefly HPV prevalence and genotype

distribution vary from population to population: differences being regional, national and global. However, some trends are seen, in particular with reference to HPV 16 and HPV 18 which are thought to be cumulatively responsible for the majority of CC and are found in high prevalence in general and HIV populations worldwide (Clifford *et al.*, 2005, Clifford *et al.*, 2006, Smith *et al.*, 2007).

An overview of the current landscape in terms of the various molecular HPV detection technologies is provided. The three technologies chosen to assess HPV prevalence and genotype distribution for this thesis included the gold standard HPV DNA test, Hybrid Capture 2 (Qiagen), the Roche Linear Array HPV Genotyping Test and the PreTect™ HPV-Proofer assay (Norchip) which specifically detects HPV mRNA from five high risk oncogenic HPV types. In general, we found hc2 to be a user friendly assay, and as a non PCR approach to HPV molecular testing has several advantages over PCR based assays which are outlined in Chapter 2 Section 2.1.1. The hc2 assay was efficient and reliable and produced good results in terms of time required to perform the assay. The Roche Linear Array HPV Genotyping Test was time consuming, and had a relatively lower through-put of samples. Also the Roche Linear Array HPV Genotyping Test was costly; but the assay produced excellent results in terms of the number of genotypes that can be identified. The PreTect™ HPV-Proofer was a relatively new assay at the onset of the study, and we found it to be extremely efficient in terms of time technical resources, skill and output.

At the outset of this project, the characterisation of HPV prevalence and genotype distribution in Ireland was limited, while no HPV prevalence studies has been carried out in HIV positive women in Ireland. The only previous HPV study carried out on an urban

population of women presenting for routine screening (Keegan *et al.*, 2007), provided limited insight into the status of HPV infection in relation to the general cervical screening population in Ireland. In chapter 4, we describe the HPV population prevalence in a larger cohort of women from the Irish cervical screening populations, incorporating the different regions of Ireland. We report world and European age standardised HPV prevalence rates of 16.8% and 15.7 % in the 25-60 year screening cohort respectively, which is in line with those rates reported previously in the UK (Cuschieri *et al.*, 2004a, Hibbitts *et al.*, 2006, Sargent *et al.*, 2008). As expected HPV 16 and HPV 18 were highly prevalent in our screening population again, following worldwide trends (Figure 4.4). However, notably HPV 16 was the most prevalent genotype found with HPV 18 only the 4th most common genotype, thus validating the need for a HPV screening analysis of the Irish population, particularly pre-introduction of a HPV vaccination program. Other HR-HPV types were prevalent, and included HPV 31 and 52, which are determined as carcinogenic sub-types worldwide. Additionally HPV 51 and 39/66 were also in our top 10 types. In addition, the top nine HPV genotypes consistently appeared in the top ten genotypes across the different regions of Ireland, but their ranks were region specific. This was discussed in Chapter 4 Section 4.4.

This information provides valuable insights into the HPV prevalence and genotypes status in Irish women. Interestingly the age standardised rates of HR-HPV prevalence are not dissimilar to those from the UK, certain European populations and the US, in particular in countries with well established screening programmes. As discussed in chapter 1 and 2, there is a move towards incorporating HPV testing into cervical screening programmes to improve detection and management of women with cervical abnormalities in a cost effective manner. The data presented here in relation the Irish population will be valuable

for CervicalCheck, should HPV testing be introduced as part of the screening approach in Ireland. CervicalCheck has been in operation in Ireland since Sept 2008, and has been a huge success with over 280,000 women screened in the first year of the programme. This resulted in detection of 13.4% low grade abnormalities and 1.4% high grade abnormalities (CervicalCheck, 2010), which were referred to colposcopy for further treatment and follow-up. The increase in colposcopic referrals has huge cost implications for health care providers and it is hoped that HPV testing will be incorporated into the screening algorithm for Irish women to reduce the overall costs and follow-up in colposcopy.

The possible role that HIV plays in relation to HPV infection is outlined in Chapter 1 Section 1.6.1. Briefly, HIV positive women suffer increased HPV infection rates, cervical abnormalities and CC compared to HIV negative populations due to interaction between the two viruses, the weakened immune response, and genetic instability.

Before the onset of the study the HPV DNA and HPV mRNA prevalence and persistence in HIV positive women residing in Ireland was unknown, along with the effects of anti-retroviral therapy on the prevalence of HPV DNA and mRNA infection. This was particularly true of HPV mRNA infection as no published data is currently available on HPV mRNA status in HIV positive women worldwide. This is the first study to examine combined HPV DNA and mRNA prevalence and persistence rates in HIV positive women. The hypothesis being that HPV DNA and mRNA infections are more persistent in HIV positive women compared to normal populations, while persistence may be lower in patients on anti-retroviral therapy. We demonstrated increased prevalence of HPV DNA (51.1%) in comparison to normal populations (~19%) and increased HPV mRNA

(21.8%) in comparison to normal populations (~ 3%-15%) (Molden *et al.*, 2005b, Molden *et al.*, 2006). This is important information in the context of using HPV testing in the management of cervical abnormalities in HIV positive women. The high prevalence of HPV DNA infection suggests that this approach would not be of benefit for management of these women, and may in fact result in over treatment. On the other hand detection of HPV mRNA may be a more useful predictor of high grade disease in this cohort. In the current study due to the small number of cases, it was not possible to fully evaluate the utility of HPV mRNA testing for management of cervical disease in HIV positive women in Ireland, but this certainly warrants further investigation. The current management guidelines for these women included annual follow-up and colposcopy referral where abnormality is detected. Given the high rate of low grade abnormalities and the relatively low rate of CC an alternative management approach could be warranted.

The effects of anti-retroviral therapy reducing HPV DNA prevalence rates compared to women not receiving anti-retroviral therapy is shown in Chapter 5 Section 5.3.3.2. While there was a significant reduction in HPV DNA prevalence rates in those women who were virally suppressed, no effect was observed with HPV mRNA prevalence rates. These issues are fully discussed in Chapter 5 Section 5.4.

The HPV DNA and mRNA prevalence and persistence along with possible effects of anti-retroviral therapy was outlined with HIV positive women living in Ireland now known to suffer higher levels of HPV infection in comparison to the general population of Ireland. It was previously understood that HIV positive women are more susceptible to HPV infection and our study demonstrates that HIV positive women in Ireland may suffer

higher rates and persistence of HPV infections, which can lead to obvious consequences for the incidence of cervical disease in this cohort of patients.

While immunosupression is one of the primary risk factors for HPV infection in HIV positive patients, we determined other factors including cytological diagnosis, years since HIV diagnosis and smoking as key risk factors for HPV infection. It is interesting to speculate that the findings relating to years since diagnosis may be in fact related to the least time on anti-retroviral therapy.

The results presented in this thesis detail the HPV profile of a large Irish population study. As such, Ireland now has a detailed specific HPV profile that substantially expands on what was previously known. In this study, we found HPV 16 and HPV 18 to be important in the Irish context however, other possibly important HPV types were also described. Additionally, the study expanded outside of an urban setting, providing a better picture of HPV genomes in the country as a whole. We also outlined the prevalence and persistence of HPV infection in HIV positive women residing in Ireland and the effects of anti-retroviral therapy on those infections. The results generated from this thesis can now be applied to inform future developments in HPV detection, vaccination and therapeutic approaches for the general cervical screening population of Ireland and HIV positive women living in Ireland.

6.1. Future work

In this thesis, I have presented two independent yet related pieces of work. This work was carried out under the umbrella of CERVIVA, which continues to carry out research on the utility of HPV testing in cervical screening. The finding from the population based type specific HPV prevalence study has for the first time provided a comprehensive report on the HPV prevalence and genotype distribution in the Irish Cervical Screening population. This information is of great importance for future generations of Irish women, where HPV vaccination will become a standard aspect of CC prevention in Ireland. It provides baseline knowledge in relation to HPV prevalence in the general screening population which will be used for future directions in terms of introducing HPV testing into cervical screening. There are a number of directions this research can take going forward.

A prospective longitudinal study on HPV prevalence and type distribution on vaccinated populations, when they reach the age for cervical screening is merited to evaluate the effect of vaccination on HPV prevalence and to assess whether non HPV 16/18 genotypes become more dominant in vaccinated populations.

One of the key findings of the population type specific study was the large proportion of HPV 66 detected in the general screening population. There is little information currently on HPV 66 prevalence in cervical intraepithelial neoplasia and cancer. Similarly, there is limited information on the HPV genotypes detected in low grade cervical disease. Future research should concentrate its efforts on genotype analysis of low grade lesions to assess whether the same HPV types detected in the screening population are persisting through to low grade lesions. This will be hugely important in future populations of vaccinated women.

Data generated from other studies underway within CERVIVA have indicated a clear role for the utility of HPV mRNA testing in the management of low grade and high grade cervical disease in colposcopy. In this project, as the numbers were not sufficient, it was not possible to clearly ascertain a role for HPV mRNA testing in the management of cervical disease in HIV. A large scale multicentre study is required to fully evaluate the utility and cost effectiveness of incorporating HPV mRNA testing into the routine management of cervical disease in HIV positive women. Another avenue to explore would be to extend the HPV prevalence and genotype analysis to include other HPV associated lesions in HIV such as anal intraepithelial neoplasia (AIN) and oral cancers.

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