Novel ‘Test of Disease’ Markers in Cervical Pre-cancer

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Declaration

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Padmaja Naik

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Summary

Each year, more than a quarter of a million women die of cervical cancer in the world. In Ireland, almost 90 deaths are due to cervical cancer. The incidence of cervical cancer can be reduced by screening. In many developed countries, cervical screening has transformed into HPV-based screening, including Ireland. The switch from cytology-based screening to HPV-based screening, whereby HPV testing has become the "test of risk" for cervical cancer screening, with cytology, the triage test acting as effectively the "test of disease", is not without its challenges. HPV testing has a high specificity – a negative test virtually rules out disease. A positive HPV test has, however, a very low PPV for high-grade disease. This is the limitation that triage is trying to mitigate, and it has opened several pathways for triaging and managing HPV-positive cases, such as extended genotyping, molecular biomarkers, and methylation markers.

The current use of reflex cytology as a 'Test of Disease' poses challenges, such as not all HPV-positive women will have an abnormal cytology. Moreover, HPV-positive results can increase anxiety in women. Although negative cytology does not exclude the presence of CIN, it is known to have a specificity between 98-99% for CIN2+. The overall risk for CIN2+ in high-risk HPV-positive women with normal cytology is reported to be 7.7% (Wittenborn et al., 2023). In almost 83% of women with low-grade intraepithelial lesions, cytology may not progress to high-grade cervical neoplasia. Changes in screening populations with vaccinated and non-vaccinated women will result in different cytology outcomes.

Finally, the availability of very few skilled and experienced scientific personnel in the cytology discipline is causing a significant impediment to the continued use of cytology as a reflex test of disease in many parts of the world. Thus, there is a need to explore alternative triage methods and, indeed, different sample types further to stratify the immediate risk of developing high-grade CIN and the overtime risk of developing risk in HPV-positive women.

This thesis aims to address some of the key challenges with (A) HPV primary screening, which is to find the optimal triage strategy to avoid unnecessary follow-up and identify those women who need immediate assessment and those at increased risk over time who need follow-up for a maximum risk for developing high-grade cervical neoplasia and (B) diagnostic challenges in identifying women with progressive disease. The project focuses on cervical screening, cervical pre-cancer diagnostics, and prognostic biomarkers. Chapter one details information regarding cervical cancer, screening and traige options, diagnosis and molecular biomarkers and an introduction to this project. Chapter two details all methods and materials used in this project. Chapters 3 and 4 explore the use of extended genotyping as a biomarker for triage in HPV-positive

women from primary cervical screening. Chapter 5 examines the potential of specific biomarkers of chromosomal abnormalities and cell cycle dysregulation, namely Hec1 and Nuf2, in cervical pre-cancer that have potential utility in cervical cancer diagnostics and prognostics.

Chapter 3 describes the use of extended genotyping in high-risk HPV-positive women enrolled in the CERVIVA Primary HPV Screening Pilot Study using the FDA-approved BD Onclarity (Becton & Dickinson, Sparks, MD, USA) HPV assay. The assay uses real-time PCR technology to test amplified DNA for the qualitative detection of high-risk types of HPV and detects 14 high-risk HPV types (16, 18, 31,33,35,39,45,51,52,56,58,59,66 and 68) and provides the capability for individually genotyping six high-risk HPV types (16,18,31,45,51,52) and three genotype groups (Group P1: 33/58, Group P2: 35/39/68 and Group P3: 56/59/66). Among those high-risk HPV-positive women, the most frequent individual HPV genotypes detected were HPV16 and 31, and HPVP2 and P3 are group genotypes. Interestingly, non-HPV16 genotypes were associated with more than half of CIN2+ cases. More than 40% of the absolute risk estimate for CIN2+ and more than 50% of the absolute risk for CIN3+ were found to be associated with non-HPV16 and HPV18.

In Chapter 4, extended genotyping was compared as an alternative triage approach to cytology and other molecular triage approaches under investigation by CERVIVA for HPV-positive women. In this chapter, the clinical performance of extended genotyping was established as a triage test compared with cytology and the biomarker p16/ki67 (CINTec Plus). In this chapter, the impact of extended genotyping was modelled as part of the screening algorithm to assess the impact on colposcopy referral rates and detection of high-grade disease. The study findings show that extended genotyping for HPV16 with HPV18 and combined with one of the other genotypes (HPVP1 or HPV31 or HPV51 or HPV52) and cytology triage offer the best model for HPV-positive women having the highest PPV and specificity. Genotype groups P2 (HPV types 56/59/66) and P3 (HPV types 35/39/68) carry less risk than some of the other HR HPV types or groups and could be the candidates for routine recall management strategy. As part of this algorithm, extended genotyping showed an impact in reducing the colposcopy referral rate, detecting more CIN2+ cases and improving specificity.

In Chapter 5, novel biomarkers of disease progression were evaluated in a cervical pre-cancer and cancer as proof of concept study. Using immunohistochemistry, this proof of concept study examined the expression of kinetochore markers Hec1 and Nuf2 in 51 cervical tissue samples from 33 LLETZ recruited through the CERVIVA HistoCerv Project. Immunohistochemistry was carried out using Rabbit polyclonal Nuf2 antibody (Abcam ab230313) and Rabbit polyclonal Hec1 antibody (Thermo-Fischer Scientific PA5-102765) and protocol based on The Hapten-linked multimer

detection kit used on the Ventana Benchmark ULTRA equipment (Roche Diagnostics). Hec1 and Nuf2 were over-expressed in cervical intraepithelial neoplasia tissue specimens (CIN1 and CIN3) compared to the normal cervical tissues. A key component of this study involved establishing the ploidy status of cells within the CIN cases to prove that the dysregulated expression patterns of the kinetochore complex (Nuf2 and Hec1) were due to chromosomal instability and aneuploidy in the pre-cancerous disease state. To achieve this, the HER2/C17 Dual-color dual-hapten in situ hybridisation (D-DISH) protocol established for her2 overexpression in breast cancer was adopted and applied in this case to high-grade cervical pre-cancer (CIN3 n=3 cases). The D-DISH protocol was carried out on Ventana Benchmark ULTRA autostainer (Roche Diagnostics). The results showed multiple copies of chromosome 17 in these CIN3 cells, indicating the presence of aneuploidy, indicating that Hec1 and Nuf2 proteins are markers of disease progression and indicators of chromosomal numeric aberration associated with aneuploidy and chromosomal instability.

In conclusion, this study highlights the message that genotypes other than HPV 16/18 should not be underestimated in organised HPV primary cervical screening for risk stratification and management of HPV women. Extended genotyping has real potential to benefit those screening programmes where cytology services are limited for reasons such as the unavailability of cytology personnel, resources, and the need for high throughput. Other secondary benefits of carrying out extended genotyping are surveillance to monitor the effectiveness of HPV vaccination and monitoring the persistence of high-risk HPV types. Finally, new biomarkers, Hec1 and Nuf2, show real potential as markers of disease progression in cervical intraepithelial neoplasia and should be further evaluated as the prevalence of CIN and cervical cancer decreases in the context of HPV vaccination. This PhD work contributes to the knowledge base and will inform future screening and diagnostic protocols as we advance and move into an era of significant change in cervical cancer prevention.

Abbreviations

°C	Degrees Celsius
AGC	Atypical glandular cells
AIS	Adenocarcinoma In Situ
ARF	ADP-ribosylation factor
ARTISTIC	A Randomised Trial in Screening to Improve Cytology
ASC-H	Atypical Squamous Cells but cannot exclude high grade
ASCUS	Atypical Squamous Cells of Undetermined Significance
ASR	The Age Standardised (World) Rate
ATHENA	Addressing the Need for Advanced HPV Diagnostics
AUC	Area Under the Curve
CA19-9	Carbohydrate antigen 19-9
CADM1	Cell adhesion molecule 1
CDKN2A	Cyclin dependent kinase inhibitor 2A
CEA	Carcinoembryonic antigen
CERVIVA	The Irish Cervical Screening Research Consortium
cGIN	Cervical Glandular Intraepithelial Neoplasia
C.I.	Confidence intervals
CIN	Cervical Intraepithelial Neoplasia
COVID-19	Coronavirus disease 2019
DAB 3,3'-	Diaminobenzidine tetrahydrochloride
DAPK1	Death-associated protein kinase 1
DNA	Deoxyribonucleic acid
E	Early gene
ELISA	Enzyme-linked immunosorbent assay
EPB41L3	Erythrocyte membrane protein band 4.1 like 3
ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
FAM19A4	Family with sequence similarity 19 (chemokine (C–C motif)-like) member A4
FDA	Food and Drug Administration
G	Gap
GHSR	Growth hormone secretagogue receptor

H&E	Haematoxylin and Eosin
HC2	Hybrid Capture 2
Hec1	Highly Expressed in Cancer 1
HER2	Human epidermal growth factor receptor 2
HIQA	Health Information and Quality Authority
HPV	Human Papillomavirus
hrHPV	High risk HPV
HPRA	Health Products Regulatory Authority
HSE	Health Service Executive
HSIL	High-Grade SIL
HTA	Health Technologies Assessment
IARC	International Agency for Research on Cancer
INK4	Inhibitors of cyclin-dependent kinase 4
k	Kappa statistic
Ki-67	Antigen Kiel 67
L	Late Region
LBC	Liquid Based Cytology
LCR	Long Control Region
LIS	Laboratory Information System
LLETZ	Large Loop Excision of the Transformation Zone
LSIL	Low-Grade SIL
M	Mitosis
MAL	Myelin and lymphocyte protein
MCM	Mini-chromosome maintenance
MEM	Minimum Essential Medium
MIR-124	MicroRNA-124
miRNA	Micro RNA
mRNA	Messenger RNA
N	Number
N:C	Nuclear to cytoplasmic ratio
NAD	No abnormality detected (cytology)

Ndc80	Nuclear division cycle 80)
NCBI	National Center for Biotechnology Information
NCRI	National Cancer Registry Ireland
NILM	Negative for Squamous Intraepithelial Lesions or Maligmanacy
NPV	Negative Predictive Value
NTCC	New Technologies in Cervical Cancer Trial
Nuf2	NUF2 Component Of NDC80 Kinetochore Complex
OR	Odds Ratio
ORF	Open Reading Frame
ORI	Origin of replication
p53	Tumour protein 53
PAP	Papanicolaou test
PAX1	Paired-box transcription factor 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHACTR3	Phosphatase and actin regulator 3
PK	Proteinase K
POBASCAM	Population-based Screening Study Amsterdam
POU4F3	POU Class 4 Homeobox 3
PPV	Positive Predictive Value
pRb	Phosphorylated Retinoblastoma protein
PRDM14	PR-domain containing protein 14
RARB	Retinoic acid receptor beta
RCT	Randomised Control Trial
RECQL4	RECQ helicase Q4
S	Synthesis
SAC	Spindle assembly checkpoint
SCC	Squamous cell carcinoma
SCJ	Squamocolumnar Junction
SIL	Squamous Intraepithelial Lesions
SOX1	SRY-Box Transcription Factor 1

SPC	Spindle Pole Body Component
SPSS	Statistical package for the social sciences
SSCA	Squamous cell carcinoma antigen
ssDNA	Single stranded DNA
SST	Somatostatin receptor type
TCH	The Coombe Hospital
TOC	Test of cure
TOP2a	Topoisomerase II alpha
TP63	Tumour protein 63
TS	Triage Strategy
TSG	Tumour suppressor gene
TZ	Transformation Zone
URR	Upstream Regulatory Region
VIA	Visual inspection with acetic acid
WHO	World Health Organisation
ZIC1	Zinc finger protein 1

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Chapter 1 Background and Introduction to the Project

1.1 The burden of cervical cancer and pre-cancer

According to the WHO statistics in 2022, cervical cancer is the fourth most common cancer among women globally, with an estimated 660,000 new cases and 350,000 deaths (https://www.who.int/news-room/fact-sheets/detail/cervical-cancer). The incidence (Fig 1.1) and mortality of cervical cancer are the leading cause of death in Eastern and Central Africa (Singh et al., 2023). The global average age at death from cervical cancer is 59 years, ranging from 45 years to 76 years and more than a third of the global burden is contributed by India and China (Arbyn et al., 2020). The incidence has decreased in developed countries due to organised population-based cervical screening programmes (Singh et al., 2023). In developing countries, cervical cancer is still causing a considerable number of deaths due to a lack of organised programmes and low resources for the implementation of vaccination (Brisson et al., 2020; Simms et al., 2019).

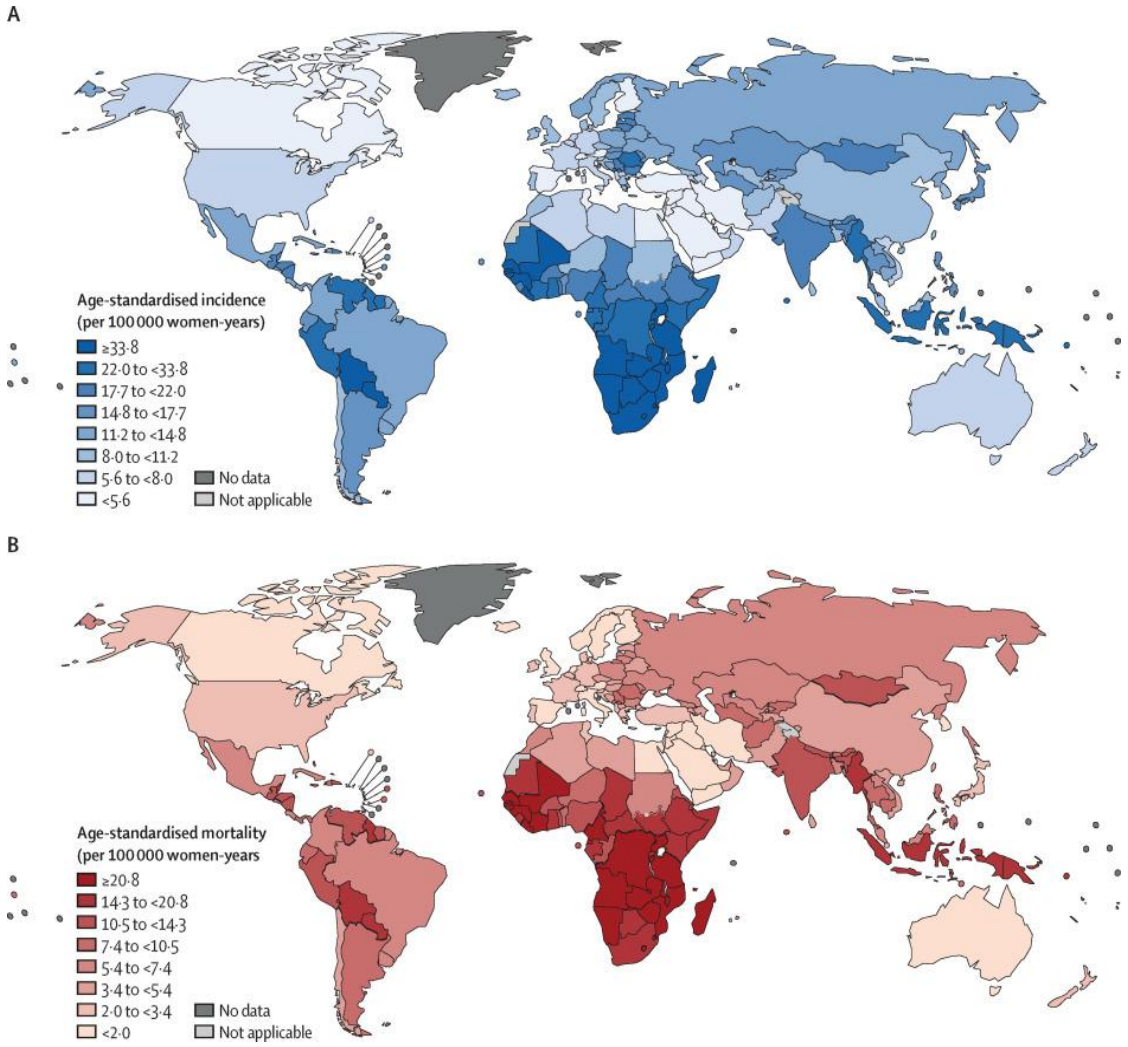


Figure 1.1 Global incidence and mortality of cervical cancer in 2020. A) Age-standardised incidence, B) Mortality rates. Adopted from Global Estimates of Incidence and Mortality of Cervical Cancer in 2020: A Baseline Analysis of the WHO Global Cervical Cancer Elimination Initiative (Singh et al., 2023).

In Ireland, the incidence of cervical cancer is 265 new cases diagnosed per annum, with 85 deaths annually (National Cancer Registry Ireland, 2024). Per year, 2927 cases of cervical pre-cancer are detected and treated in Ireland. The annual report published by the National Cancer Registry Ireland in December 2023 summarised cancer data collected up to diagnosis year 2021. There has been a decline in cervical cancer incidence since the year 2010 due to the introduction of the cervical screening programme in 2008. The mortality rate per 100,000 is 4.1% (Figure 1.2).

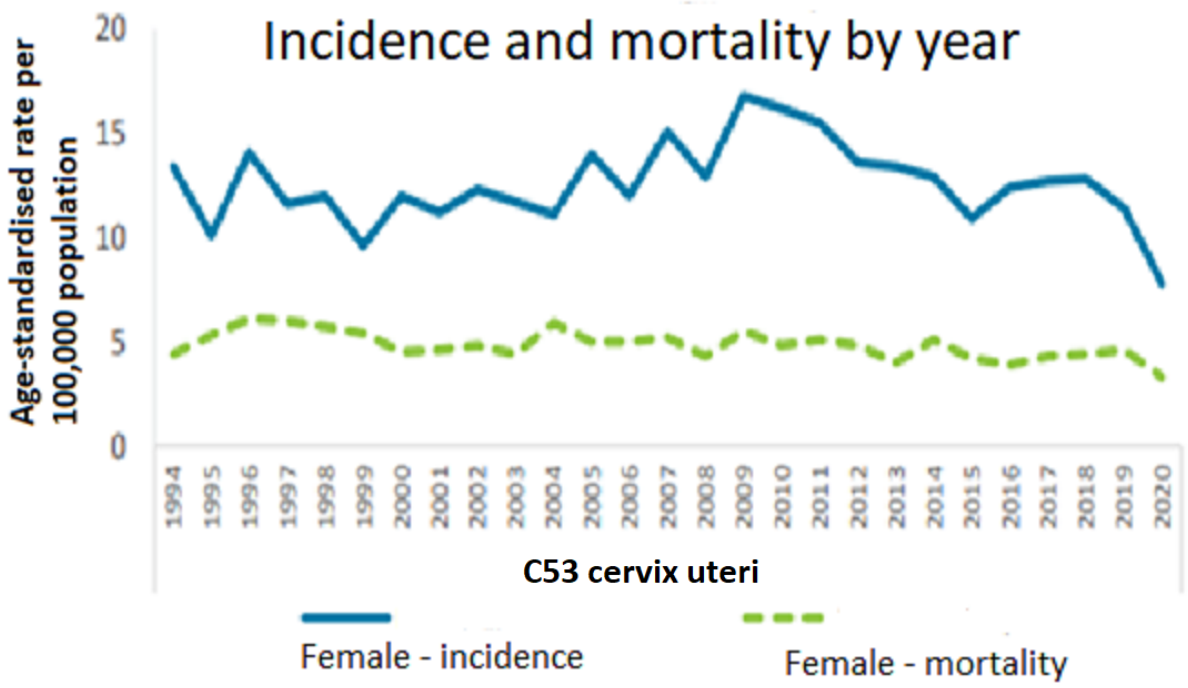


Figure 1.2 Incidence rates for cervical cancer in Ireland age-standardised using the 2013 European population. The data presented refers to cases diagnosed in 2018-2020 unless otherwise specified. (NCRI, Annual Report 2021).

1.2 Anatomy and Pathology of the Cervix

1.2.1 Anatomy and Physiology of Cervix

The cervix is the lower fibromuscular portion of the uterus, measuring 3-4 cm in length and 2.5 cm in diameter. It varies in size and shape depending on the age, parity and menstrual status of the woman. The lower part of the cervix, called the portio vaginalis, protrudes in the vagina and opens into the vagina through its orifice called the external os, and the upper half remains above the vagina (Prendiville et al., 2017).

The anatomy of the cervix can be divided into five regions, as shown in Figure 1.3. These five regions are the Ectocervix, Endocervix, Endocervical canal, Squamocolumnar Junction (SCJ), and Transformation Zone (TZ).

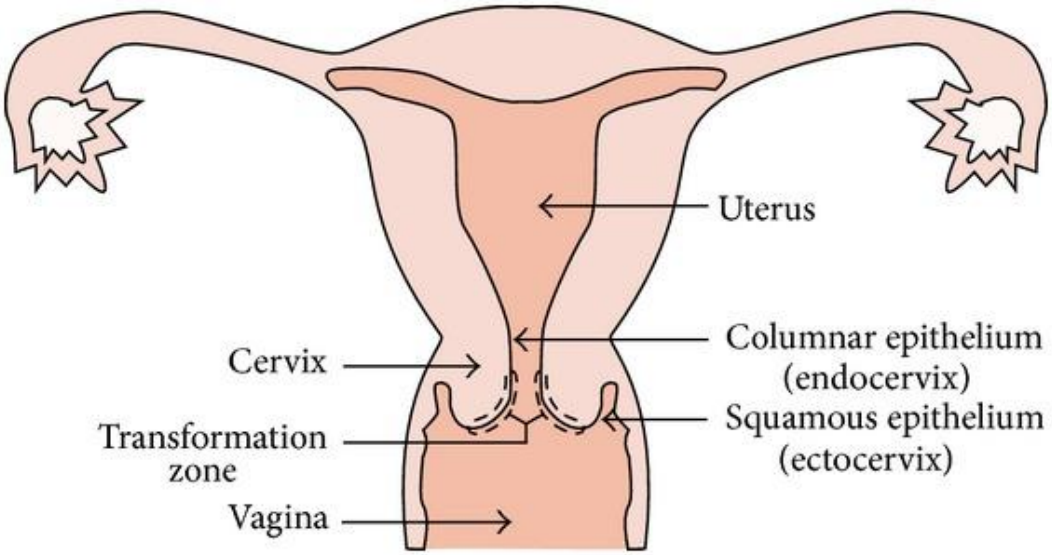


Figure 1.3 Anatomy of the cervix (Bengtsson & Malm, 2014).

The ectocervix is the outermost part of the cervix protruding into the vaginal cavity and is lined by non-keratinising stratified squamous epithelium. The endocervix is the tissue that surrounds the Endocervical canal and is lined by columnar epithelium, which secretes mucus. The area where the ectocervix lined by squamous epithelium and endocervix lined by glandular epithelium meet is known as the Transformation Zone, as shown in Figure 1.3. The original SCJ originates in the endocervical canal, but as the cervix everts during reproductive age, the SCJ lies on the ectocervix and becomes the new SCJ. In colposcopy terminology, this SCJ is the new SCJ. The epithelium between these two SCJs is the TZ or transition zone. Before puberty, the SCJ is normally at the external os, known as the original SCJ. After puberty, The lower half of the cervix called the portio vaginalis protrudes into the vagina. The acidic environment of the vagina then causes the glandular epithelium to transform into squamous epithelium, which is known as squamous metaplasia, accomplished by reserve cells of the endocervix differentiating into squamous cells instead of glandular cells (Sellors, John W. Sankaranarayanan, 2003; THOR, 1996). Squamous metaplasia develops through three stages, namely reserve cell hyperplasia, immature squamous metaplasia and mature metaplasia (Koss, 2000). This new SCJ is termed the transformation zone from which most abnormalities are thought to arise (Doorbar & Griffin, 2019).

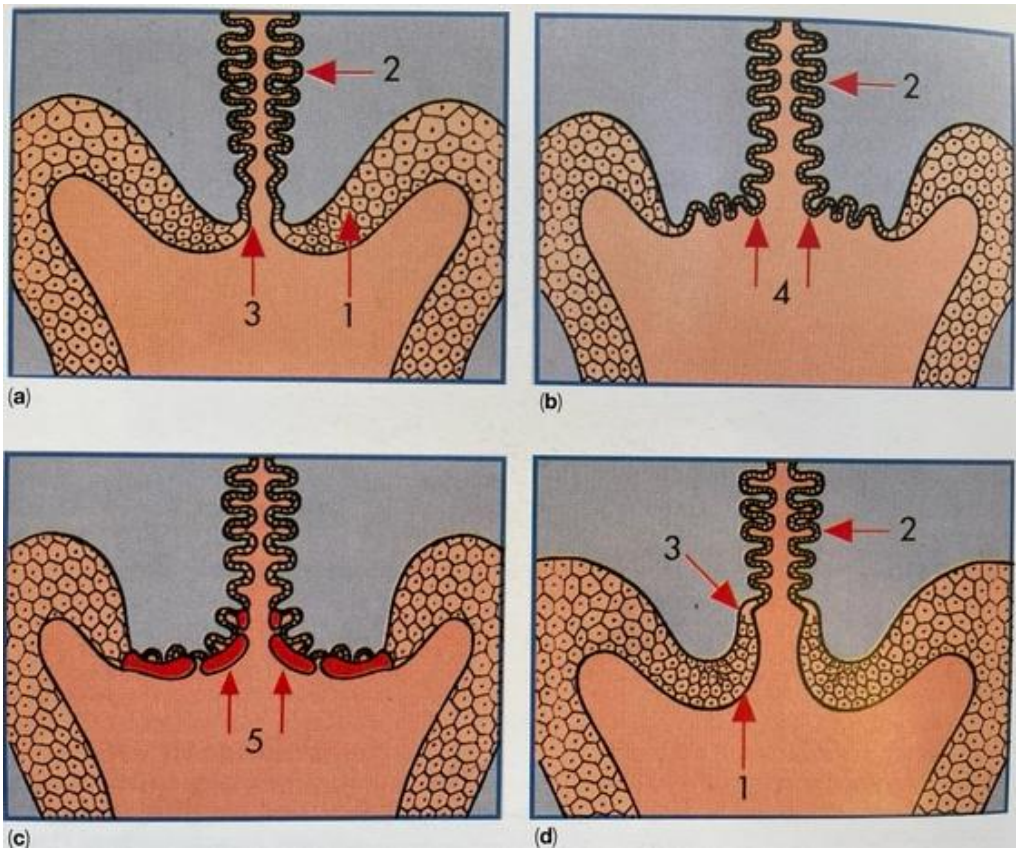


Figure 1.4 Development of squamous metaplasia in the cervix. (a) SCJ prior to puberty, (b) Eversion of the endocervical epithelium at puberty and first pregnancy, (c) Metaplastic changes in the endocervical epithelium in the transformation zone, (d) Relocation of SCJ in the endocervical canal after the menopause (Eurocytology, accessed on 24.09.2024).

The primary role of the cervix is to act as a barrier between the environment of the vaginal cavity and the uterus and allow the ascent of sperm to the fallopian tubes (Martyn et al., 2014). The vaginal environment is relatively acidic during reproductive life and pregnancy, and this acidity is thought to play a role in squamous metaplasia (Prendiville et al., 2017). The columnar cells exposed are eventually replaced by metaplastic squamous epithelium. Initially, the irritation of exposed columnar epithelium by the acidic vaginal environment results in the appearance of sub-columnar reserve cells, and these cells proliferate, producing reserve cell hyperplasia, and eventually become metaplastic squamous epithelium. The reserve cells multiply, differentiate, and eventually lift off the columnar epithelium (Figure 1.4). The exact origin of the reserve cells is not known (Prendiville et al., 2017). The atrophic cervix shrinks in the peri-menopausal period and afterwards, with the SCJ relocating within the endocervical canal after menopause Figure 1.4 (d).

1.2.2 Histology of cervix

The stroma of the cervix is composed of dense, fibromuscular tissue through which vascular, lymphatic, and nerve supplies to the cervix pass and form a complex plexus (Prendiville et al., 2017). The ectocervix and the entire length of the vagina is lined with stratified squamous epithelium, which is uniform, stratified, and non-keratinizing (Figure 1.5). The lowest level of cells in the squamous epithelium is a single layer of round basal cells with large dark-staining nuclei and little cytoplasm attached to the basement membrane. The basal cells divide and mature to form the next few layers of cells, called parabasal cells, which also have relatively large dark-staining nuclei and greenish-blue basophilic cytoplasm. Further differentiation and maturation of these cells leads to the intermediate layers of polygonal cells with abundant cytoplasm and small, round nuclei. These cells form a basket-weave pattern. Further maturation forms the superficial layers of large and markedly flattened cells with small, dense, pyknotic nuclei and transparent cytoplasm. Overall, from the basal layer to the superficial layer, these cells undergo an increase in size and a reduction in nuclear size (Sellors, John W. Sankaranarayanan, 2003).

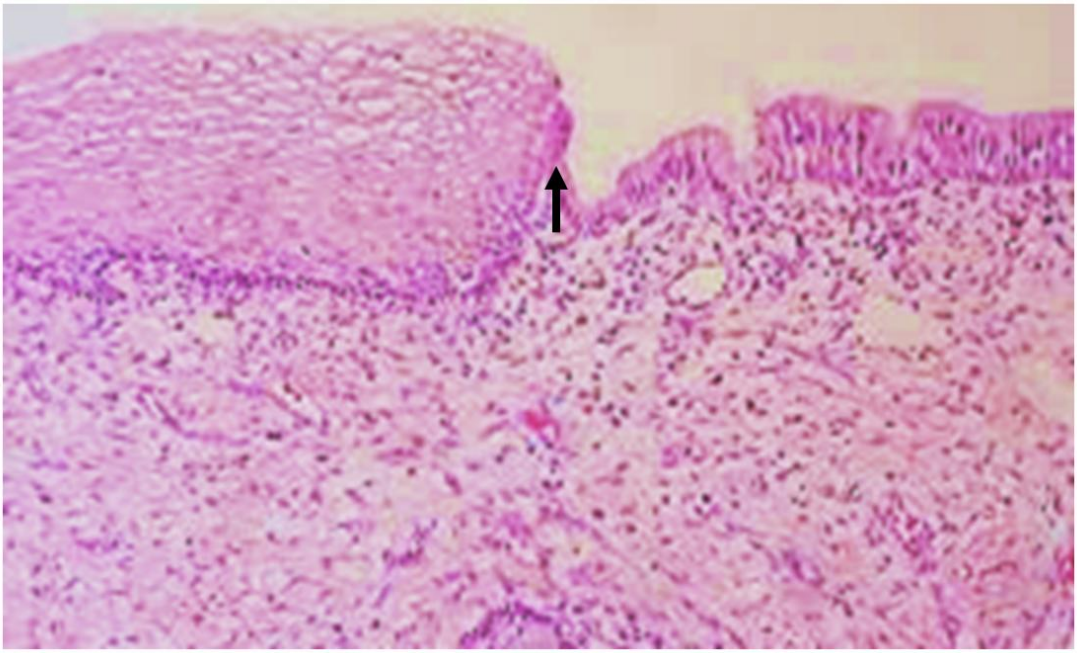


Figure 1.5 Histology of normal cervix with squamo-columnar junction indicated by the black arrow (Pierre et al., 2016)

The maturation of the squamous epithelium of the cervix is dependent on oestrogen. After menopause, the cells do not mature beyond the parabasal layer, and the epithelium becomes thin

and atrophic. The endocervical canal is lined with columnar epithelium (often referred to as glandular epithelium). It comprises a single layer of tall cells with dark-staining nuclei close to the basement membrane.

1.2.3 Squamous metaplasia

The process of squamous metaplasia has been described in detail by (Sellors, John W. Sankaranarayanan, 2003) as follows:

The first sign of squamous metaplasia is the appearance and proliferation of reserve cells (Figure 1.6 A and B). This is initially seen as a single layer of small, round cells with dark-staining nuclei situated very close to the nuclei of columnar cells, which further proliferate to produce reserve cell hyperplasia (Fig. 1.6 B). Morphologically, the reserve cells have a similar appearance to the basal cells of the original squamous epithelium, with round nuclei and little cytoplasm. As the metaplastic process progresses, the reserve cells proliferate and differentiate to form a thin, multicellular epithelium of immature squamous cells with no evidence of stratification (Figure 1.6 C). The term “immature squamous metaplastic epithelium” is used when there is little or no stratification in this thin, newly formed metaplastic epithelium. Groups of mucin-containing columnar cells may be seen embedded in the immature squamous metaplastic epithelium at this stage. As the process continues, the immature metaplastic squamous cells differentiate into mature stratified metaplastic epithelium (Figure 1.6 D).

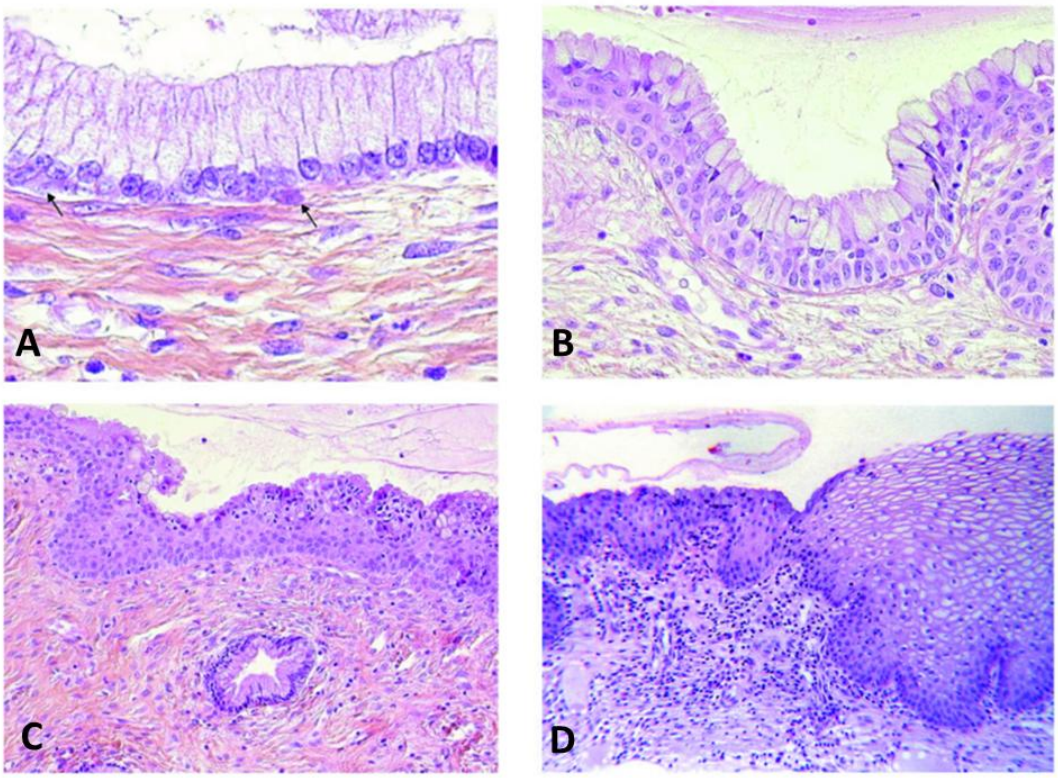


Figure 1.6Development of squamous metaplastic epithelium. (A) The arrows indicate the subcolumnar reserve cells. (B) The reserve cells proliferate. (C) The reserve cells further proliferate and differentiate. (D) Mature squamous epithelium is indistinguishable from native squamous epithelium (Sellors, John W. Sankaranarayanan, 2003).

High-risk Humanpapilloma Virus (HPV) types infect the immature basal squamous metaplastic cells. Some remain episomal and initiate productive infections. While others bypass the host immune system, integrate and proliferate into these cells. The uncontrolled proliferation and expansion of these atypical cells may lead to the formation of an abnormal dysplastic epithelium, which may regress to normal, persist as dysplasia, or progress to invasive cancer after several years, depending on whether the HPV infection is allowed to become a transforming infection (Sellors, John W. Sankaranarayanan, 2003).

1.2.4 Cervical intraepithelial neoplasia (CIN)

Carcinoma of the uterine cervix and its precursors have been well studied, researched, and presented throughout the history of cervical cancer (Koss, 2000). Rubin, in 1910, first described an early neoplastic change confined to the cervical epithelium and believed they were precursors of squamous cell carcinoma (Koss, 2000; Rubin IC, 1910). This was followed by the term carcinoma *in situ* in 1912 by Schottlander and Kermauner (GRAY et al., 1960) and in 1932 by Broders (Broders, 1932). Richart suggested the name cervical intraepithelial neoplasia for all precancerous intraepithelial abnormalities (RICHART, 1967). The term dysplasia was introduced by Reagan et al. (Reagan & Hicks,

1953; Vacher-Lavenu, 1999) in 1953 to describe these lesions, which were defined by Poulsen et al. in 1975 for the WHO (Poulsen et al., 1975). These lesions are graded and reported based on their nuclear and cytoplasmic features.

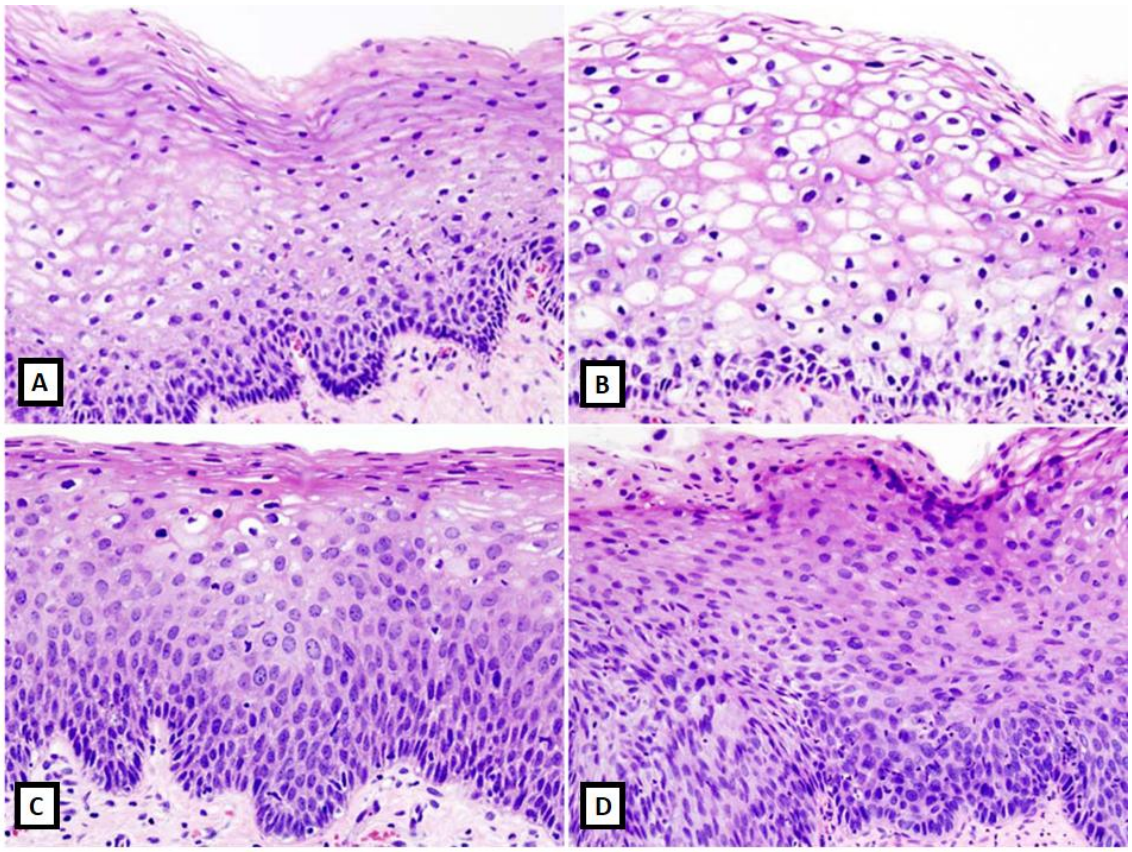


Figure 1.7 Histology of cervical intraepithelial lesions. (A) Normal epithelium, (B) CIN1, (C) CIN2, (D) CIN3 (medcell.org)

A diagnosis of CIN relies on histological features, including differentiation, maturation, and stratification of cells and nuclear abnormalities (Martin & O'Leary, 2011). Nuclear abnormalities and epithelium involvement are directly proportional to the grade of dysplasia. Cervical dysplasia is divided into three categories: Mild/CIN1 involving the lower third of epithelium (Figure 1.7 B), Moderate/CIN2 involving the lower and middle third of epithelium (Figure 1.7 C), and Severe/CIN3 involving the lower, middle and upper third of the epithelium (Figure 1.7 D). Carcinoma-in situ, which is a very early stage of cervical cancer, involves the entire thickness of the epithelium replaced by immature abnormal cells with abnormal nuclei and atypical mitoses and an intact basement membrane. During the transition from in situ to invasive carcinoma, tumour cells penetrate the epithelial basement membrane and enter the underlying cervical stroma. Once the cervical stroma is invaded, the lymphatics and blood vessels are accessible, and dissemination beyond the cervix is possible (Jhingran A, Eifel PJ, Wharton

JT, 2003). Invasive squamous cell carcinoma involves the invasion of the basement membrane and is the most common histology, accounting for most primary cervical carcinomas. According to Buckley and Fox, 1989, only 70% of these tumours are purely squamous, and the remaining 30% have a significant glandular component. Squamous cell carcinomas can be well differentiated/keratinising or poorly differentiated/non-keratinising (Scully, R. E., Bonfiglio, T. A., Kurman, R. J., Silverberg, S. G., & Wilkinson, 2012). Less than 10% of cervical cancer can represent adenocarcinoma in situ and adenocarcinoma of the cervix.

1.2.5 Cytology of cervix

Cervical cytology has been the traditional screening method of screening for cervical cancer and its pre-cancerous lesions ever since George Papanicolaou discovered the pap smear test in 1928 (Vilos, 1998). Exfoliated cells from the cervix are sampled using a cervical brush, stained using a modified Papanicolaou stain and examined under the microscope to find any abnormal changes in the nucleus of epithelial cells (Koss, 1960).

The following chart (Figure 1.8) describes normal and abnormal epithelial cells in cervical cytology with their cytoplasmic and nuclear features (Alrajjal et al., 2021).

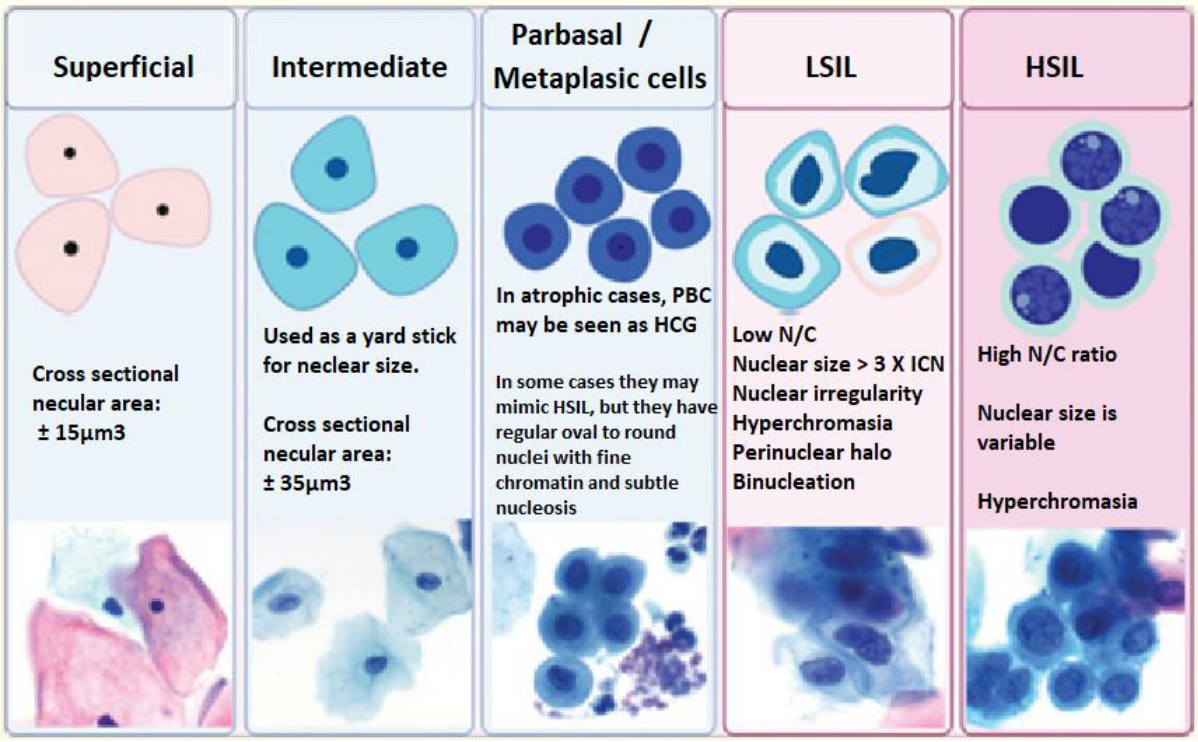
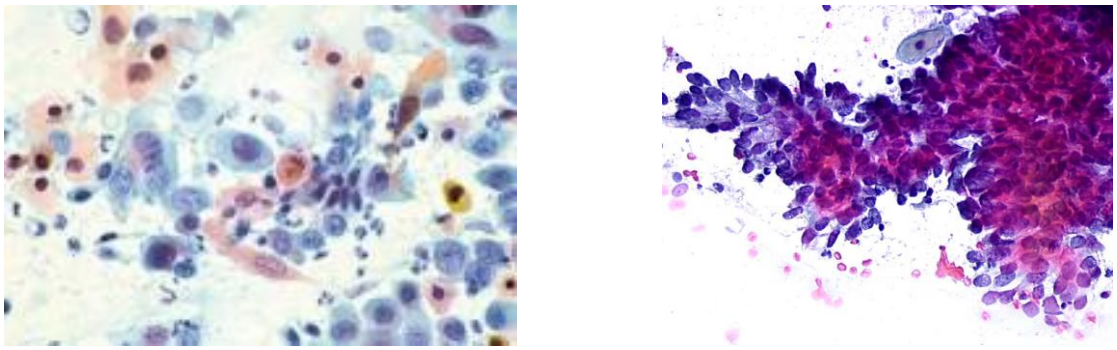


Figure 1.8 Cytology of squamous intraepithelial lesions. Adopted from Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of Uterine Cervix and Bethesda System (Alrajjal et al., 2021).

The cytomorphology of normal to squamous carcinoma changes throughout different grades. Nuclear features such as enlargement, hyperchromasia, coarse chromatin pattern, and the nuclear to cytoplasmic ratio increase as the abnormality increases (Figure 1.8). Cytoplasmic keratinisation, anisocytosis and tumour diathesis (Figure 1.9 A) becomes evident in the case of squamous carcinoma cytomorphology as opposed to the hyperchromatic three-dimensional clusters (Figure 1.9 B) with feathering features in adenocarcinoma (IARC Monograph Working Group, 2012).



A: Squamous cell carcinoma B: Endocervical adenocarcinoma

Figure 1.9 Cytology of squamous carcinoma and adenocarcinoma. (A) Cytology of squamous cell carcinoma, (B) Cytology of endocervical adenocarcinoma. Adopted from Histopathology of the uterine cervix, Digital Atlas (Frappart et al., 2014)

The reporting of cervical cytology is conducted using different reporting systems around the world, as shown in Figure 1.10 (Alrajjal et al., 2021; Nayar & Wilbur, 2017).

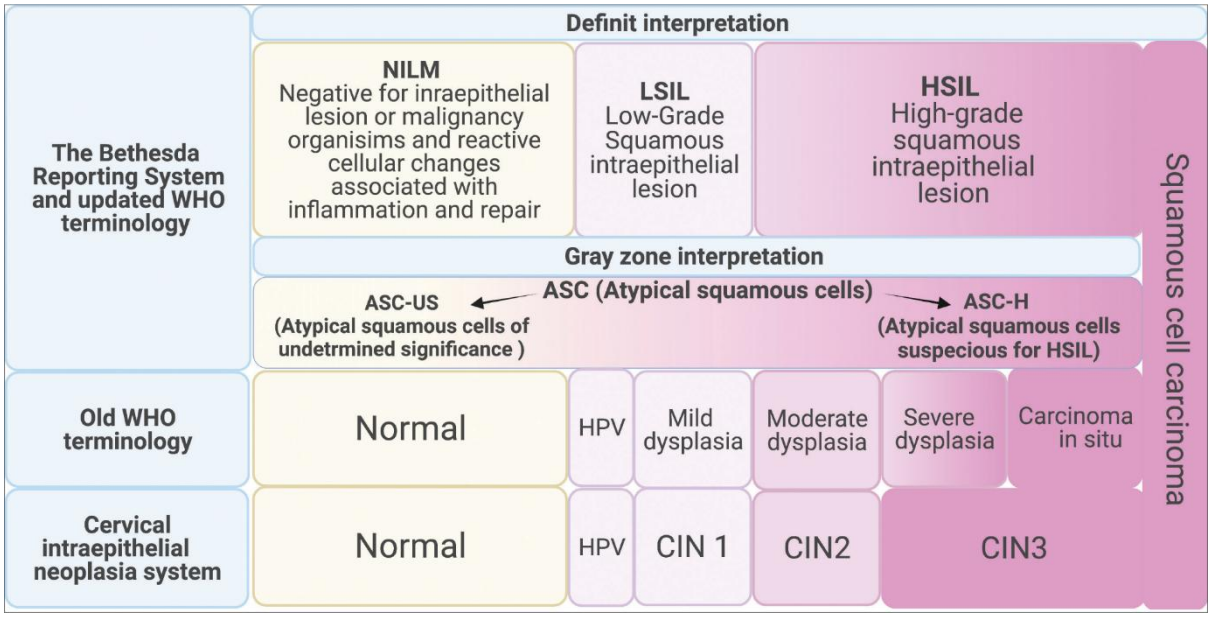


Figure 1.10 The cytology reporting of cervicovaginal specimens. Adopted from Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of the Uterine Cervix and Bethesda System (Alrajjal et al., 2021).

In Bethesda and the updated WHO system, smears are categorised as negative for intraepithelial lesions for malignancy (NILM), atypical squamous cells of undermined significance (ASCUS), low-grade squamous intraepithelial lesion and high-grade squamous intraepithelial lesion and suspicious of squamous cell carcinoma. In the old WHO terminology and CIN terminology, NILM is reported as Normal, HPV is reported on its own, LSIL is reported as mild dysplasia or CIN1 and HSIL is categorised as moderate or severe dysplasia or CIN2 and CIN3 and squamous cell carcinoma. UK-based publications and pre-Bethesda ones also use the term 'dyskaryosis' instead of dysplasia.

Glandular lesions are reported as atypical glandular cells of undermined significance (AGUS), specifying endocervical or endometrial origin and cervical glandular intraepithelial neoplasia (CGIN), specifying adenocarcinoma in situ or endocervical or endometrial adenocarcinoma. Glandular lesions also may have different terminologies, such as glandular atypia and glandular abnormalities in UK and pre-Bethesda publications. In Ireland, cervical cytology reporting is performed using The Bethesda system.

1.3 Human Papillomavirus

1.3.1 General information

Human Papillomavirus (HPV) are a large family of double-stranded, circular DNA viruses of 50-60 nm diameter enclosed in an icosahedral capsid. To date, approximately 200 genotypes have been identified (de Sanjose et al., 2010; Denny, 2018). These are divided into five different genera types:

Alpha, Nu, Mu, Beta and Gamma. The Alpha genera include genotypes that have been found in cervical cancers worldwide (Bosch & De Sanjosé, 2007; IARC, 2012; J. S. Smith et al., 2007), while Beta and Gamma genera can trigger cutaneous papilloma and skin cancer. About 5% of all cancers worldwide are attributed to HPV infection(de Sanjose et al., 2010; Denny, 2018). The cancer burden attributed to human papillomavirus infection varies from age-standardised incidence rates of 6.9 per 100,000 person-years in high-income countries to 16.1 cases per 100,000 person years in low-income countries (Figure 1.11).

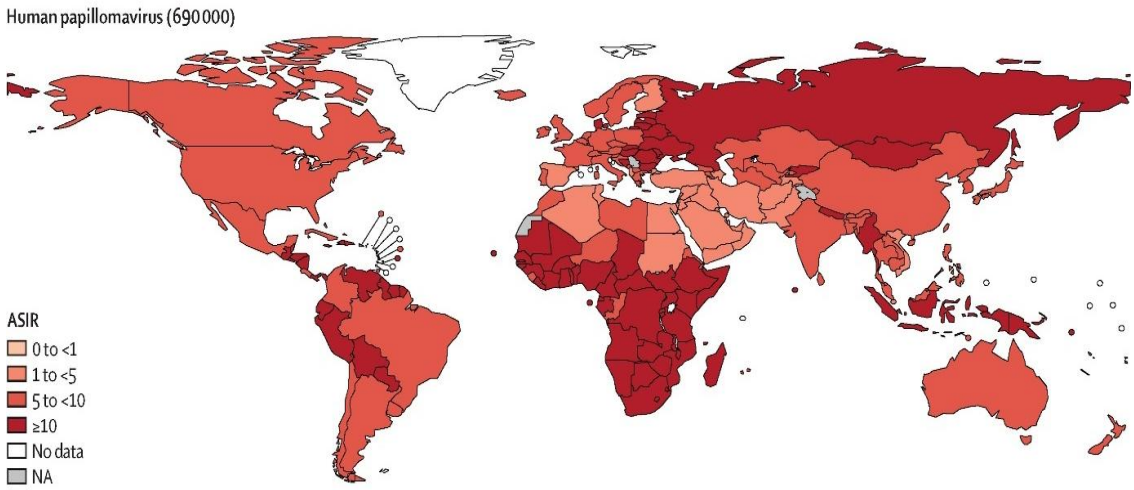


Figure 1.11 Country-specific age-standardised incidence rates (ASIR) per 100,000 person-years of cancer attributable to HPV infection. Adopted from Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis (de Martel et al., 2020).

Papillomaviruses have a broad genotypic diversity. They have been grouped alongside other biological agents according to their level of carcinogenicity by IARC in their Monographs on the Evaluation of Carcinogenic Risks to Humans (Figure 1.12).

Figure 1.12 HPV Classification of carcinogenicity used by the IARC. Adopted from Biological Agents IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100B (Bravo & Felez-Sanchez, 2015; IARC Monograph Working Group, 2012).

In total, 14 high-risk HPV types have been attributed to high risk for developing cervical cancer. HPV16 and HPV18 cause overall 70% of cervical cancer (Crosbie et al., 2013), the remaining 30% of cervical cancer are caused by other types. HPV52 and 58 are relatively more prevalent in Asia, HPV33 is prevalent in Europe, and HPV45 is more prevalent in Africa (Mishra et al., 2020).

1.3.2 HPV genome

HPV has a circular double stranded DNA genome of approximately 8 Kb size (Graham, 2010). It is divided into three main regions (IARC monogram).

A. Early region: Encodes proteins required for viral gene expression, replication and survival.
B. Late region: Encodes the viral structural proteins.
C. The Long Control Region/Upstream Regulatory Region: Regulates viral gene expression and replication.

The detailed functions of these Early genes and Late genes are illustrated in Figure 1.13.

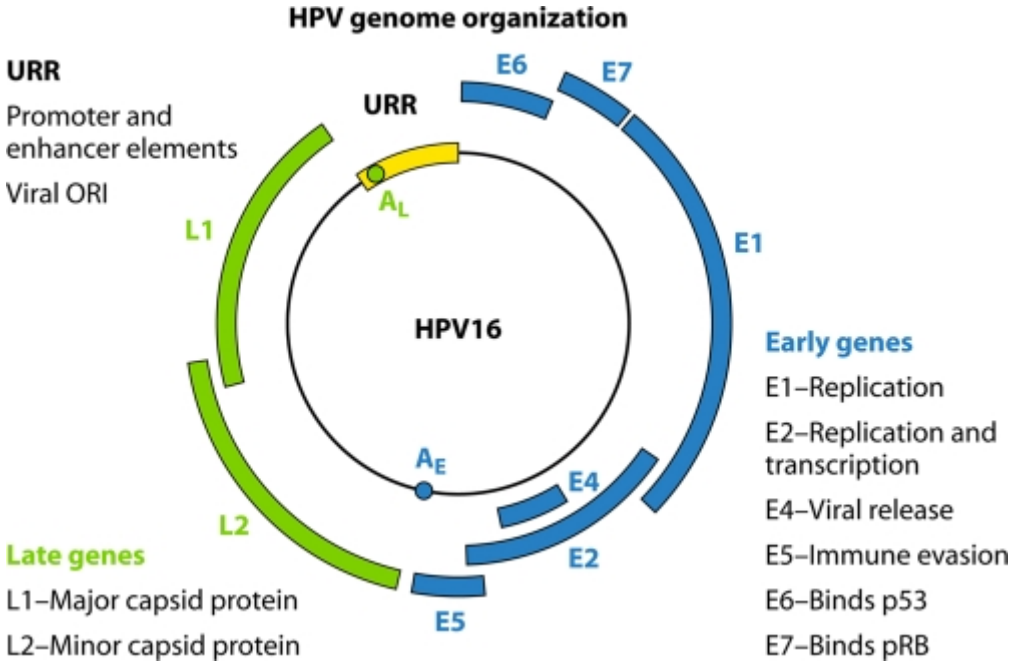


Figure 1.13 HPV genome- genomic organisation of a typical mucosal high-risk HPV (Stanley, 2010)**.** The functions of early genes are illustrated in blue, and late genes are illustrated in green.

1.4 Pathogenesis of HPV, cervical cancer screening and triage methods

1.4.1 HPV life cycle

HPV life cycle takes place within stratified squamous epithelium. HPV infection begins with the virus entry through microabrasions or microtraumas in the stratified squamous epithelium (Burchell et al., 2006). The virus then reaches the basal epithelia and attaches to cells using common cell surface molecules such as heparin sulphate proteoglycans (Shafti-keramat et al., 2003). The productive cycle of HPV involves discrete phases such as cell proliferation and episomal maintenance in the lower epithelial layers, followed by genome amplification and the expression of capsid proteins (Middleton et al., 2003). The virus then follows three life cycle stages: establishment as low copy number nuclear plasmid, maintenance through multiple cell divisions and production when the daughter cells derived from infected basal cells start differentiating (IARC monograph). The cell becomes permissive of viral replication, and hundreds or even thousands of HPV genomes are generated in a single cell (Crosbie et al., 2013). This life cycle takes place for 2-3 weeks until cervical epithelium matures and is shed off (Stanley, 2010).

Most HPV infections clear within 1-2 years. Some maintain latency where HPV is there but not actively replicating. HPV is maintained in the putative stem cells in the basal layer, remaining invisible to the immune system and HPV testing. Persistent HPV infection is the prerequisite for the development of

cervical intraepithelial lesions (Bosch et al., 2002; Lu et al., 2023; Walboomers et al., 1999). In a minority of cases, once HPV is inside the cell, uncoating occurs, and the circular virus genome is transported to the nucleus (Graham, 2010; Walboomers et al., 1999). Factors such as the use of oral contraceptives, smoking, multiple sexual partners, age of the sexual intercourse initiation, other sexually transmitted agents, host susceptibility factors and immune response contribute to and or enhance the chances of progression to cervical intraepithelial lesions and cervical cancer. The long process of carcinogenic transformation from HPV infection to invasive cancer (10-20 years) provides ample opportunities to detect the diseases at a stage when treatment is highly effective (Basu et al., 2018).

1.4.2 HPV-driven hallmarks for cervical cancer and precancer

HPV gains entry to the cervical epithelium through access to the basal lamina and the epithelial basal cells. The complex process of development of cervical cancer and the role of HPV oncoproteins at each stage is described in Figure 1.14 (Crosbie et al., 2013). Infected basal cells serve as the reservoir of infection, limiting the differentiation of the squamous epithelium and expanding the infected cell population in the infected basal layer (Doorbar, 2018). Integration of viral DNA into the host genome is considered a crucial step to initiate carcinogenesis (Crosbie et al., 2013; Faridi et al., 2011; Münger et al., 2004). The carcinogenic process starts with over-expression of oncoproteins E6 and E7 with persistent high-risk HPV infection. Degradation of tumour suppressor protein p53 by E6 leads to the derangement of the control of cell cycle arrest and apoptosis due to loss of p53 transcriptional activity (Doorbar, 2018; White et al., 2016). HPV maintains its genome replication and constant low level of its episomes with the help of E2 protein (Doorbar, 2018). Furthermore, HPV self-limits its infection by interfering with the cell expansion and cell density regulatory pathways (Egawa et al., 2015).

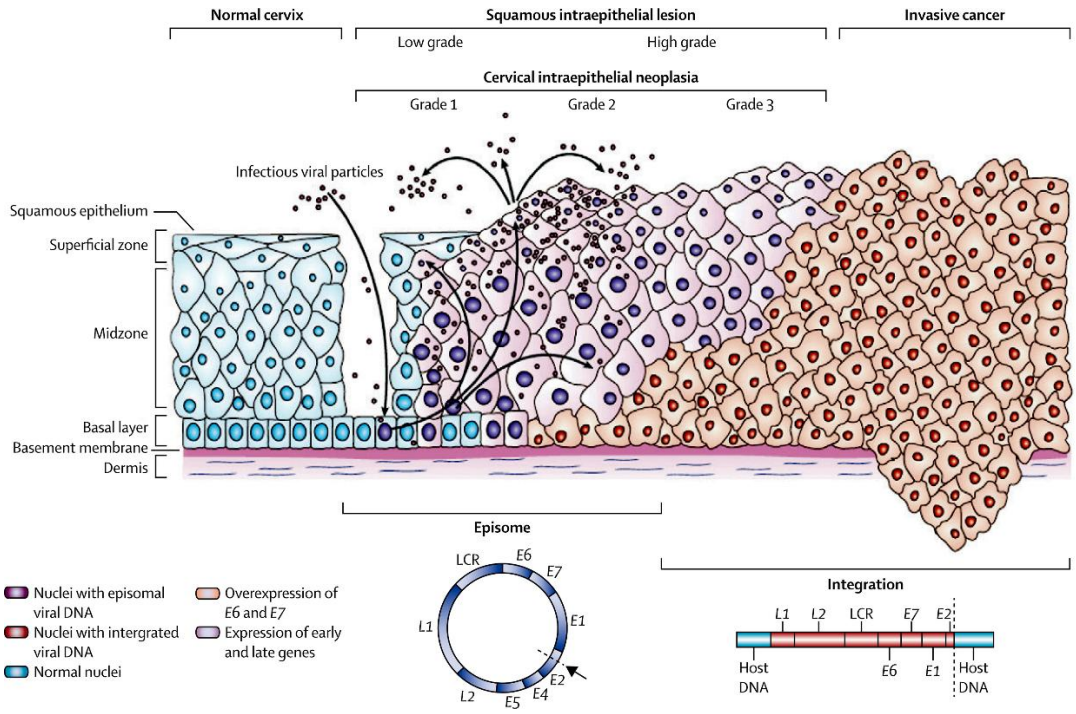


Figure 1.14 Human papillomavirus life cycle and development of cervical cancer pathogenesis (Crosbie et al., 2013).

Once these HPV infections are established over time, the HPV life cycle starts with a steady flow of infected cells from the basal layer reservoir into the upper epithelial layers. This process is accompanied by cellular differentiation with viral genome amplification. Simultaneously, sophisticated immune evasion techniques evolved by high-risk HPV types enable the virus to remain undetected for longer periods, escaping immunological surveillance (Frazer, 2009; Piersma, 2011). E7 forms a complex with the cell cycle regulator protein pRB and degrades it, releasing the E2F transcription factor to allow progression into S-phase (Crosbie et al., 2013; Doorbar, 2018; White et al., 2013). The binding ability of pRB and inhibiting transcriptional factors E2F-1 and cMyc proteins leads to the activation of some of the essential genes required for HPV DNA synthesis (Faridi et al., 2011). Simultaneously, HPV late promoters are activated, allowing the production of the E1, E4, L2 and L1 proteins. The combination of these events overrides cell cycle checkpoints, allowing the integration of HPV into the host genome and the immortalisation of infected cells. It allows for viral DNA replication in non-cycling cells, allowing DNA-damaged cells to enter the normal cell division process, thus initiating cancerous or tumourogenetic mutation (Faridi et al., 2011), and leading to the deregulation of growth control in the infected cell and the development of cancer (Stanley, 2010). The role of HPV in causing abnormal chromosomal segregation during mitosis and, hence, aneuploidy is described in detail in Chapter 5. Development of cervical pre-cancer and cancer is a slow process and

can take several years post-persistent high-risk HPV infection, depending on the host and viral gene interactions.

1.5 Cervical cancer screening

Cervical cancer satisfies the set of classic screening criteria described by Wilson and Jungner in 1968 (Basu et al., 2018). Anatomical accessibility and easy visualisation make cervical sampling relatively painless and simple, resulting in screening as an effective strategy to control cervical cancer (Basu et al., 2018).

For more than 40 years, cervical cytology/Pap smear has been the test for cervical cancer screening in most of the screening providing countries. Although cytology has been effective in reducing cervical cancer incidence and deaths, sampling errors (Hearp et al., 2007), suboptimal sensitivity (Koliopoulos et al., 2017), lack of trained cytologists and pathologists (Field, 2016) and difficulty in implementing robust quality control due to the subjective nature of cytology (Macios et al., 2023), have led to high false negative rates. The discovery of HPVs in the 80s, followed by a greater understanding of the link between HPV and cervical cancer pathogenesis, has led to the development of alternative and improved test options.

1.5.1 Cervical cancer prevention and control

The Global strategy towards eliminating cervical cancer as a public health problem, adopted by the World Health Assembly in 2020, recommends a comprehensive approach to cervical cancer prevention and control. The recommended actions include interventions that span across an individual's lifetime(https://www.who.int/news-room/fact-sheets/detail/cervical-cancer).

The WHO's comprehensive prevention and control programme includes three following independent components:

Vaccination Target: 90% of girls are fully vaccinated with the HPV vaccine by age 15.

Screening Target: by age 35 and again by age 45, 70% of women are screened using a high-performance test.

Treatment Target: 90% of women with pre-cancer treatment and 90% of women with invasive cancer are managed appropriately.

1.5.2 HPV and cervical screening

Major randomised control trials such as the NTCC trial, POBASCAM, the ARTISTIC trial in the UK, and Public Health Trial in the Netherlands contributed to the introduction of population-based HPV

primary screening (Bulkmans et al., 2004; Kitchener et al., 2009; Rijkaart et al., 2012; Ronco et al., 2014). The long process of carcinogenic transformation from HPV infection to invasive cervical cancer provides ample opportunity to detect the disease at a stage when treatment is highly effective (Basu et al., 2018).

Extensive work conducted by several researchers helped to develop and standardise HPV detection assays in clinical settings (Altobelli et al., 2016; Arbyn et al., 2016; Chrysostomou et al., 2018; Clarke et al., 2018; Cuschieri et al., 2015; Doorbar et al., 2015; Koliopoulos et al., 2017; Moss et al., 2006; Vink et al., 2015). According to the European Guidelines, as well as the World Health Organization (WHO), HPV testing is now recommended as the primary screening tool for cervical cancer (Arbyn et al., 2010; Harlfinger et al., 2023; von Karsa et al., 2015). Bruni L et al. identified recommendations for cervical screening in 139 (69%) of 202 countries and territories, of which 48 (35%) of 139 countries recommended primary HPV-based screening (Bruni et al., 2022). Australia was one of the first countries to completely transition its national cervical screening programme to primary HPV testing (M. A. Smith et al., 2022). In Europe, Finland, France, Germany, Italy, Ireland, The Netherlands, Spain and Sweden, as well as Norway, Turkey and the United Kingdom, have either started to implement HPV testing on a regional or national level (European Cancer Organisation) The Netherlands was the first country to switch to high-risk HPV screening at the national level (Aitken et al., 2019). Australia started implementing HPV primary screening in 2017 (Brotherton et al., 2023). In the first screening round of the Finnish randomized HPV screening trial, direct referral of HPV positives to colposcopy led to a sensitivity of 94% and specificity of 93%; however, disease detection by cytology and HPV screening was not significantly different. (Vahteristo et al., 2022).

1.5.3 Cervical Screening in Ireland

Since 2008, Ireland has a National Cervical Screening Programme, CervicalCheck. Each year, over 1 million women between the ages of 25 and 65 years are invited to attend cervical screening. Up to 31st March 2020, CervicalCheck operated a cytology-based screening programme with call/recall for eligible women with either a three-year screening interval for women aged 25-44 or a five-year screening interval for women aged 45-60. HPV testing was implemented as a “Test of Cure” in CervicalCheck colposcopy clinics in 2012, and it utilised a combined cytology and HPV test approach. In May 2015, HPV triage was introduced for the triage of low-grade cytological abnormalities (ASC-US/LSIL). In March 2020, the programme switched to an HPV-based primary screening for women aged 25-65 years with cytology triage for HPV-positive cases.

Since CervicalCheck started in 2008, the number of women who developed cervical cancer decreased by 7% year-on-year from 2010-2015. More recent data shows that the decreasing incidence has been

sustained as the programme matures, with a 2.8% annual percentage decrease from 2010-2018 (NCRI, 2021). From September 2008 to March 2020, CervicalCheck has provided almost 3.2 million cervical screening tests. Since 2008, 64,110 cases of high-grade pre-cancerous cells (CIN2 and CIN3) and 60,650 cases of low-grade pre-cancerous cells (CIN1) have been identified (CervicalCheck, 2022). In 2011, at its peak, the cervical cancer screening programme led to the highest detection of CIN2+ lesions and their treatment, thus preventing them from progressing to invasive cancer.

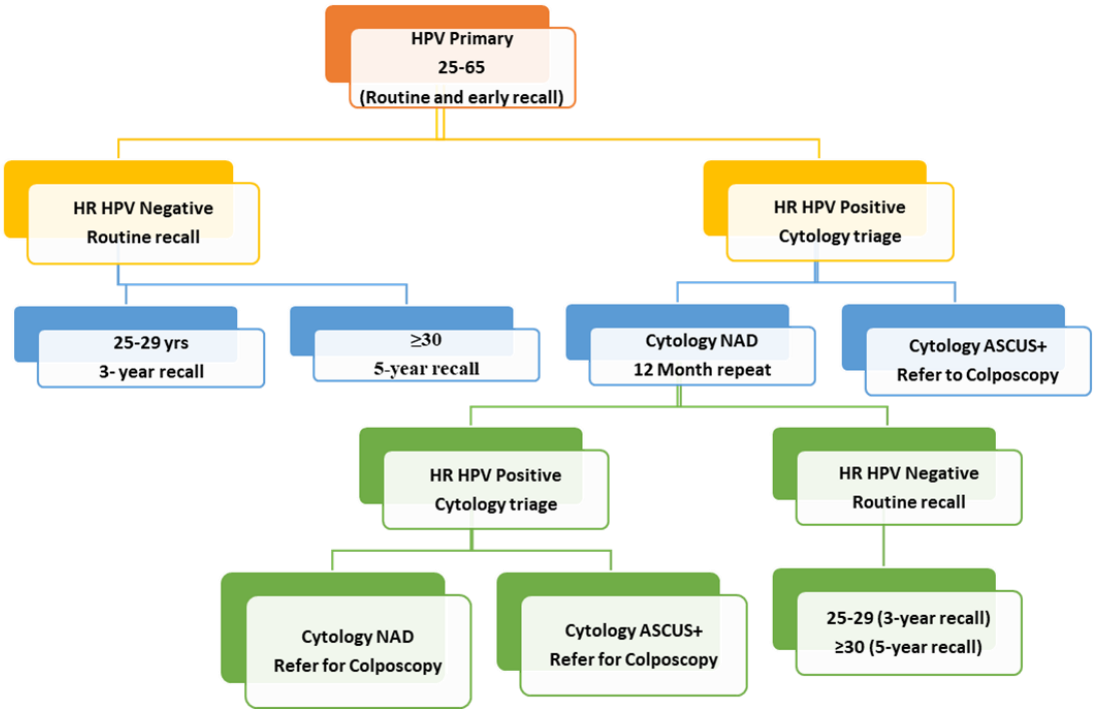


Figure 1.15 Current screening pathway in Ireland. Quality Assurance in Laboratories Providing HPV Testing, Cytology and Histopathology Services (CervicalCheck, 2020).

As per the current cervical screening pathway in Ireland, women are screened between the age of 25-65 years using HPV primary screening (Figure 1.15). HPV-negative women between 25-29 years get recalled every three years for screening, and women 30 get recalled every 5 years. HPV-positive women are subjected to cytology as a triage test. HPV-positive and cytology-negative women get a

yearly recall for screening. Women with HPV-positive and ASCUS+ cytology results are referred to colposcopy. Post HPV primary implementation, 526,816 women were screened (73% programme coverage), of which 9,575 were diagnosed with high-grade pre-cancerous cells (CIN 2/3 and cGIN), and 187 were diagnosed with cervical cancer (CervicalCheck, 2022).

1.6 HPV primary screening and triage methods

Screening, diagnosis, and prevention are the classic steps of cancer screening, where triage is interposed between screening and diagnosis to identify subpopulations at increased risk and therapy to optimise the overall management process (Solomon, 2003). In the case of cervical screening, HPV testing, cytology, and colposcopy are the screening methods, and histology is the diagnostic method. HPV testing, Cytology and Histology are also used for post-treatment monitoring. As mentioned by Solomon D., 2003, factors such as the test's performance characteristics, the target population, the prevalence of disease, the screening test, the cost of the follow-up, logistic and monitory resources and most importantly, patient compliance affect the utility of triage tests. However, it is difficult to satisfy all the above factors. Therefore, a balance between specificity and sensitivity, along with the positive and negative predictive value of the test, plays an important role. The following sections describe a number of the triage methods explored for HPV-positive women:

1.6.1 Cytology

HPV-positive samples are processed for cytology in order to make a cervical smear. The liquid-based cytology (LBC specimen) is processed using a ThinPrep processor, which gives a smear in a monolayer of cells on a glass slide. This smear is then fixed in 95% ethyl alcohol. The smear is then stained using Pap stain consisting of haematoxylin as a nuclear stain and EA 50 and OG6 as cytoplasmic stains. These smears are then screened under the microscope to see if abnormal cells are present in the smear and grade them accordingly. Women with HPV-positive and abnormal cytology results are then referred to colposcopy.

Cytology has the advantage of being an established method to detect morphological abnormalities in cervical cells, does better in sensitivity with prior knowledge of HPV positivity and has been tried and tested for many years. In most countries, cytology is used as a triage test following a positive HPV screening result (Chrysostomou et al., 2018; Maver & Poljak, 2020) with varying degree of sensitivity for the detection of abnormalities. A Swedish study performed in an HPV-positive subset of women showed cytology-based triaging identified 60.8% of CIN2+ cases (Schreiberhuber, L., Barrett, J.E., Wang, 2024). A cross-sectional study in Cameroon exploring triage of human papillomavirus (HPV)-positive women showed a sensitivity of 80.0% for manual cytology and 84.8% for automated cytology (Vassilakos et al., 2021). A sensitivity of 85.6% was seen in informed cytology in a sub-study nested in

the NTCC Randomized Controlled Trial (Bergeron et al., 2015). However, cytology results such as ASCUS will still lead to colposcopy referral, where finding cervical lesions can be complex, thus causing over referral and anxiety among women.

1.6.2 Partial and Extended HPV genotyping

HPV genotyping is emerging as one of the potential triage methods. HPV-positive specimens are subjected to extended genotyping where individual or groups of HPV genotypes can be identified. Depending on the presenting genotype of HPV, women are either sent to colposcopy or to routine recall. The specific genotype of HPV infection is used to determine the risk of cervical cancer (Adams & Mbatani, 2018) in many developed countries. The use of genotyping as a triage of primary HPV infection is supported by several country-specific guidelines, e.g. Australia (Cuschieri et al., 2019) and Sweden (Portnoy et al., 2024). According to Adams and Mbatani (2018), all women with HPV 16 and/or 18 positives should be referred to colposcopy, and those with other HR-HPV subtypes can be followed up with cytology or biomarker tests to determine further risk.

However, a recent study in the US by Wright TC et al. indicates that women with HPV 16 had the greatest risk for developing >/ CIN 2 or >/ CIN3, followed by those with HPV 31 in most instances (Wright et al., 2019). The recommendation by (Stoler et al., 2019) is that hierarchical genotype groupings should be considered in future review of guidelines for co-testing and HPV primary screening. This is supported by Bruni L et al. (2010), where the most common high-risk HPV types in Eastern Europe are not only HPV16 and HPV 18 but also HPV 31, HPV 33, and HPV 39, with a prevalence rate of 21.4% (Bruni et al., 2010). Their recently published 15-year study of cervical cancer screening experience with multiple rounds of HPV testing (Hammer et al., 2020) corroborates previous findings that current HPV positivity, particularly when prevalent rather than new, is associated with the highest rates of CIN3+. In a screening program implementing HPV testing, most CIN3+ is detected at the first HPV-positive test. IARC published a triaging algorithm for hrHPV-positive women using a combination of partial genotyping and cytology based on the WHO guidelines in 2023 (Figure 1.16)

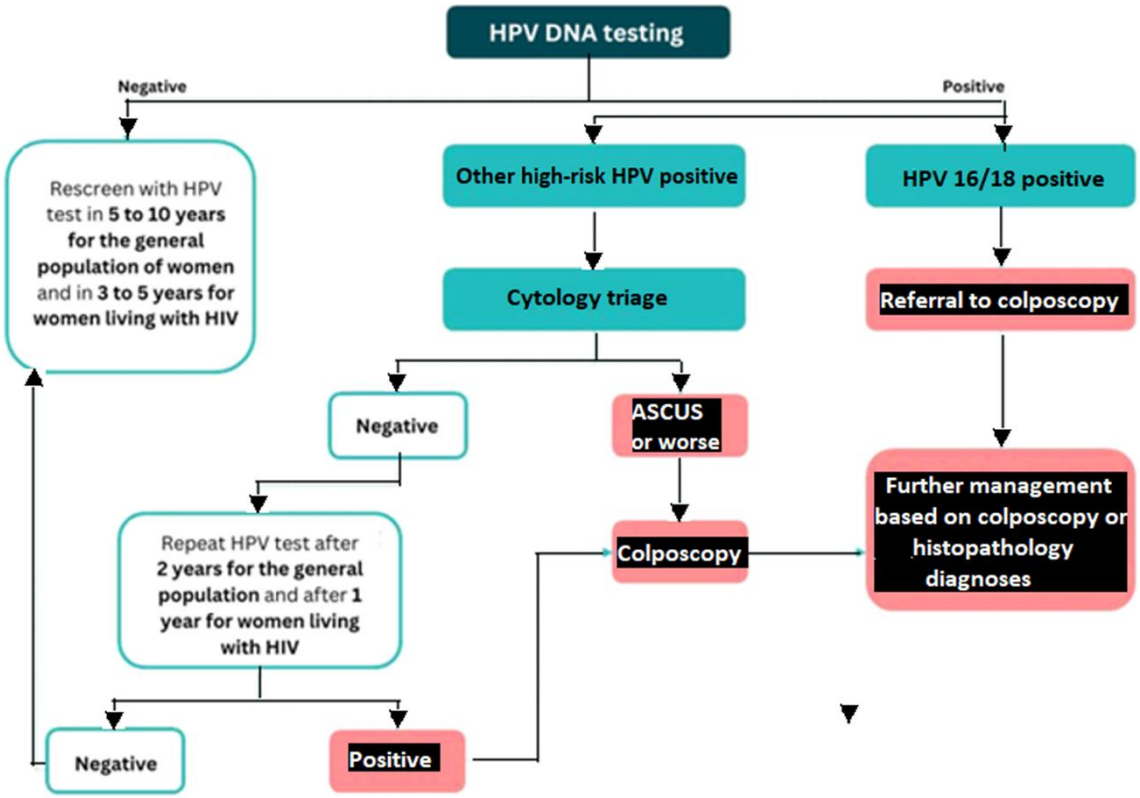


Figure 1.16 Using HPV tests for cervical screening and managing hrHPV-positive women – a practical online guide (International Agency for Research on Cancer, 2021)**.**

1.6.3 Biomarker assay- p16/Ki-67 Dual staining

p16INK4a (p16) is a tumour suppressor protein that is a cyclin-dependent kinase inhibitor and is essential in regulating the cell cycle (Rabban et al., 2010). Ki-67 is a nuclear antigen that can be detected in the non-G0 phase of the cell cycle, marking the process of cell proliferation (Carreras et al., 2007). p16 over-expression is caused by increased E7 oncoprotein activity (correlated with persistent HPV infection), and Ki-67 is a marker of tumour proliferation. The test is considered positive when both markers are expressed within the same cell (Secosan et al., 2022). The test had been initially developed for histology and tissue-based diagnostic, but CINTec PLus Cytology has now been developed specifically for use in cervical cytology samples. In this triage method, HPV-positive specimens are subjected to preparation of the smear using the Thin Prep processor and the smears made are stained using the FDA-approved CINTec PLUS dual staining p16/Ki-67 (FDA, 2020). HPV-positive and dual stain-positive women are then referred to colposcopy. A number of studies done using p16/Ki-67 dual staining showed the utility of this marker as a possibility of one of the triage methods (Clarke et al., 2019; Ebisch et al., 2017; Øvestad et al., 2023; Uijterwaal et al., 2015; Wright et al., 2017). Y. Hu et al. evaluated p16/Ki-67 dual staining in triaging HPV-positive women among women ages >/ 21 in China (Y. Hu et al., 2020). They found that p16/Ki-67 dual staining, either alone or combined with HPV 16/18 genotyping, showed a good stratification with high specificity and

comparable sensitivity for HPV-positive women. Other protein assays for cervical cancer include CEA, SSCA, and CA19-9 measured in serum by ELISA test (Güzel et al., 2021); however, these are more frequent markers of cancer rather than pre-cancer.

1.6.4 Methylation markers

The methylation test detects the addition of a methyl group to the fifth position of the Cytosine preceding Guanines (CpG) to form 5-methyl-cytosine (Banila et al., 2022; Bowden et al., 2019; Lorincz, 2016). This emerging method involves HPV-positive specimens subjected to methylation markers analysis such as DAPK1, CADM1, and RARB (Wentzensen et al., 2009). A number of studies have been done to see if methylation marker positivity can predict the risk of cervical neoplasia in HPV-positive women (Bonde et al., 2021; Chang et al., 2021; Salta et al., 2023; Van Keer et al., 2021; van Leeuwen et al., 2019). Methylation makers such as FAM19A4, GHSR, PHACTR3, PRDM14, SST, and ZIC1 have been studied for cervical cancer screening (Snoek et al., 2019). Studies on histological samples of cervical pre-cancer and cancer showed hypermethylation in a number of genes such as CADM1, MAL, MIR-124-2, FAM19A4, POU4F3, EPB41L3, PAX1, SOX1 (Clarke et al., 2018; Kelly et al., 2019). Methylation testing has the advantage of requiring a minimal amount of DNA and is highly reproducible. (Clarke et al., 2019) and can be automated.

1.7 Colposcopy referral and diagnosis of cervical cancer and pre- cancer

HPV-positive women detected using HPV primary screening and triage positive method are referred to colposcopy procedure. Cervical lesions are identified by the application of glacial acetic acid and/or iodine application during this process. A cervical biopsy is taken from aceto-white areas or iodine-negative areas and subjected to histopathology diagnosis for the confirmation of cervical cancer and pre-cancer. Both HPV detection and colposcopy referral rates vary due to several factors such as study design- observational or randomised control trial, methodology used for HPV detection, adherence to patient care compliance procedures and population-based risk factors in different geographical areas and interpersonal and inter-laboratory variation in cytology reporting (Hashim et al., 2020). The referral rate for HPV-positive and ASCUS+ cytology varies from 27% to 46% in different studies (Demarco et al., 2017; Katki et al., 2011; Wright et al., 2017). This does have an impact on the risk and detection of CIN2+ in the colposcopy referral population.

To date, histology is considered a gold standard for the diagnosis of cervical cancer and pre-cancer. Depending on the accuracy of histology grading of cervical intraepithelial lesions, women are followed up at certain intervals or subjected to large loop excision of the transformation zone. However, histology diagnosis has limitations due to the inter and intra-observer reproducibility of CIN grade evaluation among pathologists (Martin & O'Leary, 2011). Cervical intraepithelial lesions are extremely

heterogeneous and have complex morphological features accompanied by processes such as reserve cell hyperplasia, immature metaplasia (Jenkins, 2007), inflammation, repair, atrophy and hormonal changes (Van Baars et al., 2015). Although CIN2 is the treatment threshold, it is not reproducible (Carreon et al., 2007); it is managed conservatively in young women due to its high regression rate (Lycke et al., 2023). The accuracy of histology diagnosis of cervical intraepithelial neoplasia can be aided by using prognostic and diagnostic molecular markers.

1.8 Prognostic and diagnostic molecular biomarkers in cervical cancer and pre-cancer

Biomarkers may be defined as quantifiable indicators of a biological, pathological or therapeutic response process and include molecules such as messenger RNA and proteins. Molecular biomarkers may be present in blood, other body fluids and tissues, and they may be useful in elucidating both normal and abnormal processes in the body. In cervical cancer, progression biomarkers may be useful for identifying and predicting the prognosis of low-grade cervical lesions. The development of invasive cervical cancer depends on the persistence of HPV infection, initiating oncogenic changes in epithelial cells at the cervical transformation zone, progressing to pre-cancerous changes, and finally developing into invasive cancer. The process depends on the various viral and host cellular interactions that take place over a period of a few years involving both epigenetic and genetic changes in oncogenes and tumour suppressor genes. Epigenetic changes affect gene expression by mechanisms other than changes in the underlying DNA sequence, whereas genetic changes are considered adaptations in the DNA sequence itself (Wilting & Steenbergen, 2016). These viral and cellular biomarkers (Figure 1.17) can be detected by using viral markers such as HPV DNA detection, HPV oncogenic mRNA and HPV integration markers such as E6/E7. Methylation markers can be detected in both viral and host DNA. Whereas p16, p16/Ki-67 dual staining, mini chromosome maintenance proteins and chromosomal instability markers can be detected in host cells.

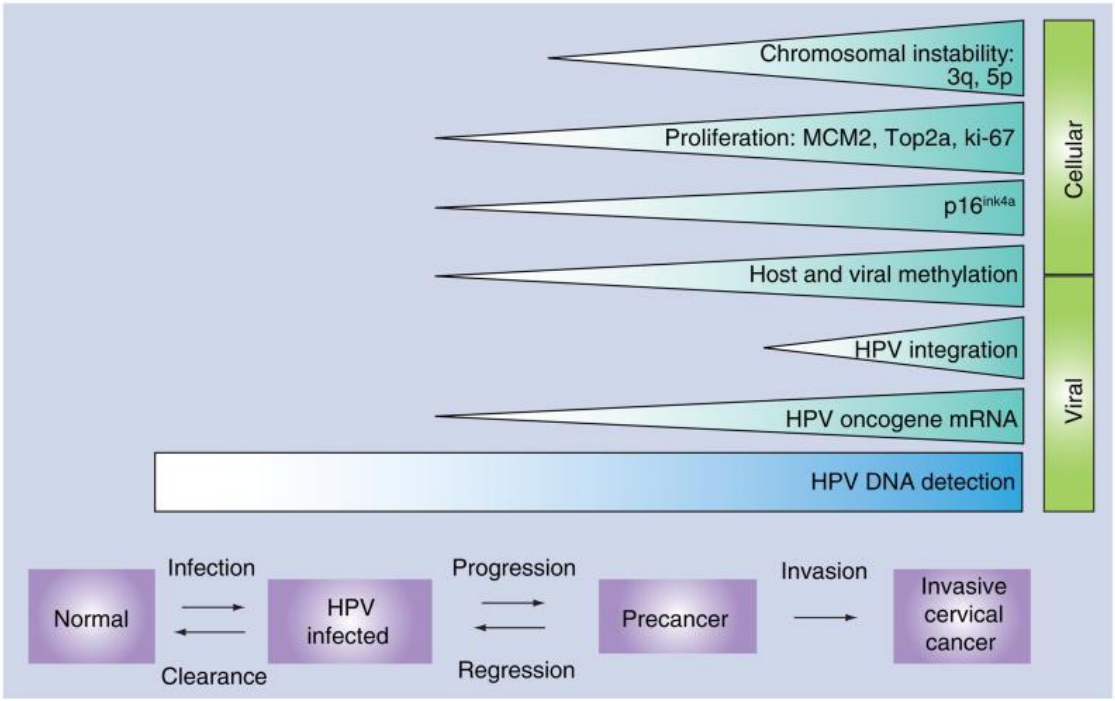


Figure 1.17 Human papillomavirus natural history and cellular and viral biomarkers used in cervical cancer screening (Sahasrabuddhe et al., 2011).

p16 is a 16 kDa protein, encoded by CDKN2A, within the INK4/ARF tumour suppressor locus on Chromosome 9p21 (J. Li et al., 2011). p16INK4A immunostaining is the most used tissue biomarker as an addition to H&E-based diagnosis to differentiate difficult CIN lesions or in the case of a professional disagreement in histological specimen interpretation of cervical pre-cancer and cancer diagnosis (Silva et al., 2017). Expression of the HPV E7 oncoprotein triggers de-methylation of the p16 promoter, which, as a result, causes upregulation of p16 expression (McLaughlin-Drubin et al., 2011). Several studies involving the use of p16 have shown the significance of p16 immunostaining providing more accurate and reproducible diagnostic results in the interpretation of cervical biopsies and patient management (Izadi-Mood et al., 2012; Klaes et al., 2001; Krishnappa et al., 2014; Leeman et al., 2020; Miralpeix et al., 2017; O'Neill & McCluggage, 2006; Stoler et al., 2018).

The monoclonal antibody Ki-67 was first described in 1983 by Johannes Gerdes and colleagues and is used as a marker of proliferation in pathology (Ross & Hall, 1995). Ki-67 is expressed in all active phases of the cell cycle (G1, S, G2, M) except the G0 phase (Popiel-Kopaczyk et al., 2023). As per Keating et al., the expression of Ki-67 is restricted to one-third of the basal layer of the epithelium, but in dysplasia and cancer, it is expanded above the basal and parabasal layers, and the number of positive cells increases (Keating et al., 2001). Ki-67 overexpression was found to be present in 100% of invasive cervical cancer samples (Popiel-Kopaczyk et al., 2023) and is a useful prognostic factor in patients with stage I–II cervical cancer (Tu et al., 2022).

The utility of the p16/Ki-67 immunostaining has been well-established in HPV-related cervical biopsies (Iaconis et al., 2007; Keating et al., 2001; Mandal et al., 2020; Miranda et al., 2023; Walts & Bose, 2008). p16/Ki-67 dual staining was used as a novel biomarker in triaging Pap cytology negative and HPV-positive cervical cancer screening (Petry et al., 2011). A number of studies followed after this showed the use of p16/Ki-67 dual staining using ThinPrep cytology samples (Benevolo et al., 2017; Carozzi et al., 2013; Ikenberg et al., 2013; Wentzensen et al., 2014, 2015; White et al., 2016; Wright et al., 2017).

Nuclear protein DNA topoisomerase type IIa (TOP2A) is responsible for enzymatic uncoupling during the replication of DNA strands, playing a significant role in S-phase regulation (Peres et al., 2016). Gene expression profiling studies have shown increased expression of TOP2A in cervical cancer cell lines and invasive cervical cancer (Martin & O'Leary, 2011), and a few studies have confirmed over-expression of TOP2A in high-grade cervical intraepithelial lesions in cervical cancer (Branca et al., 2006; C. A. Brown et al., 2012; Dixon et al., 2017; Zuberi et al., 2021). A panel of TOP2A/MCM2, p16INK4a and cyclin E1 expression levels was found useful as a panel of biomarkers that allow the identification of cervical lesions with a higher risk for progression to cervical cancer (Del Moral-Hernández et al., 2021).

Minichromosome maintenance complex (MCM) is a family of six proteins (Mcm2–7, molecular masses of 101, 91, 97, 82, 93, and 81 kDa, respectively) with highly conserved amino acid sequences between the six different polypeptides and are essential for cell viability and are needed for the initiation phase as well as for replication fork progression (Kelman & Kelman, 2013). The role of MCMs in cervical cancer and their correlation with the clinical parameters of cervical cancer was explored by Das M et al., 2013. Their study indicated that MCM 2, 4, 5, 6, 7, 10 and RECQL4 were significantly over-expressed in cervical cancer (Das et al., 2013). A strong expression of MCM2 protein in HSIL was observed using Human Transcriptome Array 2.0 and validated by Counter® PanCancer Pathway NanoString Array (Kaur et al., 2019). In a Brazilian study, the expression levels of p16, Ki-67, and MCM7 protein in normal and CIN tissue demonstrated that MCM7 is a more efficient and sensitive marker for assessing disease progression in the uterine cervix (Lobato et al., 2012).

Immunohistochemical staining of 336 cervical cancer tissue microarrays for BCL-2, HER2, CD133, CAIX, and ERCC1 expression showed that these biomarkers could be predictive protein markers for clinical outcomes in early cervical cancer patients treated primarily with radical surgery with or without adjuvant radiation (Jeong et al., 2021). In the independent validation of miRNAs by RT-qPCR of FFPE tissues from hrHPV-positive women, deregulated expression of microRNAs led to the prediction of CIN2+ and CIN3+ (González-Ramírez et al., 2023).

Programmed death ligand 1 (PD-L1) is a protein capable of strongly modulating the adaptive arm of the immune system, and its expression can reduce the immune response in both infectious diseases and cancers (Mezache et al., 2015). PD-L1 is overexpressed in some cervical intraepithelial neoplasia and squamous cervical cancer (Gu et al., 2019; Mezache et al., 2015).

In recent years, numerous review studies (Garrido et al., 2023; Güzel et al., 2021; Jafari et al., 2023) on the available database of biomarker studies have shown that biomarkers have the potential to play an important role in cervical pre-cancer and cancer. These include studies done on KRT17 and CRISP2, ANT3 and FBLN1 (Hao et al., 2021), and EREG (T. Li et al., 2023). Desmoglein-3 (Dsg3) is a transmembrane glycoprotein which is preferably found in desmosomes of keratinocytes in squamous epithelium, and both its loss and upregulation of Dsg3 have been implicated in cancer progression (Viehweger et al., 2022). Dsg3 expression predominates in squamous cell carcinomas of different organs of origin, such as oral carcinoma (Troeltzsch et al., 2022).

The published EU-funded study "AUTOCAST", performed in part by researchers of the CERVIVA consortium, described novel panels of diagnostic and prognostic gene expression biomarkers in cervical cancer (Varga et al., 2017). The study showed the potential of diagnostic panels (PIK3AP1, TP63 and DSG3) to distinguish cytologically normal hrHPV+ women from hrHPV+ women with CIN2+ and the ability of prognostic gene panels (KRT78, MUC5AC, BPIFB1 and CXCL13, TP63, DSG3) to differentiate hrHPV+ CIN1 and carcinoma cases. In order to develop a prognostic or diagnostic antibody-based test that can be performed directly on the woman's histology or cytology slides, it is necessary to investigate the performance of these expression biomarkers at the protein level in tissues of disease. Numerous other biomarkers have been identified in the literature as potential molecular biomarkers for further risk stratification of HPV-positive women or to aid in the prognosis of cervical disease, including but not limited to HEC2, NUF1, p16/Ki-67, MCM5, TP63, DSG3, HPV E1/E4 (Doorbar & Griffin, 2019; Husain & Ramakrishnan, 2015; Tornesello et al., 2013).

Molecular biomarkers can potentially enhance the accuracy and efficiency of screening and triage (Taghavi et al., 2023). Molecular biomarkers may be useful in the identification of HPV-positive women who should be followed more closely than their negative counterparts. The role of HPV oncoproteins E6/E7 in cervical carcinogenesis is described in several studies (Boccardo et al., 2010; Ferrera et al., 2019; Hanahan & Weinberg, 2011; McBride & Warburton, 2017; Moody & Laimins, 2010). These oncoproteins deactivate p53 and pRB host proteins involved in tumour suppression (Boccardo et al., 2010; Moody & Laimins, 2010), causing genomic instability and cell mutation. Thus, detecting these oncoproteins can help detect those lesions that can potentially progress to cervical cancer. Currently, the OncoE6 Cervical Test is available in some countries to detect oncoprotein E6 expressed by HPV16

and HPV18, but it has a varying sensitivity, and insufficient evidence has been generated through large clinical trials. In addition to these molecular tests, there is a move to detect viral load due to evidence presented by (Basu et al., 2016). Studies by (Basu et al., 2016; Zhou et al., 2024) show viral load measurement is directly proportional to increased HSIL lesions. The most recent publication done by Taghavi et al, 2023 suggested that triaging methods using molecular technology such as partial and extended genotyping, methylation tests, detection of E6/E7 proteins, and hrHPV viral load in the same sample as the hrHPV test may improve the prediction of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and invasive cancer, offering more precise, efficient, and cost-effective screening regimes.

1.9 HPV vaccination and its impact on cervical screening

The extensive knowledge of cervical cancer pathogenesis and the role of HPV in the development of cervical cancer and pre-cancer has led to the development of prophylactic HPV vaccination. These vaccines contain HPV L1 self-assembling virus-like particles (Crosbie et al., 2013). The HPV vaccine was first developed by the University of Queensland in Australia by Professors Ian Frazer and Jian Zhou (Frazer, 2019). Currently, there are three bivalent, two quadrivalent and one nonavalent prophylactic HPV vaccine available worldwide (WHO, 2022). Bivalent vaccine Cervarix (GlaxoSmithKline; Wavre, Belgium), Cecolin (Xiamen Innovax Biotech Co Ltd; Xiamen, Fujian Province, China), and Walrinvax (Walvax Biotechnology Co; Kunming, Yunnan Province, China) target the two most carcinogenic types, HPV16 and HPV18 (Castle, 2023). Two quadrivalent vaccines, Gardasil (Merck & Co, Kenilworth, NJ, USA) and Cervavac (Serum Institute of India (SII), Pune, India), target HPV16, HPV18, and HPV6 and HPV11 (Castle, 2023). The Gardasil 9 (Merck) nonavalent vaccine targets HPV6, 11, 16, 18, 31, 33, 45, 52, and 58.

Some of the earliest evidence of the benefits of HPV vaccination comes from Scottish data, which showed women undergoing cervical screening from the age of 20 showed a dramatic reduction in preinvasive cervical disease (T. Palmer et al., 2019) among those HPV vaccinated women (T. Palmer et al., 2019). Clinical trials of both the bivalent and quadrivalent vaccines demonstrated a level of cross-protection against certain closely related HPV types (Donken et al., 2018; Kavanagh et al., 2014; Mesher et al., 2018). A systematic review of the cross-protective effect of HPV vaccines based on data from 23 randomized clinical trials and real-world evidence in 33 observational studies shows that cross-protection is inconsistent across non-vaccine HPV types and is primarily driven by HPV 31 and 45 (D. R. Brown et al., 2021). The Costa Rica HPV Vaccine Trial has documented cross-protection of the bivalent HPV vaccine against HPV31/33/45 for up to 7 years after vaccination (Tsang et al., 2020).

Additionally. studies from Sweden (Lei et al., 2020), Finland (Luostarinen et al., 2018)s, Denmark (Kjaer et al., 2021), England (Falcaro et al., 2021) and Scotland (T. J. Palmer et al., 2024) showed that HPV vaccination significantly reduces the incidence of cervical cancer. However, the success of vaccination depends on the individual country's national immunisation programme, choice of the vaccine type, enrolment policy and the uptake. The national HPV vaccination programme implementation has progressed up to 80% in high-income countries, but the rate of these programmes is 41% in low and middle-income countries (Bénard et al., 2023). In a review by Shankarnarayan et al., 2016 of a pooled analysis of 20 eligible studies involving 140-million-person years of follow-up after vaccination, HPV 16 and 18 infections showed a significant decrease between the pre-and post-vaccination periods in high-income countries with 50% or more HPV vaccination coverage by 68 per cent (Sankaranarayanan et al., 2016). A systematic review and meta-analysis including data from 60 million individuals and up to 8 years of post-vaccination follow-up showed compelling evidence of the substantial impact of HPV vaccination programmes on HPV infections and CIN2+ among girls and women and on anogenital warts diagnoses among girls, women, boys, and men (Drolet et al., 2019). An updated systematic review of the quadrivalent HPV vaccine demonstrates statistically significant decreases in vaccine-type HPV genital, oral and anal infections and prevention of recurrent respiratory papillomatosis, anal and cervical lesions (W. Wang et al., 2022).

More than a decade has passed since the first prophylactic virus-like particle-based HPV vaccine was registered (Brotherton and Bloem., 2018). In 2018, the WHO recommended that all countries proceed with the implementation of the HPV vaccine. The success of these vaccination programmes depends on their safety, immunogenicity, duration of protection, effectiveness, coverage, and ability to overcome the barriers to coverage, such as awareness, implementation, accurate data, finance, and performance of other genotypes.

In Ireland, the vaccination programme using Gardasil 4 was rolled out through schools, starting in 2010 in those aged 12–13 years in the first year of secondary school and age equivalent in non-secondary schools). A catch-up vaccination followed this commenced in 2011/2012 for girls in the sixth year of secondary school and their age equivalent in non-secondary schools (HPSC, 2013). Early evidence shows that HPV vaccination has reduced the prevalence (3.7% to 1.5%) of high-grade cytology among 25-year-old women (Rourke et al., 2024). As of September 2019, Gardasil 9 is the HPV vaccine currently used in Ireland (HPRA, 2020).

1.10 Chromosomal instability and biomarkers in cervical cancer and precancer

Chromosomal instability is found in over 90% of solid tumours and in blood cancers and can occur due to the gain or loss of whole chromosomes (aneuploidy) or gain of an extra set of chromosomes

(polyploidy) known as numerical instability or gain or loss of chromosome segments due to deletion, amplification, inversion, and translocation known as structural instability (Hosea et al., 2024). Figure 1.18 shows the causes of chromosomal instability, which leads to errors in DNA replication.

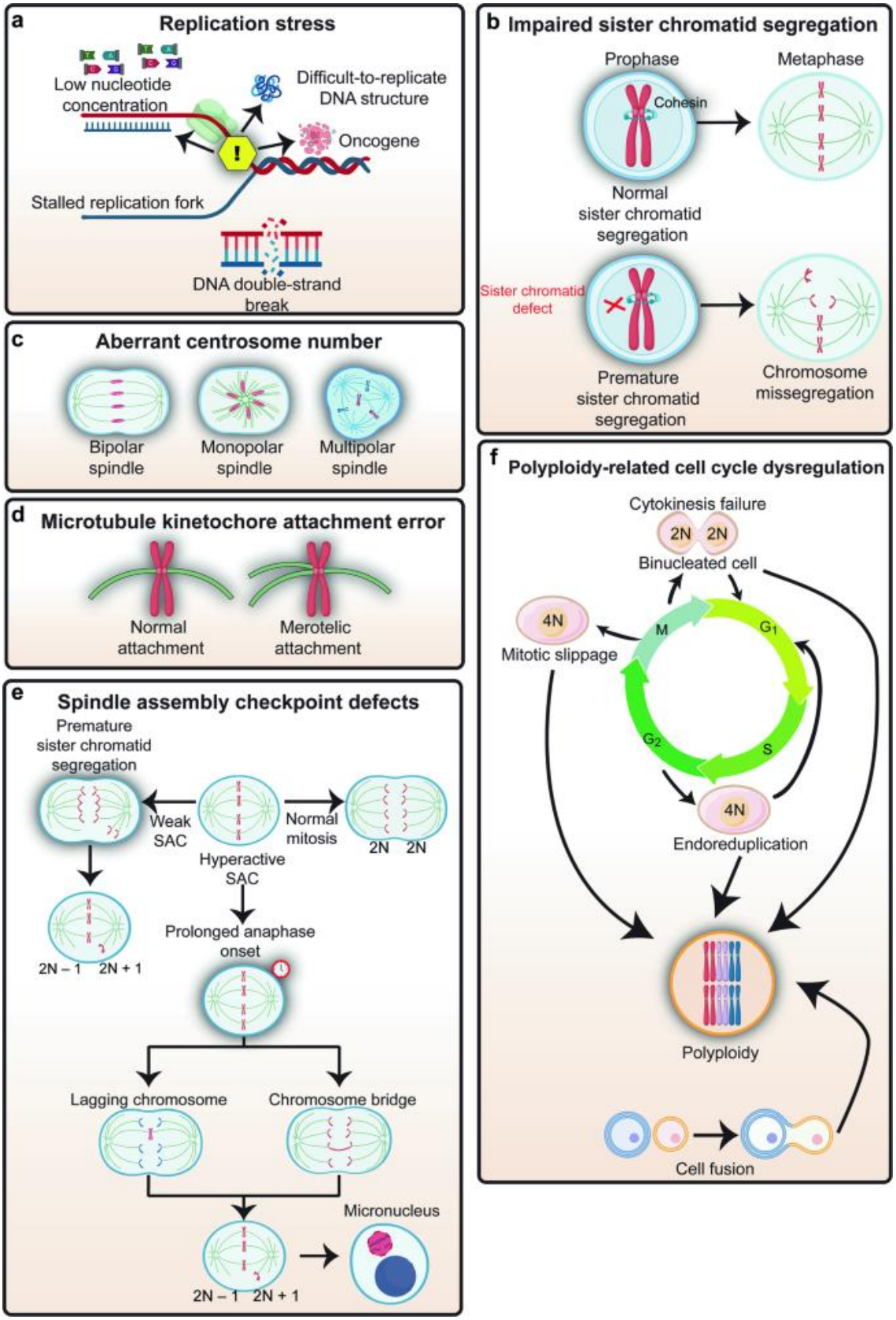


Figure 1.18 Causes of chromosomal instability. a) Replication stress leads to stalling and collapse of replication forks, which results in double-strand breaks. b) Sister chromatid defect allows premature separation of sister chromatids before full alignment, leading to chromosome missegregation. C)

Aberrant centrosome numbers, such as monopolar spindle and multipolar spindle, could lead to aneuploidy. d) Microtubule kinetochore attachment error causes failure to form bi-orientation, where each kinetochore is attached to microtubules from only one spindle pole, leading to chromosome missegregation. e) Aberrant SAC could lead to aneuploidy, as weakened SAC causes premature chromatid separation, while hyperactivated SAC results in a lagging chromosome. f) An extra set of chromosomes, as seen in polyploidy, could arise from cytokinesis failure, mitotic slippage, or endoreduplication (Hosea et al., 2024).

HPV oncoproteins E6 and E7 are known to induce DNA damage, centrosome abnormalities and chromosomal segregation defects, thereby leading to chromosomal instability (Duensing & Münger, 2004; Moody & Laimins, 2010). As per Wilting & Steenbergen; 2016, a transforming infection by HPV contributes to the deregulation of the DNA methylation machinery, gives rise to DNA methylation mediated silencing of tumour suppressor genes and, along with HPV-induced mitotic defects, results in chromosomal instability and aneuploidy (Figure 1.19).

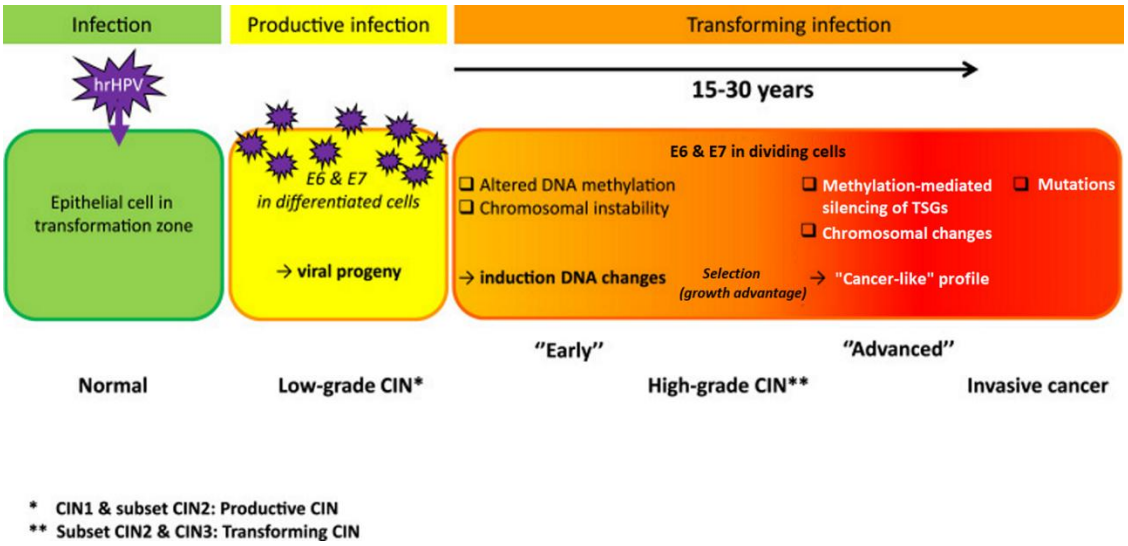


Figure 1.19 Schematic representation of HPV-mediated cervical carcinogenesis. CIN: cervical intraepithelial neoplasia; TSG: tumour suppressor gene. (Wilting & Steenbergen, 2016).

Chromosomal gains at 3q26, 5p15 and 20q13 have been described in cervical precancer and cancer (Luhn et al., 2013). 3q gains were most frequently found in HPV16-positive cervical squamous cell carcinoma of the cervix (Thomas et al., 2014). The human TP63 gene is located on chromosome 3q27-28 within a region that is frequently amplified in squamous cell carcinomas (Moll & Slade, 2005), head and neck cancer (Hibi et al., 2000), and nasopharyngeal carcinoma (Crook et al., 2000).

Gain of 17q was more common in HPV18-induced adenocarcinoma compared to other HPV types, whereas other common alterations include loss of chromosome 3p and 11q, observed in 36% and 33% of squamous cell carcinoma (Wilting & Steenbergen, 2016). The HPV-16 E7 oncoprotein contributes to the induction of genomic instability by induction of centrosome duplication errors (Münger et al., 2004). Chromosomal instability can be assessed by indicators such as lagging chromosomes, chromosome bridges, micronuclei, aneuploidy, and polyploidy (Figure 1.20).

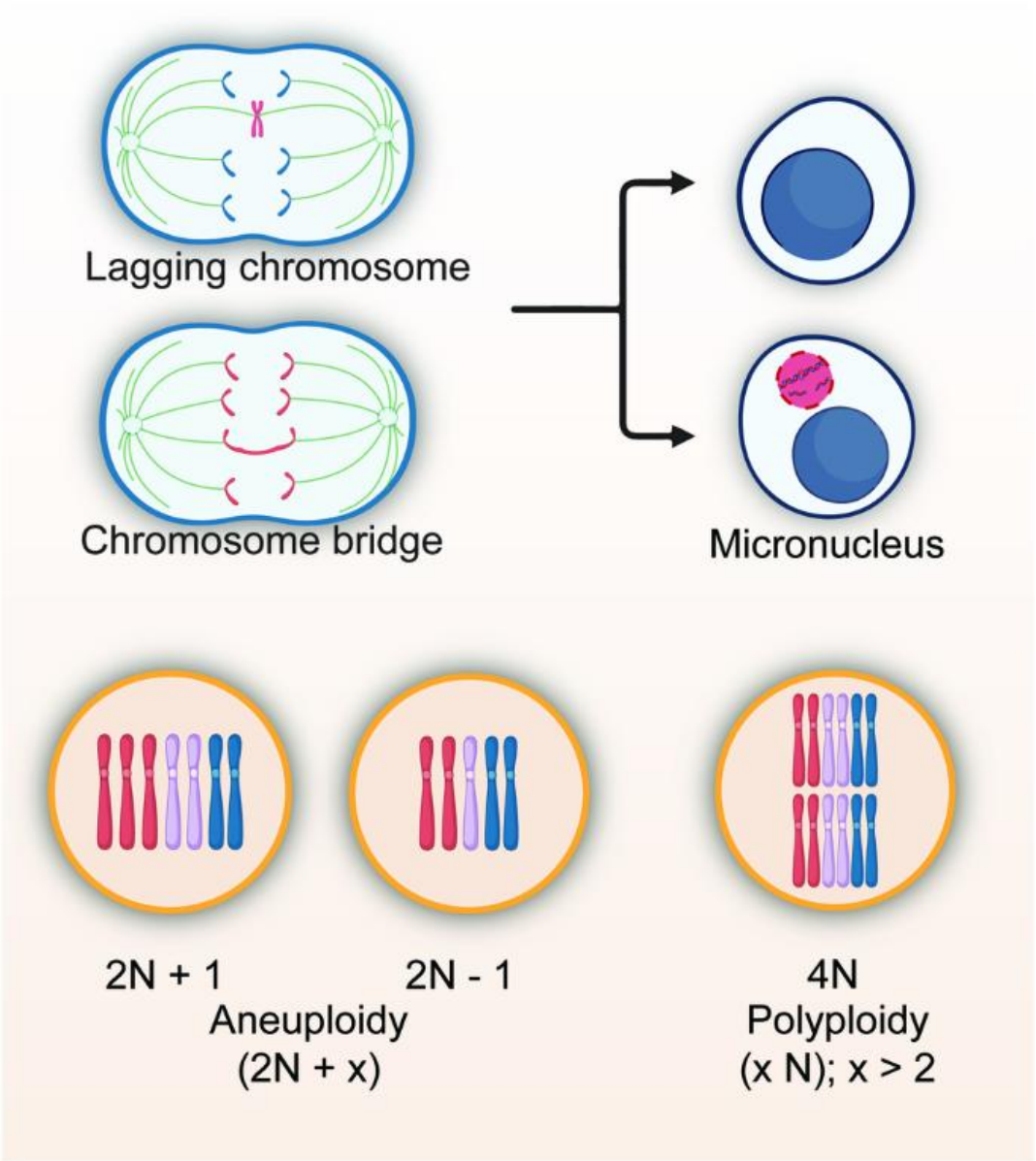


Figure 1.20 Indicators of chromosomal instability. Examples of indicators commonly used to assess chromosomal instability include lagging chromosomes, chromosome bridges, micronuclei, aneuploidy, and polyploidy (Hosea et al., 2024)

Chromosome 17 loss was reported in a few studies. In 2001, Herrington et al; concluded that the loss of sequences on the short arm of chromosome 17 is a late event in squamous carcinoma of the cervix (Herrington et al., 2001). Whereas, as per Olaharski et al., tetraploidy and chromosomal instability occur during the early stages of cervical carcinogenesis, causing the formation of aneuploidy frequently involving the loss of Chromosome 17 (Olaharski et al., 2006). Human EGF receptor 2 (HER2/neu/ErbB2) is a protein involved in normal cell growth and is located on the long arm of human chromosome 17 (17q12) (Brandt-Rauf et al., 1994). Somatic mutations in this gene were observed in 5% of 79 primary squamous cell carcinoma of the cervix (Ojesina et al., 2014). In another study, an association between expression of HER-2/neu and poor prognosis, metastatic potential and aggressive biological behaviour was reported in 200 cases with a diagnosis of cervical intraepithelial neoplasia, squamous cell carcinoma, adenocarcinoma (M Vijayalakshmi et al., 2022; Varshney et al., 2020). Immunohistochemistry in tissue microarray demonstrated the prevalence of HER2 in the cell membrane in 3.6% of 412 human cervical cancer tissues (Oh et al., 2015). Various percentages of HER2 overexpression have been reported in breast, gastric, oesophageal, ovarian, lung and other cancers.

Aneuploidy and chromosomal instability are characteristic of many human cancers and have been implicated in the development of high-grade, invasive tumours (Giam & Rancati, 2015). Aneuploid cells, i.e. cells containing an abnormal chromosome number, are common in cervical and other cancers (Weaver & Cleveland, 2008). In addition, it has been demonstrated that many of these cancers also show evidence of chromosomal instability, a phenomenon in which frequent rates of missegregation occur at each mitotic division, leading to chromosomal loss or gain, thus further altering the chromosomal number in these cancer cells. The Ndc80 complex, which forms part of the outer kinetochore, is composed of two heterodimers, Hec1(Ndc80)-Nuf2 and Spc24-Spc25 and plays a crucial role in kinetochore-microtubule interactions (Malvezzi et al., 2013). Studies suggest that Nuf2/Hec1 depletion results in a damaged outer kinetochore with decreased microtubule attachment (Ciferri et al., 2005). Redundant or “extra” molecules of the NDC80 complex concentrate in the cytoplasm, where they bind and sequester confirmed and putative loop ligands together with their binding partners. This prevents Nuf2/Hec1 from regulating microtubule–kinetochore attachments and provokes chromosomal defects in daughter cells (Ustinov et al., 2020). As a result, the dysregulated expression of these sequestered proteins promotes further proliferation of aneuploid cells, some of which eventually become malignant. Overexpression of Hec1 and Nuf2 is reported in gastric, colorectal and pancreatic cancer (P. Hu et al., 2015; Meng et al., 2015; Qu et al., 2014; H. Wang et al., 2015). Overexpression of these genes causes hyperactivation of these genes (Diaz-Rodríguez et al., 2008), leading to prolonged tetraploidy.

Based on the above background knowledge, a previously unpublished study done in our research lab, 'Altered expression of kinetochore proteins Nuf2 and Hec1 in cervical pre-cancer and cancer,' found a direct biological interaction between high-risk HPV oncoproteins and the nuf2-Hec1 complex and chromosome aneuploidy. The study concluded that Nuf2 and Hec1 are overexpressed in premalignant tissues (Figure 1.21).

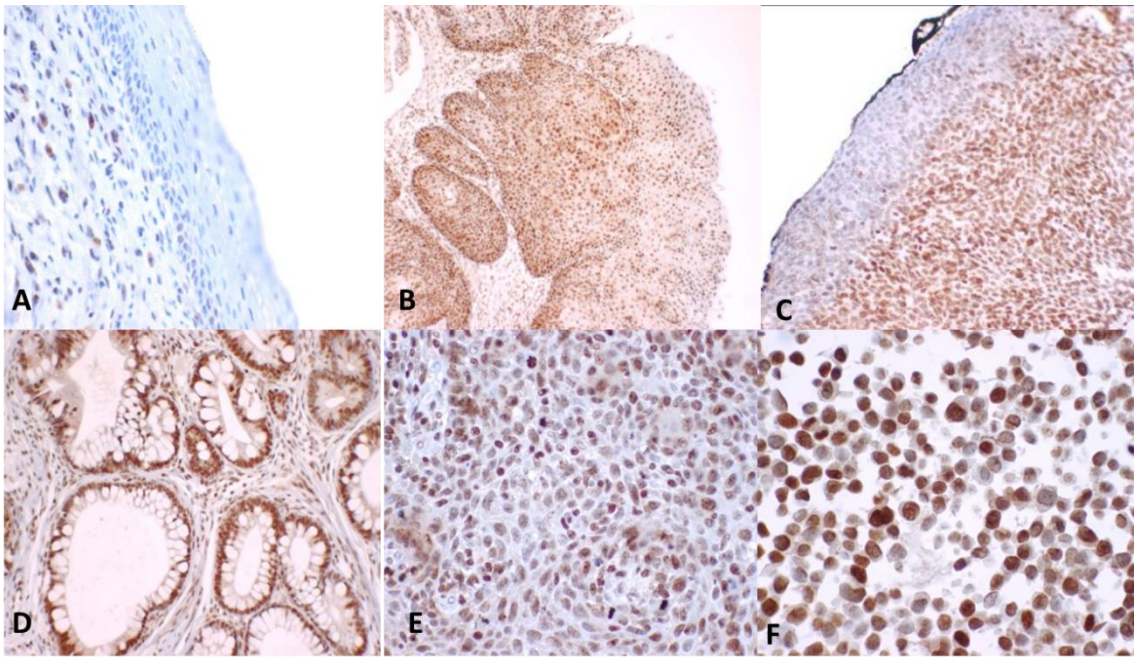


Figure 1.21 Immunohistochemical staining for HEC1. A) Normal cervical epithelium B) CIN2 C) CIN3 D) cGIN E) SCC) HeLa cells (Atbury K, Martin C, personal communication-unpublished).

1.11 Information about the CERVIVA consortium

The Irish Cervical Screening Consortium was established by Professor John O'Leary and Professor Cara Martin in 2005. CERVIVA collaborates with various academic institutions, hospitals, and industrial partners to carry out research projects in the area of HPV-associated diseases. The consortium has been involved in research projects related to the development of biochip technology, virtual slide technologies, the impact of vaccination, social impact and acceptance of vaccination, education and outreach programmes, HPV in immunodeficient patients, the development of automated screening technologies, HPV primary screening and triage methodologies.

The current project is an extension of the CERVIVA CARG project and will focus on HPV genotyping, diagnostic, and prognostic biomarkers in cervical cancer and pre-cancer.

1.12 Hypothesis

I hypothesise that different biomarker approaches will improve the performance of screening and diagnostics for cervical pre-cancer and cancer.

To that end, my project is focussed on two specific areas: A. cervical screening and B: cervical pre-cancer /cancer diagnostics and prognostic biomarkers.

The thesis is divided into two parts.

Part A will focus on the use of extended genotyping as a biomarker for triage in HPV-positive women from primary cervical screening.

Part B will focus on specific biomarkers of chromosomal abnormalities and cell cycle dysregulation in cervical pre-cancer that have potential utility in cervical cancer diagnostics and prognostics.

1.13 Aims

For Part A: the specific aims of this project were to:

- To detect specific high-risk HPV genotypes using the BD Onclarity™ HPV Assay in HPV- positive Primary Screening Population. (Chapter 3)
- To establish the absolute risk associated with individual HPV types or groups of HPV subtypes of CIN2+ disease. (Chapter 3)
- To establish the clinical performance of the BD Onclarity™ HPV Assay for detection of CIN2+ and CIN3+. (Chapter 3)
- To compare the clinical performance of extended genotyping to other triage methods (Chapter 4) as a triage method for colposcopy referral and detecting high-grade cervical abnormalities.
- To assess the impact of extended genotyping as an alternative to current triage methods (Chapter 4) on colposcopy referral rates and detection of CIN2+ disease.

For Part B: The specific aims were

- To technically validate and evaluate the performance of NUF2 and HEC1 biomarkers in cervical pre-cancer. (Chapter 5).
- To evaluate the expression of Nuf2 and Hec1 biomarkers in tissue samples. (Chapter 5)
- To establish the ploidy status in cervical pre-cancer to confirm that mitotic spindle formation is dysregulated in cervical pre-cancer.

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Chapter 2 Materials and Methods

Overview

This chapter details all the Materials and Methods used in this project. The chapter is divided into two sections:

- **Section A:** Methodology section for part 1 of the project:- HPV genotyping using the BD Onclarity HPV Assay.
- **Section B:** Methodology section for part 2 of the project: - Altered expression of kinetochore proteins NUF2 and HEC1 in cervical pre-cancer and cancer.

The subsequent results chapters will briefly refer to these methods where applicable.

2.1 Section A: Methodology section for Part 1- HPV genotyping using BD Onclarity HPV Assay

2.1.1 Study Overview

The CERVIVA HPV primary screening study was undertaken in partnership with CervicalCHeck, the Irish cervical screening programme. An overview of the study protocol has been published previously (O'Leary et al., 2018; White et al., 2024). In 2016, CERVIVA (the Irish Cervical Screening Research Consortium), in partnership with CervicalCheck (The National Cervical Screening Programme), undertook the CERVIVA HPV primary screening study. The study was a prospective observational cohort study that aimed to investigate various molecular approaches to triage women with HPV-positive primary screening cervical test results (Figure 2.1). Women attending GPs, family planning clinics, and women's health centres for their routine CervicalCheck smear tests were invited to participate in the study. Women who agreed to participate in the study provided written informed consent. Following routine cytology screening analysis at The Coombe Hospital, the residual smear samples were tested for HPV DNA using the Cobas® 4800 HPV Test (Roche, Pleasanton, CA, USA). HPV-positive samples were then subjected to a series of second-round molecular tests, including methylation biomarkers, HPV mRNA, p16ink4a/ki67, and the test which was one of the focuses of this PhD thesis - **extended HPV genotyping using the BD Onclarity™ HPV Assay** (Figure 2.1). Women enrolled in the CERVIVA HPV Primary Screening Study are being observed prospectively through the CervicalCHeck screening programme for up to 10 years. The follow-up data on women includes subsequent HPV, cytology and histology where available. My PhD study focused on extended HPV genotyping using the BD Onclarity HPV Assay on HPV-positive samples from women enrolled in the study.

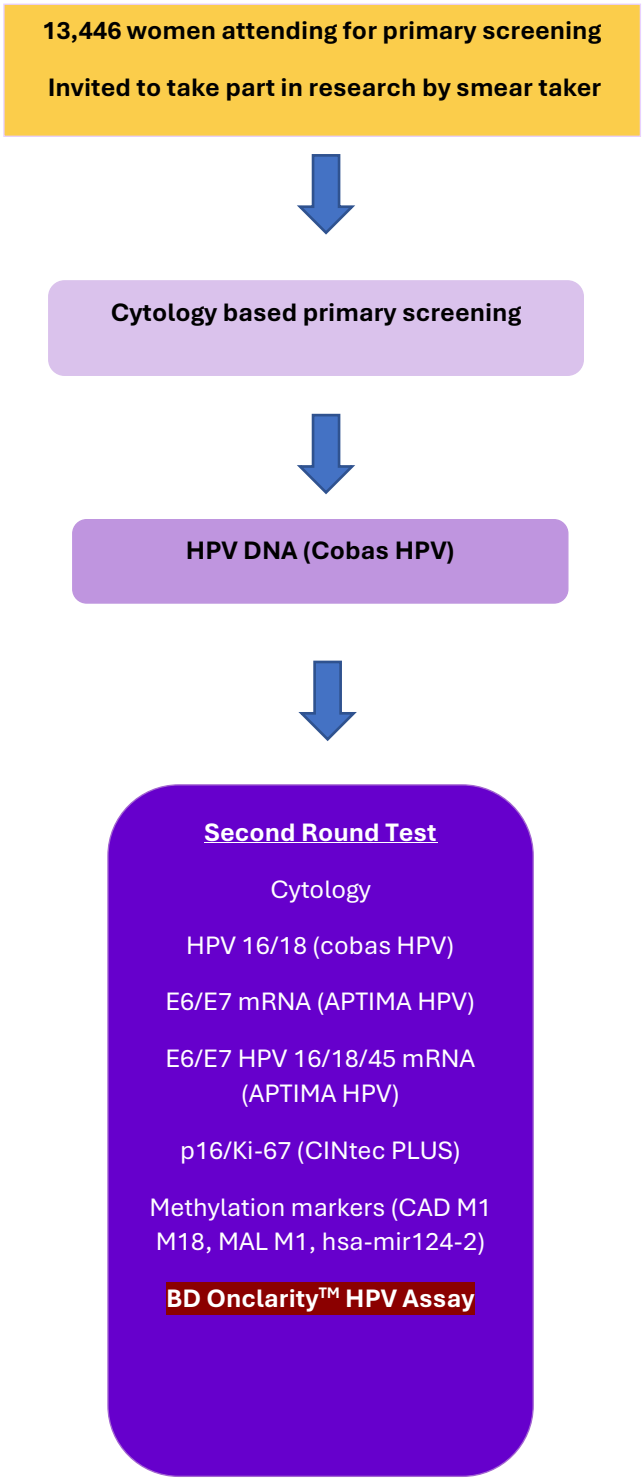


Figure 2.1 CERVIVA HPV Primary Screening Pilot Study Design, including the BD Onclarity Extended Genotyping Assay under investigation in this thesis.

In total, 13,446 women were enrolled in the CERVIVA Primary Screening Study (White et al., 2024). The routine CervicalCheck management protocol for these women at the time of recruitment into the CERVIVA Primary Screening Study (2016-2018) was as follows (CervicalCheck, 2022). Cytology-based screening was performed on ThinPrep samples, and routine follow-up was performed as per CervicalCheck guidelines at that time. Women between the ages of 25 and 44 years were screened at three yearly intervals. Women over 45 years old with two consecutive negative smears were screened every five years. Women with no intraepithelial lesions or malignancy (NILM) cytology results (no abnormality detected) were returned to routine screening at 3 or 5 yearly intervals, depending on age. Women with low-grade squamous intraepithelial lesions (LSIL) or atypical cells of undetermined significance (ASCUS) cytology results were triaged by HPV testing. HPV-positive women were referred to colposcopy, and HPV-negative women were returned for routine recall. Women with HSIL and above cytology results were referred directly to colposcopy. Colposcopy investigations were performed under standard CervicalCheck guidelines (CervicalCheck, 2022). If an area of abnormality was seen during colposcopy, a biopsy was taken. Women referred with a high-grade cytology and a suspected high-grade lesion on colposcopy received excisional treatment. The study was observation and did not alter routine management at the time.

2.2 Ethical Approval

Research Ethics approval for the CERVIVA HPV Primary Screening Study was obtained from the Irish College of General Practitioners Research Ethics Committee. Subsequently, following the introduction of the new Health Research Regulations in Ireland in 2018, a Health Research Consent Declaration was obtained from the Health Research Consent Declaration Committee (www.hrcdc.ie; Ref ID: 19-016-AF2). All participants gave written informed consent for participation in the CERVIVA Primary Screening Study.

Of the 13,446 women enrolled in the CERVIVA HPV Primary Screening Study, 12,771 were eligible to participate. The remaining 675 were excluded for various reasons such as duplicate samples, no vial available for testing, vial rejection, consent not provided, or incomplete consent form. HPV DNA analysis was performed on all cases using the Cobas 4800 HPV Test. Of these, 1,863 Cobas HPV-positive cases were available for this PhD work, which focused specifically on extended genotyping analysis with the BD Onclarity HPV Assay. As a control, 108 Cobas® 4800 HPV-negative cases were included, which consisted of two randomly selected Cobas HPV-negative samples per batch. So, a total of 1971 cases were used as a study population for this part of the study.

2.3 BD Onclarity HPV Assay

The FDA-approved BD Onclarity (Becton & Dickinson, Sparks, MD, USA) HPV assay uses real-time PCR technology to test amplified DNA for the qualitative detection of high-risk types of HPV (Bottari & Iacobone, 2019). The assay detects all high-risk HPV types (16, 18, 31,33,35,39,45,51,52,56,58,59,66 and 68) and provides the capability for individually genotyping six high-risk HPV types (16,18,31,45,51,52) and three genotype groups (Group P1**:** 33/58, Group P2: 35/39/68 and Group P3: 56,59/66).

The BD Onclarity HPV Assay is based on two main processing steps (BD Onclarity™ HPV Assay, 2018):

1. Automated specimen preparation to homogenise the matrix, lyse cells and extract cellular DNA.
2. PCR amplification of target DNA using sequencing primers and fluorescently labelled detector probes for both HPV and human beta-globin

Human beta-globin amplification and detection are included as an internal control in the BD Onclarity HPV Assay to differentiate HPV-negative specimens from those that do not exhibit HPV signal due to insufficient cellular material in the specimen. The human beta-globin serves as an internal control for the entire test by concurrently assessing specimen processing, extraction and amplification. The BD Onclarity HPV Assay uses HPV target regions for primers and probes (E6/E7 oncogenes) that provide robust detection of genotypes (Tjalma & Depuydt, 2013), reducing the potential risk for lack of detection due to nucleic acid deletions or mutations.

The automated specimen preparation for the BD Onclarity HPV Assay is completed in the BD pre-warm Heater and BD Viper LT System. Cervical specimens are extracted using the BD Fox Extraction reagent to release cellular DNA. The purified cellular DNA solution from each extracted specimen is transferred into PCR tubes containing PCR reagents, which are then sealed to prevent contamination.

The BD Onclarity HPV Assay reagents are provided lyophilised in three individual PCR tubes that can detect 14 high-risk HPV genotypes and a specimen-derived internal control, consisting of a fragment of DNA from the human beta globin gene. These genotypes are reported individually (16, 18, 31,45, 51, 52) or as a genotype group (Group 1: 33/58, Group 2: 59/56/66, and Group 3: 35/39/68). Each of the three PCR tubes contains specific oligonucleotide sets to detect HPV genotype DNA and an oligonucleotide set to detect a region of the human beta globin gene.

2.4 The Principle of Real-Time PCR

Real-time PCR is the technology that merges the polymerase chain reaction chemistry with fluorescent reporter molecules to monitor the production of amplification products during each cycle of PCR

reaction (Navarro et al., 2015). Using real-time PCR technology, the detection of target DNA is accomplished using TaqMan DNA probes (Figure 2.2) that include a fluorescent dye at the 5' end and a quenching molecule at the 3' end of the oligonucleotide (Liu et al., 2009). TaqMan probes derive their fluorescence signal from the hydrolysis of the probe by the Taq polymerase 5' to 3' exonuclease activity. During amplification, hydrolysis separates the fluorescein moiety from the quenching dye and results in an increase in the fluorescence signal (Rasmussen, 2001). A quencher will absorb the energy from the first reporter and emit it as heat rather than light, leading to a decrease in the fluorescent signal. A fluor will absorb the energy and emit it at another wavelength through fluorescence resonance energy transfer (FRET), resulting in decreased fluorescence of the energy donor and increased fluorescence of the energy acceptor. The change in fluorescence is proportional to the accumulation of PCR product (Didenko, 2001). The BD Onclarity HPV Assay uses 15 probes labelled with one of four fluorescent dyes. Each dye is paired with one of two Black Hole Quencher (BHQ) molecules. Fluorescent detection of amplification occurs in four separate optical channels of the BD Viper LT system.

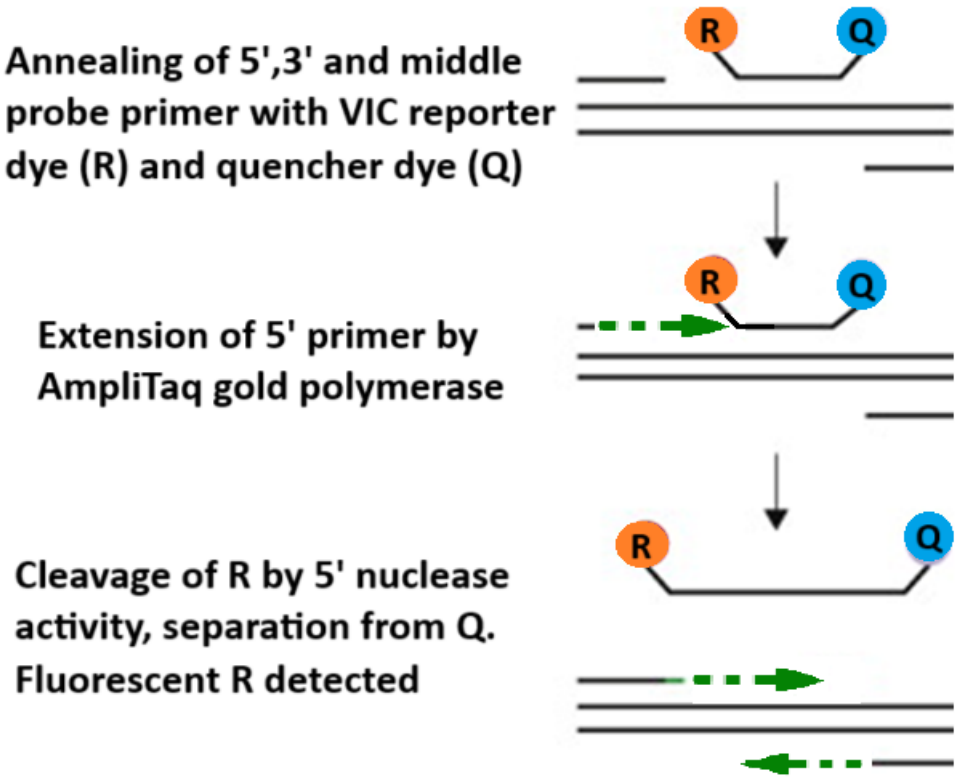


Figure 2.2 Schematic of the TaqMan Quantitative Polymerase Chain Reaction Assay (Liu et al., 2009).

2.5 Instrument set-up and processing steps

2.5.1 Sample Preparation for BD Onclarity HPV Assay

Frozen aliquots of the residual cervical samples collected in PreservCyt were thawed for 1-1.5 hr. Samples were vortexed, and 500 µl of the sample was transferred into a labelled BD Onclarity LBC Diluent tube containing LBC Specimen Diluent. The diluent tube was inverted 3-4 times to ensure the sample and diluent were appropriately mixed.

2.5.2 BD Onclarity HPV Assay Set Up and Quality Control

Each run of 30 samples included a positive and negative control provided separately in the assay kit and 28 samples. Controls were prepared as follows:

One BD Onclarity HPV Positive and one BD Onclarity HPV Negative Control were barcoded and added for every batch of 30 samples. These controls are provided as part of the BD Onclarity HPV Assay kit, in addition to an internal control - the human β-globin is measured in all samples. Controls were prepared by pouring the entire content of the diluent tube into each control tube. Control tubes were inverted 3-4 times to make sure that the controls and the diluent were appropriately mixed. Controls and specimens were pre-warmed for 1 hr using a BD Pre-warmer Heater. The batch run was carried out using BD Viper™ LT, Instrument:1, VLT0209 (Figure 2.3 A) for BD Onclarity HPV Assay according to the manufacturer's protocol (Becton, 2020).

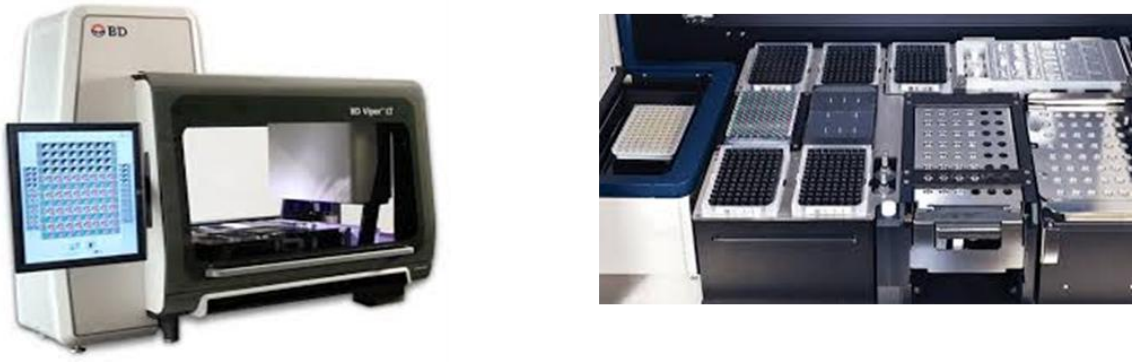


Figure 2.3 (A) BD Onclarity Viper LT™ Instrument (B) BD Onclarity Viper LT reagent platform
Adopted from BD Viper™ LT Instrument User's Manual BD Onclarity™ HPV Addendum (Becton, 2020).

2.5.3 Processing steps for the specimen and creating a rack on the BD Viper LT

The "Rack Operation" icon was clicked from the home screen, and the "Sample Login" icon was clicked to create a new rack. The Rack barcode was scanned, and the HPV assay was selected from the

dropdown menu. The positive and negative QCs prepared as per section 2.4.2 (positions A1 and B1) were scanned. Position A1 was clicked, and then the 2D barcode on the tube was scanned with the blue positive control (Blue cap tube). The process was repeated for position B1 and the negative control (white cap tube). The lot number of the diluent tube was entered by scanning the diluent tube box barcode. Sample entry was done from position C1 by manually entering the specimen number.

2.5.4 Pre-warm Procedure Steps

The Scanned BD Viper LT Specimen Rack with controls and the specimen was inserted into the BD Pre-Warm Heater. The BD Onclarity HPV Assay pre-warm protocol was selected on the BD Viper LT Instrument for 1 hour. The BD Pre-warm heater pre-warms the specimens and controls automatically according to the BD Onclarity HPV Assay pre-warm protocol. The BD Viper Specimen Rack was removed after 1 hour from the heater and loaded into the BD Viper LT instrument.

2.5.5 Reagent and Instrument Set Up

The system startup and maintenance procedures followed the instructions in the BD Viper LT manual BD Viper™ LT Instrument User's Manual BD Onclarity™ HPV Addendum (Becton, 2020). The Rack Login Display was accessed to log in to the rack barcode, and the HPV test was selected. The "Rack Operations" icon was accessed from the home screen. A new rack was created by clicking the "Modify Rack" icon. The lot number was entered during "Rack Login", followed by clicking on the "extraction tube" icon to see the layout of the extraction tube rack. The Positive and Negative Control tubes in the first two positions (A1 and B1) and the HPV LBC Diluent tubes lot number were logged in. Specimen tubes were logged in by typing each study number in the Specimen Login window. Extraction tube QC information was logged in by tapping the "extraction lot" button, and extraction tubes were loaded where indicated on the Extraction Tube Lot Login display. The plate layout button was tapped to view the Plate Layout Display. The colour-coded PCR tubes were loaded into the PCR Plate. PCR tubes were colour-coded as follows:

- Blue= BD Onclarity G1 PCR Tubes.
- Green= BD Onclarity G2 PCR Tubes.
- Orange=BD Onclarity G3 PCR Tubes.

The extraction rack was assembled with BD Fox Extraction Tubes with lot numbers, and placed in the instrument (Figure 2.3 A). Other accessories, such as a PCR Extraction Reagent Trough with a piercing Tool, tip boxes, tip waste bin, liquid waste bottle, and sealing plate, were organised in the reagent platform (Figure 2.3 B) per the manufacturer's instructions.

Final checks were done to ensure specimens had been logged in and pre-warmed and the BD Viper LT instrument had been prepared. The run was initiated by tapping the start run" button on the main status display.

2.6 Result Interpretation

Quality control: The run must pass initial QC checks and be deemed valid for any further analysis to be completed. A QC failure is flagged if any one of the positive or negative controls fails to pass the QC, resulting in no results for the whole batch.

The BD Onclarity HPV Assay uses the real-time polymerase chain reaction to detect the presence of Human Papillomavirus (HPV) in clinical specimens. All calculations are performed automatically by the BD Viper LT software. The presence or absence of clinically relevant HPV DNA is determined by the PCR threshold cycle (Ct) at which the signal crosses a pre-established threshold. The assay extracts, amplifies and detects a fragment of the human beta globin gene as an internal control to assess specimen processing, extraction, and amplification and to indicate the presence of PCR inhibitors. The human β-globin gene served as the internal control for sample adequacy and assay performance. BD Viper LT software automatically performed the interpretation of results. The presence or absence of clinically relevant HPV DNA was determined by the number of PCR cycles (CT), which was then compared with a pre-established threshold. The positivity threshold was 38.4 CT for HPV16 and 34.2 CT for other HPV types and the internal control. Specific genotypes and combined genotypes appeared in columns. Each PCR well include an internal control, which validates the reaction. Genotypes and wells specifications are as follows:

- G1 Blue= 16, 18, 45
- G2 Green= P1 (33 and 58), 31, P2 (56, 59 and 66)
- G3 Orange= 51, 52 P3 (35, 39 and 68)

Results were printed and signed for each batch with the date and initials of the operator. The instrument was maintained by cleaning it down using 70% isopropanol, 3% (w/v) hydrogen peroxide (H_2O_2), and soaking racks in 1% (v/v) sodium hypochlorite (bleach). Care was taken to follow the manufacturer's instructions for using hydrogen peroxide or bleach with water.

2.7 Section B: Methodology Section for Part 2: - HISTOCERV: Prognostic and Diagnostic Biomarkers for Cervical Cancer Screening

The second part of this thesis focuses on evaluating novel biomarkers for use in cervical cancer diagnosis/prognosis. The methodology that follows relates specifically to that study.

2.7.1 Study Design

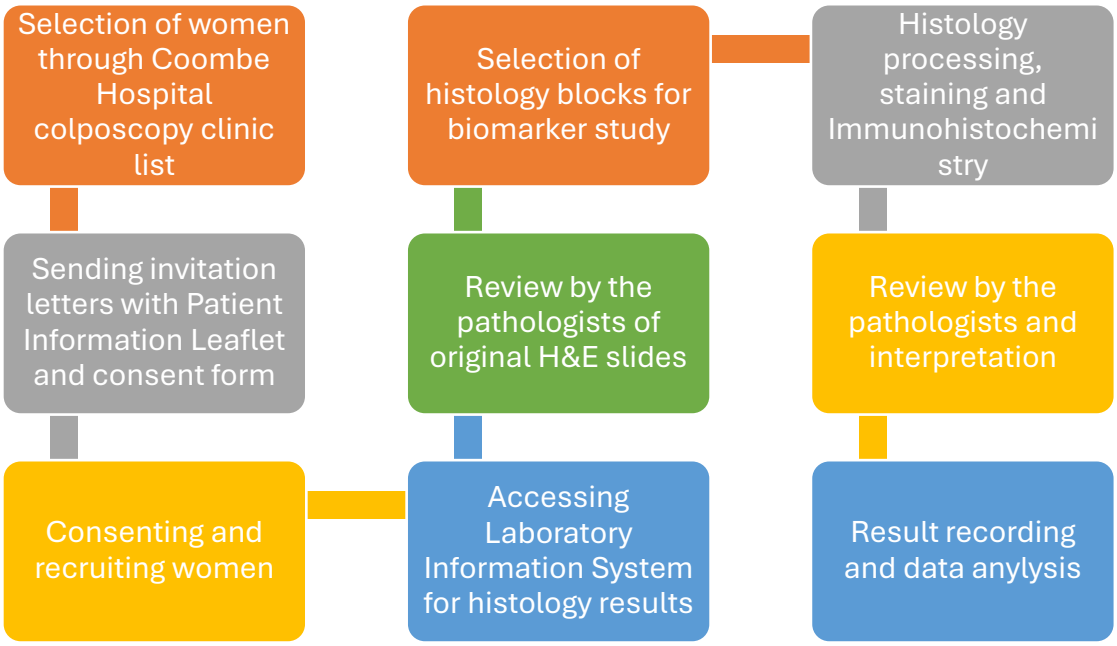


Figure 2.4 Process flow of HistoCerv Biomarker Study

Women attending the colposcopy clinic at the Coombe Hospital for a LLETZ treatment were invited to participate in the HistoCerv study (Figure 2.4). Pregnant women and women with previous history of cervical cancer or pre-cancer were excluded from this study. An invitation letter (Appendix I- HistoCerv Invitation letter), a consent form (Appendix II-Histocerv consent form), a copy of the patient information leaflet (Appendix III- HistoCerv patient information leaflet) was sent to the women before their scheduled appointment. Upon arrival and registration at the colposcopy clinic, eligible patients were asked if they had a chance to review the information sent and if they would like to speak to the research team for further information. The researcher then spoke to each patient individually and privately, explaining the study and answering any questions the patient may have. Once the woman was happy to participate, she was asked to sign a consent form. A copy of the signed consent form was placed in the patient files. CERVIVA maintained the original form in a locked cabinet, and the second copy was given to the woman for her records. A sticker containing the Study Name (HistoCerv 2019) and unique study identifier was placed on the three copies of her consent form. The code for

the unique identifier was kept in a file in a restricted access folder on the hospital's common G drive. This file was the HistoCerv Study Master File, into which clinical data from the Laboratory Information System (LIS) was recorded. A coded version of this file (The Study Database) was used in the Molecular Pathology Laboratory PC to record the histology results if a histology sample was taken during colposcopy. Updates to the data in the Study Master File were done on the G drive every month, and the Study Database was updated from this. All data was held in concordance with GDPR.

2.7.2 Study Cohort

Approximately 332 invitations were sent between July 2022 and December 2023. In 55 cases, there was either no show or no cover for the recruitment, and in a few instances, appointments were rescheduled. In 38 cases, women declined to participate in the study. Clinical recruitment resulted in the enrolment of 239 women in the HISTOCERV biomarker project. Out of these 239 women, 205 had LLETZ procedures performed and were eligible for the biomarker study. The histology breakdown consisted of 123 CIN1, 35 CIN2, and 42 CIN3. The remaining five consisted of no CIN, no results available, no diagnostic, and no slides available for the review. Most cases were CIN1 (60%) comprising of small LLETZ biopsies. The selection of cases for the biomarker assessment was on the basis of (1) a consensus review by two pathologists, (2) availability of sufficient material, and (3) an appropriate representation of the histology grades. CIN2 is the least reproducible area for pathologists and the most contentious area for clinical management. In this proof of concept study, careful consideration was given not to include CIN2 cases due to the subjective nature of this diagnosis, often differences of opinion on interpretation by the reviewing pathologist. No " true" normal tissue blocks were available. The normal tissue sections were obtained from individual blocks within CIN cases, which showed no evidence of CIN. The study was conducted to test the proof of concept of the Ndc80 complex proteins Nuf2 and Hec1 (Sundin et al., 2011) described in Chapter 1 (section1.10); 51 cervical tissues were selected for immunohistochemistry. These included 13 Normal tissue sections obtained from one CIN0 LLETZ (two sections from two separate blocks) and 11 tissue sections from CIN LLETZ with separate blocks showing no CIN. 15 CIN1 tissue sections were obtained from 10 LLETZ (two LLETZ with three separate blocks each and one with two separate blocks). 23 CIN3 tissue sections were obtained from 15 LLETZ (5 LLETZ with two separate blocks each and one case with four separate blocks). Three of 23 CIN3 cervical tissues were selected for HER2 D-DISH staining to check the chromosome ploidy status.

2.7.3 Ethical Approval

The study was approved by the Coombe Hospital Research Ethics Committee (Study No 2019_19), and all women gave written informed consent to participate.

2.8 Case selection/Tissue block Selection and Slide Processing

Using the HistoCerv Study Master File reference, the laboratory information system in Histopathology was accessed to obtain histopathology results, including block number, diagnosis, block location, and availability. Histological grades were used for biomarker analysis, including normal, CIN1, CIN2, CIN3, AIS or cGIN, and cancer cases. The selection was based on the quality of the tissue sample, i.e., the presence of intact epithelium, the absence of an artefact, and the good representation of the diagnosis. H&E slides were initially re-reviewed by three pathologists for each patient sample to determine which histology block for that patient contained the optimal tissue material representative of the given histologic diagnosis. Histology blocks from the selected cases were collected, and 5-6µm thickness sections were cut from each block. H&E stained Sections were mounted onto labelled Bond Leica frosted glass slides, placed onto the HE600 staining tray & placed onto the Ventana HE600 (Roche Diagnostics) for staining. IHC sections were cut onto charged slides (Superfrost Plus, Menzel Glaser, Germany) for analysis, followed by de-paraffinisation, IHC, microscopy, and interpretation by analysing scientists and pathologists. Sections were labelled with an 'H' number. H&E staining was used for the first tissue section; the second section was used for immunohistochemistry using Nuf2 antibody; the third section was used for immunohistochemistry using Hec1, and H&E staining was used for the last section to ensure that all the tissue sectioned was of the same overall histological grade.

2.9 Histopathology Methodology

2.9.1 Use of Microtome for Cutting Tissue Sections

Microtomy was performed on the SLEE microtome (SLEE Medical GmbH, Nieder-Olm, Germany). Blocks were trimmed to remove excess paraffin wax to expose a full-face representative tissue section sample. Using a new blade, sections were cut at 5-6µm thickness, forming a ribbon of sections. The ribbon was placed in a cold-water bath to remove folds in the sections and then in a warm water bath before one section was fixed on a glass slide. Leica Bond Plus slides were used for H&E staining. TOMO IHC Adhesive Glass slides were used for IHC. H&E slides were left on the edge of the water bath to dry before being put into racks for the H&E staining. IHC slides were placed in a metal rack and incubated between 67° - 73°C in a Memmert Incubator for 1 hour.

2.9.2 H&E staining

2.9.2.1 H&E Staining Principle

Haematoxylin is a natural dye that, when combined with a mordant and in an acidic solution, has an affinity for nucleic acids, staining them deep red. Excessive nuclear staining may be removed with a

dilute acid, the HE600 Differentiator. The red haematoxylin staining changes to blue when rinsed with the HE600 Bluing reagent. Eosin is an acidic synthetic dye that has an affinity for basic tissue elements (cytoplasm and most connective tissue fibres) and stains these in various shades of pink, red, and orange.

Pre and post-immunohistochemistry, all sections were subjected to H&E staining with Gill's Haematoxylin solution (regressive stain) and Eosin supplied by Ventana using the automated Ventana HE600**.**

2.9.2.2 Ventana Reagents

All the Ventana HE600 reagents come prepared and provided by Roche Inc.

1. Ventana HE600 Wash

2. Ventana HE600 Haematoxylin

3. Ventana HE600 Bluing

4. Ventana HE600 Eosin

5. Ventana HE600 Organic Solution

6. Ventana HE600 Differentiating Solution

7. Ventana HE600 Transfer Fluid

8. Ventana HE600 Cleaning Solution

Ventana HE600 Coverslipper Activator

Ventana HE600 Glass Coverslips

2.9.2.3 Method

The Ventana HE600 (Roche Diagnostics) stains slides using an automated Haematoxylin and Eosin staining method by utilising an individual slide staining system. Fresh reagents are dispensed onto each slide, alleviating tissue cross-contamination in the H&E staining process. It combines the incubator, stainer, and coverslipping process into a single system. Xylene and Alcohol are replaced from the H&E process, reducing the risk of exposure to hazardous chemicals. Ventana HE600 Gill's Haematoxylin is used as a nuclear stain, and the Ventana eosin is used as a counterstain.

Tissue sections measuring 5µm were cut, placed on a Ventana staining slide tray, and loaded immediately onto the HE600 for staining. The HE600 system allowed for the incubation of the sections

followed by the simultaneous staining, dehydrating and coverslipping of slides by selecting the validated HE protocol, "Protocol 7" on HE600. Tissue sections were brought to water through a selection of reagents. The haematoxylin used was a Gill's Haematoxylin solution (regressive stain) supplied by Ventana (Roche Diagnostics) as the neat working solution. Differentiation was attained using an Acid Differentiator supplied by Ventana. Haematoxylin was 'blued' using the Ventana Bluing agent. The Ventana Eosin was used as an acidic dye to stain basic tissue elements (cytoplasm and most connective tissue fibres), stained in shades of pink, red, and orange. Once stained the sections were coverslipped using the VENTANA HE 600 Coverslip Activator (Roche Diagnostics) on pre-glued coverslips. Once the staining process was complete, slides were removed from the HE600 and checked for H&E staining quality.

2.9.3 Antibody Selection

Before commencing immunohistochemical analysis of Nuf2 and Hec1, a substantial review was carried out to source the most appropriate commercially available antibodies. The data sheet review led to the selection of Rabbit polyclonal Nuf2 antibody (Abcam ab230313) and Rabbit polyclonal Hec1 antibody (Thermo-Fischer Scientific PA5-102765).

2.9.4 Control Material for IHC Studies

Stocks of HeLa cells (HPV 18 positive cervical cancer cell lines, obtained from the American Type Culture Collection (ATCC), stored in liquid nitrogen, were thawed to 37°C. Cells were cultured in buffered Minimum Essential Medium (MEM) (Sigma-Aldrich Cat No. M4655-500ml). In 500 ml of MEM media, 50 ml of Foetal Bovine Serum (FBS) (Sigma-Aldrich Cat No. 12103C), 10 mls of Penicillin/streptomycin (5000 units of Penicillin, 5mg/mL of Streptomycin in 100ml.) (Sigma-Aldrich) and 4ml of L-Glutamine (200 nm stock concentration) (BioWhittaker) was added to form the buffered MEM.

Cells were initially cultured in T25 cell culture flasks. 1ml of thawed HeLa cells were reconstituted with 10mls of Buffered MEM media. Flasks were incubated at 37°C and cHecked daily for confluency. When confluency reached 80%+, the cells were split. The media in the T25 flask was aspirated gently and removed. 4ml of TrypLE (1X) (Gibco) was added over the cells and incubated at 37°C for 5 minutes. The flask was viewed under an inverted microscope (Zeiss, Axiovert 35 Inverted Phase Contrast Microscope) to confirm the cells detached from the flask surface. 10mls of Buffered MEM was added to the flask to halt the Trypsinisation process. Cells were brought up to T175 for subsequent culture. Cell pellets were collected from each passage by centrifugation of cell suspension at 3000 rpm for 5 minutes at 4°C. The supernatant was removed, and 5mls of PBS (1X, 7.4PH, Ambion™) was added to

wash the cells. The cells were spun again at 3000 rpm for 5 minutes, and the supernatant was removed. Cell pellets were stored at -80°C for later.

2.9.5 Preparation of Cell Line FFPE blocks

Cell line suspension, HeLa (HPV 18 positive cell line with confluency of 80-90% was used. The cell suspension was washed with PBS twice and then trypsinised. The cell suspension was incubated at 37°C for 5 min and checked for cell flow. The cell suspension was neutralised by adding an equal amount of media. The cell suspension was centrifuged at 5000 rpm for 10 min. The molten agar was added to the above cell pellet to re-suspend the cells. The suspension was centrifuged at 5000 rpm for 10 min. The solidified pellet was cut and put into cassettes for block preparation by processing overnight in the Leica HistoCore Pegasus Dual Retort tissue processor and embedded in paraffin wax using the Sakura TissueTek Tec6 (Sakura Fintek, Europe).

2.10 Immunohistochemistry (IHC)

2.10.1 The Principle of Immunohistochemistry

Immunohistochemistry is a technique for identifying cellular or tissue constituents by means of antigen-antibody reaction, the site of the antibody being identified either by direct labelling of the antibody or by use of a secondary labelling antibody. This stepwise process involves de-paraffinisation of the FFPE tissue section, followed by antigen retrieval, the addition of primary antibody, the application of secondary antibody and finally, the detection of antibody by detection reagent (Magaki et al., 2019). Antigen retrieval is done by heat-induced epitope retrieval (HIER), the most commonly used method, enzyme digestion, oxidation using hydrogen peroxide, or by using a denaturant or detergent (D'Amico et al., 2009). Primary antibodies can be monoclonal, targeting a single epitope or polyclonal, targeting many different epitopes (Cartun et al., 2019). Various primary antibody concentrations are used according to the manufacturer's guidelines to optimise primary antibodies. A secondary antibody is used to enhance the amplification, depending on the primary antibody's choice. The secondary antibody is labelled with the enzyme horseradish peroxidase. Chromogens such as DAB are used as detecting agents. This study applied the following immunohistochemistry protocol based on The Hapten-linked multimer detection kit used in the Ventana Benchmark ULTRA equipment principle (Figure 2.5)

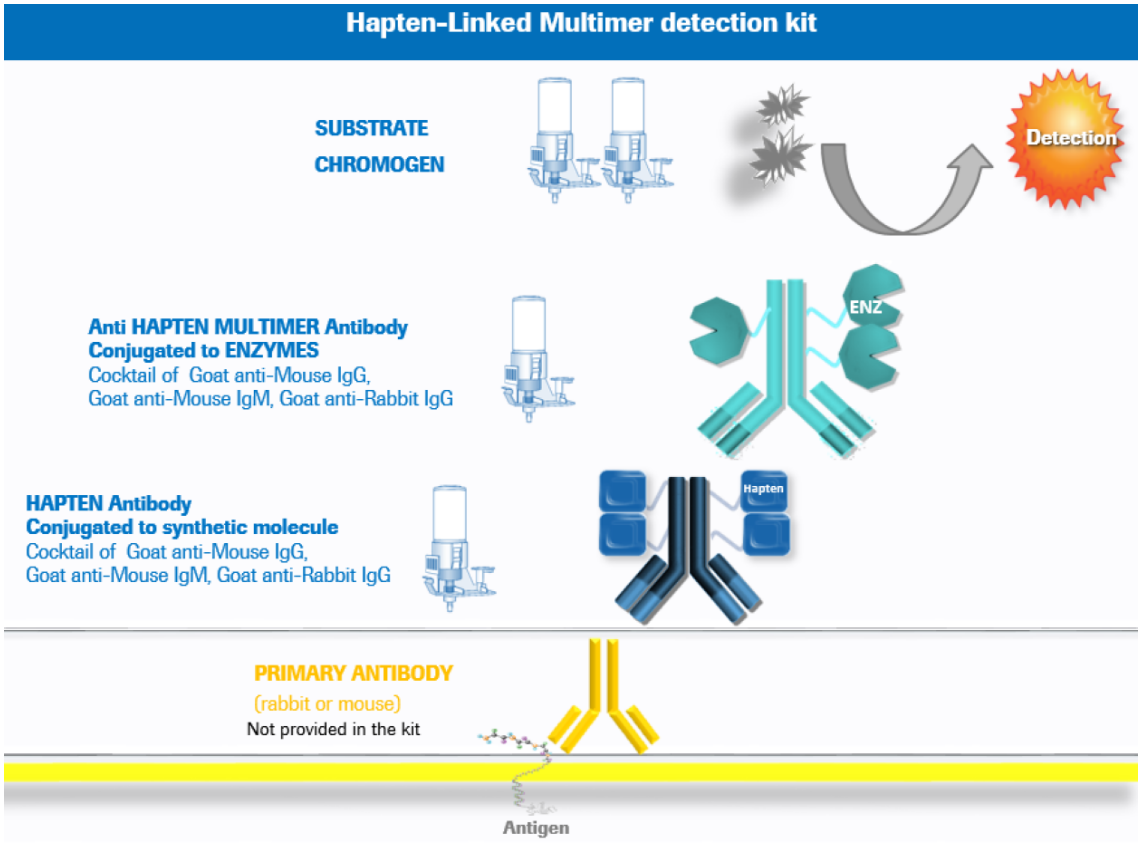


Figure 2.5 The basic principle of immunohistochemistry illustrated with the Hapten-linked multimer detection kit used in Ventana Benchmark ULTRA equipment and adopted from the BenchMark ULTRA User Training manual (Ventana Medical Sytems, 2012)(Roche Diagnostics).

Haptens are small, relatively inert organic molecules that can be attached to DNA without disrupting the DNA's hybridisation properties (Genome Exploration Laboratory Msstate). For indirect labelling, probes are labelled with modified nucleotides that contain a hapten. The two most common haptens employed in FISH are biotin and digoxigenin. A hapten-linked antibody is conjugated to numerous copies of synthetic and nonendogenous hapten molecules. The Hapten-linked multimer detection kit allows the detection of any primary mouse or rabbit antibody. The cocktail of anti-mouse and anti-rabbit hapten-conjugated antibodies will target the primary mouse or rabbit antibodies. It will be detected by the anti-hapten multimer that carries an enzyme. In the presence of their substrate and a chromogen provided by the detection kit (Figure 2.6), the multimer conjugated enzymes will generate an insoluble precipitate.

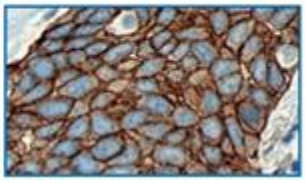
Product name	Size	Hapten	Enzyme	Chromogen/ Substrate	Staining example
OptiView DAB	250 tests	HQ Hydroxyquinoxaline	HRP Horseradish Peroxidase	DAB (Diaminobenzidine) H_2O_2	

Figure 2.6 OptiView DAB detection kit is a hapten-linked multimer detection kit used in the Ventana Benchmark ULTRA and adopted from BenchMark ULTRA User Training manual (Ventana Medical Sytems, 2012)(Roche Diagnostics).

2.10.2 D-DISH

Accurate chromosome segregation during meiosis and mitosis leads to correct chromosome complement in daughter cells, and chromosome segregation errors lead to aneuploidy. Aneuploidy is defined as a state of abnormal chromosome numbers that deviates from a multiple of the haploid complement (Godek & Compton, 2018). Human epidermal growth factor receptor-2 (HER2/neu, c-erbB2) is one of a family of four membrane tyrosine kinases (Gutierrez & Schiff, 2011). HER2 gene signalling has been reported to have a functional role in mediating and sustaining chromosomal instability through chromosome missegregation during mitosis, leading to aneuploidy (Burrell et al., 2010). Dual-color dual-hapten in situ hybridisation (D-DISH) is a test for assessment of HER2/neu gene overexpression on light microscopy (Gajaria et al., 2020).

2.10.3 D-DISH Principle

Dual In-Situ Hybridisation (DISH) (INFORM, Ventana) is a fully automated ISH technique (Roche, 2020a) carried out on the Ventana Benchmark ULTRA autostainer. The DISH incorporates an oxide-reduction reaction, silver deposition technology and a chromosome 17 (C17) probe. The method is a pre-validated technique, optimised by Ventana on 10% buffered FFPE tissue sections on the BenchMark ULTRA immuno-stainer. DISH quantifies the number of centromeric C17 signals by conventional bright-field light microscopy. DISH combines light microscopy with full automation and molecular analysis. Chromosome 17 is detected with DIG- labelled probe and appears as a red signal. HER2 is detected by the DNP-labelled probe and appears as a discrete black signal (Figure 2.7).

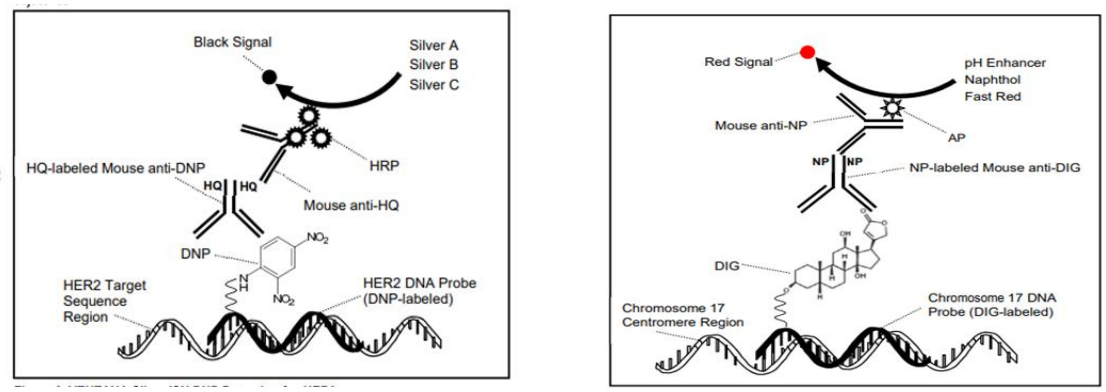


Figure 2.7 VENTANA Silver ISH DNP Detection for HER2 and Red ISH DIG Detection for C17H

(Roche, 2020b)

2.10.4 IHC Materials

Staining was carried out using:

1. The Ventana BenchMark ULTRA System.
2. The HE600 Instrument (Roche Diagnostics) (for H&E stain and IHC coverslipping).
3. TOMO IHC Adhesive Glass.
4. Finn Pipettes- Ranging in volume: 10µl (x2), 5 - 50µl (x2), 10 - 100µl (x2), 30 - 300µl and 100 - 1000µl.

The Ventana BenchMark autostainers incorporate onboard de-paraffinisation, antigen retrieval and staining using the Optivew DAB Detection Kit. The Immunostainers can perform fully automated staining runs and semi-automated/titration runs. Titration runs are performed for any non-Ventana/Roche antibodies purchased neat/pre- diluted.

2.10.5 Reagents Used for Immunohistochemistry Using Ventana BenchMark ULTRA

1. EZ Prep Detergent was used during de-paraffinisation of slides. Slides were heated to melt the paraffin. EZ Prep allowed the melted paraffin to float to the surface of a liquid and be removed by rinsing.
2. Reaction Buffer- Tris-based buffer solution, pH 7.6, was used to rinse the slides between steps of the immunostaining procedure.
3. Liquid Coverslips were used as a hydrophobic reagent to slides to provide a barrier between air and reagents, thus preventing liquid evaporation of reagents throughout the assay.
4. CC1- Cell Conditioning 1, a Tris buffer solution with high pH, was used at 32 Minutes at 95°C during HIER to break the covalent bonds made in formalin-fixed tissues.

2.10.6 Extra Reagents for D-DISH

1. SSC: A sodium salt citrate buffer used for hybridisation and stringency (Add 8L distilled water to 1 bottle of Bulk reagent)
2. UltraView Silver Wash II: Acidic citrate-based buffer formulation at pH 4, for silver signal retrieval, 2L pre-dilute is placed in Option bulk bottle.

2.10.7 Refrigerated Reagents

1. An OptiView DAB detection kit: This works on the Hapten-linked multimer detection principle.
2. Ventana antibody diluent: Buffered, proteinaceous solution used to dilute antibodies.
3. Haematoxylin: counterstain to allow for the visualisation of DAB-stained cells.
4. Bluing Reagent: used to blue the haematoxylin counterstain.

2.10.8 Extra Reagents For D-DISH

1. Protease 3: Unmasks the nucleic acids hidden due to the effects of formalin fixation. Protease 3 is the mildest pre-treatment available for ISH, with 0.02 units/m enzymatic activity.
2. C17 probe conjugated with DNP (5 dinitrophenols): Hybridises to a complementary repeating sequence specific to the centromeric region of Chromosome 17.
3. Her2 Dual ISH DNA Probe cocktail: This cocktail enables the Her2 gene and chromosome 17 centromere to be co-hybridised and visualised on the same slide.
4. CC2: Citrate buffer at a slightly acidic PH, which at raised temperatures hydrolyses the covalent bonds formed by formalin in tissue.
5. Monoclonal rabbit anti-DNP antibody binds to the Chromosome 17 DNP probe, allowing visualisation.
6. VENTANA Silver ISH DNP Detection Kit: Her2 is detected by the DNP-labelled probe and appears as a discrete black signal.
7. Red ISH DIG Detection Kit: Chromosome 17 is detected with DIG- labelled probe and appears as a red signal
8. Hematoxylin II, nuclear counterstain.

2.10.9 Performing an IHC Run

IHC Run was performed using the Ventana BenchMark ULTRA (*BenchMark ULTRA*, Roche Diagnostics) analyser. Once the instrument was turned on, NEXES software was started. All the reagents were registered by referring to 'Registration Wand for Kits and Ventana Products ULTRA Manual. Labels were created for each slide by referring to 'Slides and Slide Labels' in the ULTRA Manual. Appropriate labels were applied to each slide, and the slides were mounted on the slide trays (Figure 2.8). All

reagent dispensers and the reagent trays on the reagent carousel were mounted, ensuring the reagent meniscus was present at the tip of the dispenser nozzle. Bulk fluid bottles were filled in using prepared bulk fluids. The waste containers were checked by inspecting the waste module bottle. All the drawers and the reagent hood were closed, and the staining run was started from the BenchMark ULTRA home screen by accessing the Instrument View and clicking the Running mode button. The ULTRA system reads the reagent and barcodes and calculates the run time and the time at which primary antibody titration should be applied. The countdown to titration is visible on the screen. The machine alarms when the titration is due to be applied. The relevant slide drawer is opened. Titrations of antibodies were pipetted (100µls) using a Finn Pipette and applied onto the appropriate slides. All open slide drawers were closed by pressing the button on the instrument slide control panel and holding it for two seconds (until the two beeps and the unit light stops flashing). The IHC staining resumed automatically shortly after the drawer closed. Slides were removed from Ventana Benchmark ULTRA and rinsed briefly in soapy water to remove liquid coverslipper, followed by tap water to remove residual soap. Slides were then placed on the Roche HE600 Coverstainer for dehydration and coverslipping by selecting the appropriate protocol and coverslipping for IHC, ISH and Special Stains.

2.11 Antibody Optimisation

Initial optimisation was performed using HeLa cell blocks and antigen retrieval in CC1 buffer for 32 and 56 minutes. Hec1 with 1:200 dilution and Nuf2 with 1:300 dilutions were used. Following a review of these, the HeLa cell block was chosen with the Hec1 antibody utilised at dilutions titrated from 1:200, 1:100, 1:50, 1:35 and 1:25, and the Nuf2 antibody was utilised at a dilution of 1:300, 1:200, 1:100, 1:75 and 1:50 along with negative cervical tissue and positive cervical tissue for optimisation. Antibodies were optimised, and tissue samples were processed using the Benchmark Ultra immunostaining system (Benchmark LT, Ventana, Tucson, Arizona, USA) and the Optiview DAB detection Kit (Ventana, USA).

Optimised Hec1 protocol using the Benchmark ULTRA immunostainer as follows:

CC1 for 32 minutes at 95°C, primary antibody (1:35), manual titration, 1 hr, Hematoxylin counterstain 4 min, bluing reagent 4 min.

Optimised Nuf2 protocol using the Benchmark ULTRA immunostainer as follows:

CC1 for 32 minutes at 95°C, primary antibody (1:75), manual titration, 1 hr, Hematoxylin counterstain 8 min, bluing reagent 4 min.

Figure 2.8 Ventana benchmark LT immunoslide platform (Ventana Medical Sytems, 2012)

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2.12 Histopathological Review and Interpretation

Two pathologists performed an IHC slide review. Histopathology review was conducted as follows:

1. An initial H&E review was performed to confirm the disease before and after sections that were used for IHC.
2. IHC review was performed on positive and negative controls. IHC staining pattern/localisation and staining intensity were noted.
3. Scoring of Nuf2 and Hec1 biomarkers was based on H-scoring, which includes a combination of scores for immunostaining intensity of positive cells and percentage of tumour cells displaying positivity. The pathologist scored the intensity of Nuf2 and Hec1 biomarkers on an ordinal score of 0-3. The percentage of tumour cells displaying immunostaining was estimated within a 10% increment. The H score (Table 2.1) was derived from the cross product of the intensity score (0 to 3) and the percentage of tumour cells staining (0 to 100%)
4. D-DISH staining was evaluated for the presence of diploid and aneuploidy nuclei in HeLa tissue, negative cervical tissue and CIN3 cervical tissue.

Table 2.1 H score system for immunohistochemistry results				
Staining intensity	Score	**% Positivity**	**Score**	**H Score***
Negative	0	>10	0	0
Weak	1	>40	1	1
Moderate	2	>70	2	4
Strong	3	100	3	9

*An 'H' score of 4 or more was considered overexpression of the biomarker.

2.13 Sample Size

The sample size estimates for the different elements of my study were as follows:

Part A: Extended Genotyping in Primary Screening Population In a study population of HPV-positive women from primary screening, we estimate, based on data obtained within the CERVIVA HPV primary screening study, the prevalence of CIN2+ in HPV-positive individuals is approximately 15% (Dudding & Crossley, 2013). The sensitivity of current triage methods, such as manual cytology in HPV-positive cohorts, ranges from 70-90%. Therefore, we have estimated the sample size required to achieve, for example, 90% sensitivity; assuming 90% specificity at a precision of 5%, we would require at least 922 HPV-positive samples. For example, in HPV 16/18 genotyping triage, a sensitivity of 80% assuming 90% specificity at a precision of 5%, we would require at least 1640 HPV-positive samples. Therefore, the study cohort is an adequate sample size to evaluate the performance.

Part B: Biomarkers for cervical cancer diagnostics; this was an exploratory and proof of concept study, so sample size estimates were not applicable. A number of cases representative of high grade disease (CIN3) and low-grade disease (CIN1) were selected to prove the dyresgulation of the proposed biomarkers compared to normal tissues. The proof of concept for dysregulation of HEC1 and NUF 2 was assessed in 15 CIN3 cases compared to 9 CIN1 and 9 normal cervical tissues.

2.14 Data Analysis and Statistics

For Chapters 3 and 4, an analysis was carried out using the agreement proportion calculation with 95% confidence intervals (CIs) to determine the percentage of agreement between the Cobas 4800 test and the BD Onclarity HPV Assay. HPV genotype prevalence and cytology outcome were calculated using proportion and percentage in positive BD Onclarity HPV assay.

Clinical performance for the Onclarity was assessed by calculating sensitivity, specificity, PPV and NPV for the detection of high-grade disease adapted from https://slideplayer.com/slide/1no4308641/ (Table 2.2).

Table 2.2 Clinical Performance Indicators

Clinical Performance Indicator	Definition
Sensitivity	Ability to detect people who have a disease
Specificity	Ability to detect people who do not have a disease
Positive Predictive Value (PPV)	The likelihood that a person with a positive test result truly has a disease
Negative Predictive Value (NPV)	The likelihood that a person with a positive test result truly does not have a disease

Sensitivity and specificity are intrinsic properties of the test itself, independent of disease prevalence and inversely proportional. PPV and NPV vary depending on the prevalence of the disease in the population being tested. The high-grade disease was classified as CIN 2 or greater (CIN 2+). Microsoft Excel 2017 was used for statistical analysis. SPSS Version 28.0.0.0 was used to detect statistical significance. The risk matrix was created to compare the extended genotyping with cytology and CINTec staining methods assessed within the CERVIVA HPV primary screening pilot study (outside of this thesis) by the calculation (Figure 2.10) of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with the associated 95% CI for the detection of both CIN 2+ and CIN3+. The absolute risk was calculated using the proportion and percentage of CIN2+ or greater (CIN2+) in the prevalence of each HPV genotype.

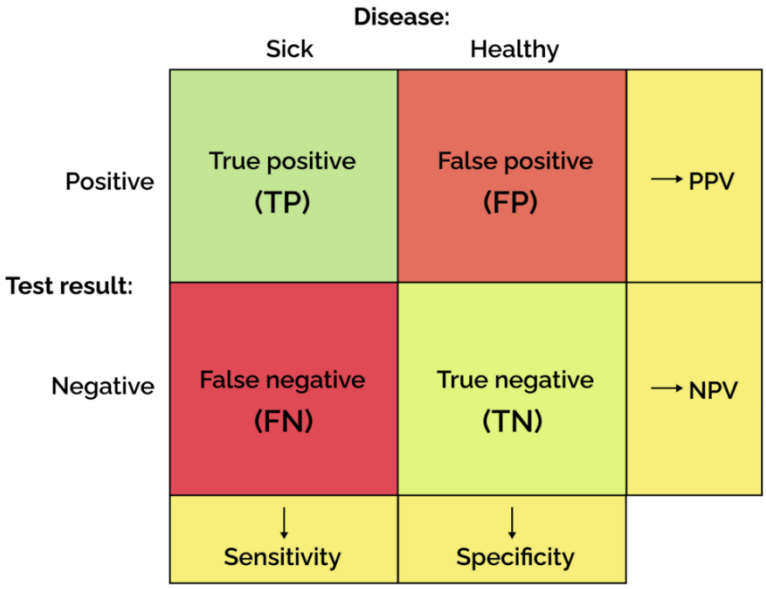

Figure 2.9 Key measures used in evaluating the performance of a diagnostic test

(Statistics - Sensitivity Specificity PPV NPV - GP Exams, 2018)

For Chapter 5, H scores for different grades of disease were aggregated and compared to determine any differences, as detailed in Chapter 5 (section 5.4.4).

2.15 References

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Chapter 3 Extended Genotyping for HPV in the CERVIVA Primary Screening Study Population using the BD OnclarityTM Assay

3.1 Introduction

The vast majority of cervical cancers are associated with persistent HPV infection with one of 13–14 high-risk human papillomavirus (HPV) genotypes (GLOBOCAN, 2018; Harlfinger et al., 2023). High-risk HPV genotypes are those which are associated with a higher risk of developing cervical cancer and other cancers such as vulval, vaginal, anal, penile and oropharyngeal cancers. These high-risk types include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. HPV16 is the most common HPV type, which, together with HPV18, accounts for 70% of all cervical cancers (WHO, 2017; Ramakrishnan et al., 2015). In recent years, cervical cancer screening has moved to primary HPV testing as the preferred test (Bruni et al., 2022; Serrano et al., 2022). HPV testing is more sensitive than cytology as a screening test with a high negative predictive value (Gilham et al., 2019; Hoffman et al., 2020; Ramírez et al., 2023; Swid & Monaco, 2022).

A number of HPV molecular testing platforms have been FDA-approved for use in cervical screening, including Hybrid Capture [HC2], Cervista (Hologic), the Cobas® HPV test for use on the 4800 and 6800/8800 (Roche), The BD Onclarity™ HPV Assay, the Abbott Alinity m HR HPV and the Aptima HPV Assay (Hologic). The Cobas® HPV Test, The BD Onclarity™ Assay and The Abbott Alinity Test are FDA-approved as a single test; the other three are approved for use in co-testing (Arbyn et al., 2021; U.S. Food and Drug Administration, 2024). The Aptima test is in use in many countries for primary screening without cotesting and has been shown to have equivalent sensitivity to, and higher specificity than, the approved DNA tests (Ibáñez et al., 2021; Ratnam et al., 2011). These assays have been validated for primary screening using established non-inferiority criteria (Meijer et al., 2009). Many other tests have passed the modified Meijer criteria and/or VALGENT criteria and are used in primary screening settings (hpvworld.com, 2024). A number of these HPV tests have partial or extended genotyping capabilities, as shown in Figures 3.1, 3.2 and 3.3 (BD Cervical cancer, 2024).

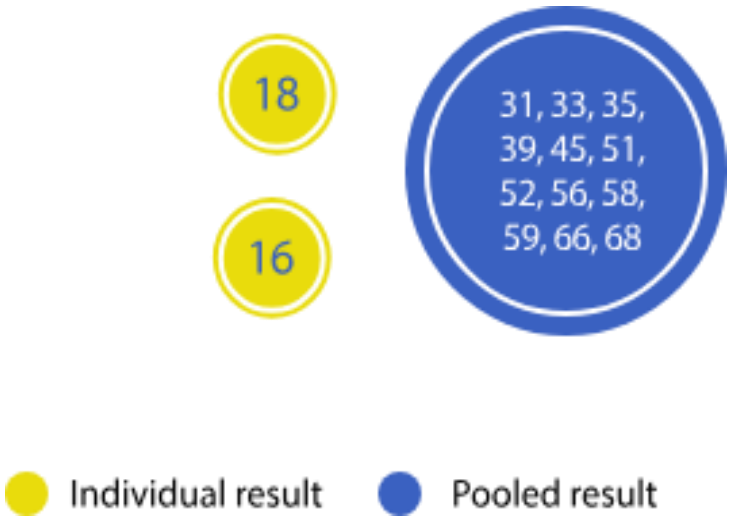

Figure 3.1 Partial genotyping involves HPV16 and 18 are reported individually, and the remaining 12 hr HPV genotypes are detected and reported as a group.

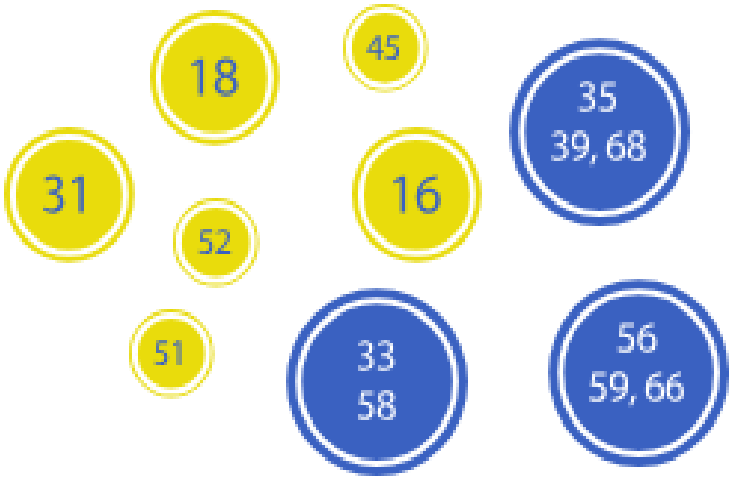

Figure 3.2 Extended genotyping involves at least six genotypes are individually detected and the remaining eight are reported as three groups.

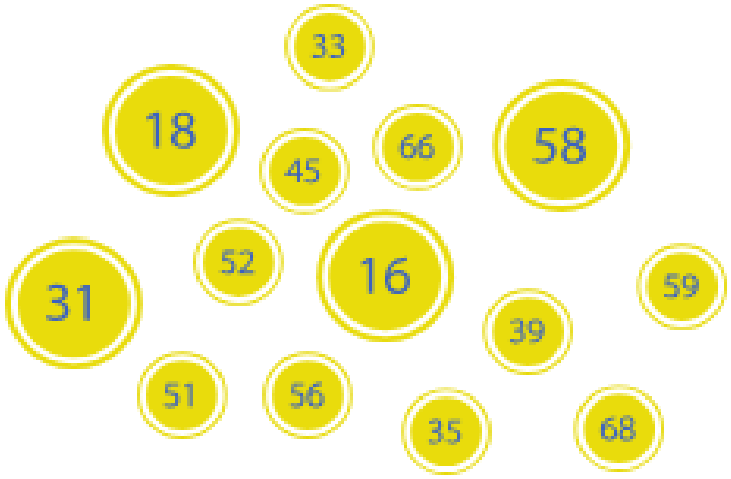

Figure 3.3 Full genotyping involves detecting all high-risk HPV genotypes individually.

For example, the Cobas® HPV test detects the 14 high-risk HPV types while specifically genotyping for types 16/18. The BD Onclarity™ Test simultaneously detects the 14 high-risk HPV types with individual genotyping for the E6/7 coding regions of HPV16, 18, 31, 45, 51, and 52, with pooled results for types 33/58, types 35/39/68 (pooled result), and 56/59/66 (pooled result).

HPV tests that include some form of genotyping for HPV provide additional information about the specific HPV types in the sample (Song et al., 2022). This can be useful for stratifying patients at higher risk of developing disease (Rohner et al., 2020). Moreover, it can provide useful epidemiological data to inform cancer prevention policies and practices in different regions in the context of HPV vaccination (Song et al., 2022; Su et al., 2023).

Some limited data is available on extended HPV genotyping analysis using the BD Onclarity™ HPV Assay in different patient cohorts from varied geographical areas. A study (Cuschieri et al., 2015) in a Scottish screening population showed that HPV16 and HPV31 were the most common HPV types associated with CIN2+. The Predictors studies, led by Professor Cuzick, showed very nicely the prevalence of different HPV types in CIN2 and CIN3, and also how the distribution changed between grades (Cuzick et al., 2014). Further afield in the U.S., a study on the risk of high-grade cervical disease in women with NILM cytology (Stoler et al., 2019a) found that HPV16, HPV52, HPV35/59/68 and HPV56/59/66 were the most prevalent genotypes. This study recommended the addition of HPV31 to HPV16 and HPV18 genotyping as a part of the US HPV primary screening algorithm. Prior to this, a study on archived samples from a multicentred U.S. clinical trial using BD Onclarity™ (Wright et al., 2014) found the relative risk of CIN3 disease in women positive for HPV31 or HPV33/58 (combined) was comparable to that of HPV18.

Across Europe, the prevalence of HPV genotypes other than HPV16, which remains the most common HPV genotype, varies considerably. The most common high-risk HPV types in women with HSIL in a

Belgian screening population were HPV16, 31, 51,52 (Schmitt et al., 2013). In the Danish screening population, the HPV genotypes HPV16, 52, 31, 18, 45, and 51 were identified in descending order of prevalence (J. H. Bonde et al., 2020). The baseline phase of the BD Onclarity™ trial by Stoler et al. was conducted to determine the screening performance of the assay for detecting cervical cancer and pre-cancer (≥CIN2) during triage of women ≥21 years with ASCUS cytology. The findings showed that the screening population in this group had 14.7% HPV positivity, and the prevalence order was HPVP3, HPVP2, and HPV16, where HPV16, 18, and 31 were more significant for ≥CIN3 (Stoler et al., 2018). In Japan, HPV 52 was noted as the second most prevalent HPV type after HPV16 in a comparative study between BD Onclarity™ and Digene HC2(Nakamura et al., 2019). A study done in China recently highlighted the prevalence and genetic polymorphism data of HPV68 (He et al., 2022).

Overall, there is increasing evidence that while HPV16 infections infer the highest risk for CIN2+ and CIN3+, HPV genotypes other than HPV16 and HPV 18 are emerging that have increased attributable risk for developing CIN2+, in particular, HPV genotypes 31 and 33 (Cuzick et al., 2021). In some parts of the U.S. and Australia (Smith et al., 2022), HPV 16/18 genotyping is currently used as part of the screening algorithm to stratify HPV-positive women with normal cytology who may be at increased risk of underlying cervical intraepithelial neoplasia (Saslow et al., 2012).

Extended genotyping may also play a role in monitoring persistent infection with high-risk genotypes over time. In a recent review, evidence was presented from the review of 10 articles (2000-2019) to support the clinical utility of genotype persistence to improve risk discrimination of high-grade CIN (J. Bonde et al., 2021). Similar findings were shared (Andrews, 2019) in a systematic review of 36 research articles that examined HPV genotyping combined with cytology. This review highlighted the promising aspect of extended genotyping for follow-up type-specific persistence versus clearance and supporting risk-based clinical action steps. Reporting genotyping can also provide discrimination of current and future CIN2+ and CIN3+ risk (Andrews, 2019).

As the circulating genotypes are changing worldwide due to the success of HPV vaccination programmes, with the possible emergence of some other HPV types, some of which are reported to have higher positive predictive values (PPVs) than HPV18 for high-grade lesions (Cuzick & Wheeler, 2016). A retrospective analysis of over 1700 cases of CIN diagnosed between 2011 and 2017 in women younger than 25 in Scotland indicated the relative contribution of non-established high-risk types, including those considered low-risk, was greater among vaccinated women with CIN2+ vs unvaccinated women with CIN2+ (Cuschieri et al., 2023). Risk stratification of high-risk HPV-positive women is vital since less than 10% of women who become persistently infected lead to the development of cancer due to high-risk HPV genotype infection (Bodily & Laimins, 2011).

In Ireland, cytology had been the primary cervical screening method until March 2020, before HPV primary screening was introduced. HPV-based primary screening in Ireland reports HPV positivity but not any specific HPV genotyping. In addition to this, the genotype prevalence of HPV-positive women previously vaccinated since 2008 using Gardasil for HPV16 and HPV18 is unknown. Extended HPV genotyping information may be used to refine the management pathways for HPV-positive women in an Irish population. The results of this study could help in the management, prevention, and follow-up of HPV-positive women.

3.2 Hypothesis

I hypothesise that extended HPV genotyping using the BD Onclarity™ HPV Assay will improve risk stratification for HPV-positive women from primary screening.

3.3 Aims

- To detect specific high-risk HPV genotypes using the BD Onclarity™ HPV Assay in HPV-positive primary screening population.
- To establish the absolute risk associated with individual HPV types or groups of HPV subtypes of CIN2+ disease.
- To establish the clinical performance of the BD Onclarity™ HPV Assay for the detection of CIN2+ and CIN3+.

3.4 Methodology

Extended genotype analysis was carried out in 1863 Cobas HPV DNA positive, and 108 Cobas HPV DNA negative specimens obtained from CERVIVA HPV Primary Screening Pilot study as described in Chapter 2 (section 2.1) using the BD Onclarity™ HPV assay detailed in Chapter 2 (section 2.2-2.5). The FDA-approved BD Onclarity™ HPV assay is a real-time PCR DNA assay targeting the E6 and E7 DNA regions of 14 oncogenic HPV genotypes (Bonde et al., 2020). It detects six individual genotypes (16, 18, 31, 45, 51, 52) and eight genotypes distributed over three groups (33/58, 56/59/66, 35/39/68). All calculations were performed automatically by the B.D. Viper L.T. software to detect the presence of genotypes. HPV genotyping data was presented as simple proportions. Concordance between results by the different methods was determined by proportion and Kappa values where appropriate. Statistical significance was determined using the Fisher's Exact test in SPSS version 28.0.0.0. Clinical performance for the BD Onclarity™ HPV Assay was assessed by calculating sensitivity, specificity, PPV and NPV for detecting high-grade disease. The absolute risk was determined by the proportion of CIN2 or greater (CIN2+) in each genotype-specific prevalence. The

high-grade disease was classified as CIN 2 or greater (CIN 2+). Full details are provided in Chapter 2, Section 2.15.

3.5 Results

A total of 1,863 Cobas® HPV positive specimens were tested for HPV using BD Onclarity™ HPV Assay as described in Chapter 2A. The mean age of the women included in this study was 34 years (age range 23-76 years). Out of 1,863 women, 717 (38.5%) were <30 years of age, with 86% HPV positivity for BD Onclarity™. The remaining 1,146 (61.5%) were 30+ years of age, with 76.8% HPV positivity for BD Onclarity™. Out of 1,863 Cobas® HPV positive specimens, 1497 tested positive using BD Onclarity™ HPV Assay (Table 3.1).

3.5.1 Overall HPV positivity with BD Onclarity™ Assay compared with Cobas® HPV Assay

The aim of this study was to evaluate the BD Onclarity™ HPV Assay detection to understand the prevalence of high-risk HPV genotypes among HPV-positive cases. Cobas® HPV DNA Test positive 1863 samples tested using the BD Onclarity™ HPV Assay. A cohort of 108 Cobas® negative samples were included in the study as controls.

Table 3.1 Agreement of Cobas® HPV test and BD Onclarity™ HPV Assay

Test Platform	Cobas® Positive	Cobas® Negative	Total
B.D. Positive	1497	05	1502
BD Negative	366	103	469
Total	1863	108	1971

The overall agreement between the BD Onclarity™ HPV Assay and the Cobas® HPV Test for the detection of HPV was 81.2% (95% C.I. 0.794-0.83); Kappa= 0.294 (95% C.I.: 0.248 to 0.340.) (Table 3.1) It is important to note that it was not a primary objective of this study to look at the agreement between the BD Onclarity™ HPV Assay and the Cobas® HPV Test. The study specifically was focused on extended genotyping of a cohort of HPV-positive women from primary screening. Therefore, the overall agreement is biased because only Cobas® HPV positive samples were selected for the study. There were 366 (19.6%) BD Onclarity™ negative specimens out of 1863 Cobas® positive cases. The nature of the study population can partly explain the 19.6% discordance between the two tests, i.e. only Cobas® positive samples were processed by BD Onclarity™. Out of 366 BD Onclarity™ negative

cases, 89% (327/366) had NILM cytology with CIN2+ follow-up in three cases, 8% had ASCUS/LSIL cytology with CIN2+ follow-up in two cases, 1 had HSIL with no histology follow up, and nine were either unsatisfactory or with no cytology.

As part of the CERVIVA HPV primary screening study, data was available on all samples for HPV mRNA as detected using the Aptima HPV test from Hologic. To investigate the discrepancies further, we subsequently analysed the APTIMA mRNA results for the 366 Cobas positive and BD negative specimens. Out of 366 specimens, 329 were negative for the HPV mRNA Aptima test, confirming what is generally accepted as the Cobas 4800 overcalls as compared to all other HPV test platforms.

At the time of review, Ct values were only available in 114 specimens out of 366 (Instrument changeover). The Cobas 4800 HPV test Ct value for discordant specimens ranged from 27.1 to 40.5, with an average of 37.3, and 81% had a Ct value >35. This suggests that different cut-off values for the different testing platforms can influence the test results.

3.5.2 Individual HPV16 and HPV18 Types Specific Prevalence with the BD Onclarity™ HPV Assay and Compared with the Cobas® HPV Test

Detection of HPV16 and HPV18 by any HPV test is extremely important since both these genotypes are associated with 70% of cervical cancers. The Cobas® HPV Test and the BD Onclarity™ HPV Assay detect these genotypes individually. The study data was analysed to verify the detection rate by both these tests (Table 3.2).

Table 3.2 HPV 16 and 18 concordance between BD Onclarity™ HPV Assay and the Cobas® HPV test

HPV 16	Cobas®(+)	Cobas® (-)	Total		HPV 18	Cobas®(+)	Cobas® (-)	Total
BD (+)	368	36	404		BD (+)	118	10	128
BD (-)	18	1075	1093		BD (-)	19	1350	1369
Total	386	1111	1497		Total	137	1360	1497

There was 96.4% (95% C.I. 0.953-0.973) agreement between Cobas® HPV test and BD Onclarity™ HPV test for detection of HPV16, (Kappa= 0.907, S.E. of kappa = 0.012, 95% C.I. 0.883-0.931) and 98%

(95%C.I. 0.972-0.987) for detection of HPV18 (Kappa= 0.880, S.E. of kappa = 0.022, 95% C.I.: 0.837-0.923) for the detection of HPV18. The BD Onclarity™ HPV Assay detected 36 cases of HPV16, which the Cobas® HPV DNA Test missed, and the Cobas® HPV DNA Test detected 18 cases of HPV16, which the BD Onclarity™ HPV Assay missed. Similarly, BD Onclarity™ HPV Assay detected an additional 10 cases of HPV18 and Cobas 4800 DNA Test detected an additional 19 cases of HPV18.

3.5.3 3.5.3 HPV Genotype Distribution among BD Onclarity™ HPV Positive Cases

The prevalence of individual HPV genotypes and group genotypes among those high-risk HPV-positive cases (n=1497 BD BD Onclarity HPV positive) was determined. All infections observed were counted regardless of whether they were present as single or multiple infections. The distribution of HPV genotypes detected using the BD Onclarity™ HPV assay was as follows across the BD Onclarity HPV positive specimens (n=1497) (Figure 3.1): HPV16: 27% (404/1497), HPVP2: (56/59/66) group: 26.6% (398/1497), HPVP3(35/39/68)group: 19.4% (290/1497), HPV31: 16% (240/1497), HPVP1(33/58) group: 13.6% (203/1497), HPV52: 12.6% (189/1497), HPV51: 10.7% (160/1497), HPV18: 8.6% (128/1497) and HPV45: 8.1% (122/1497). (Figure 3.1).

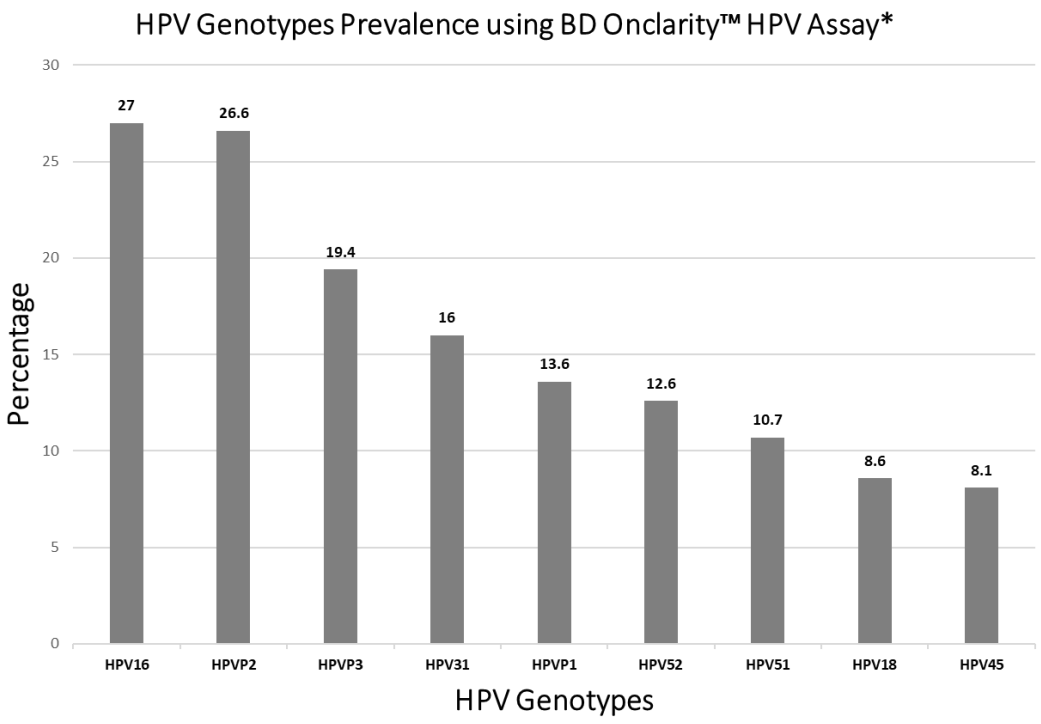

Figure 3.4 Distribution of HPV Genotypes in HPV-positive women. *All infections observed were counted regardless of whether they were present as single or multiple infections.

Where the individual genotype prevalence could be determined, HPV16 was the most prevalent, followed by HPV31, HPV52, and HPV51, while HPV18 and HPV45 were the least represented in terms of individual genotypes. Among the pooled groups, HPVP2 (56/59/66) had the highest prevalence, followed by HPVP3 (35/39/68). Group HPVP1(33/58) had the lowest prevalence.

3.5.4 HPV genotyping across different age groups

Given the higher prevalence of HPV among younger women <30 years, we looked at the specific genotype patterns among younger (< 30 years) and older women (30 and > 30 years) (Table 3.3).

Table 3.3 HPV Genotype Prevalence and Distribution in < 30yrs and >30yrs

HPV Type	Prevalence (%) 95% C.I. n=1497*	Prevalence <30 yrs 95% C.I. n=617	Prevalence ≥30 yrs 95% C.I. n=880	P value
HPV16**	27.0% (404/1497) 24.7-29.3	30.1% (186/617) 26.6-34.0	24.8% (218/880) 22.0-27.7	< 0.001
HPV18**	8.5% (128/1497) 7.1-10.0	10.8% (67/617) 8.6-13.6	7.0% (61/880) 5.3-8.8	< 0.001
HPV45	8.0% (122/1497) 6.8-9.6	8.6% (53/617) 6.5-11.1	7.8% (69/880) 6.0-10.0	0.146
HPVP1**	13.6% (203/1497) 11.8-15.4	15.0% (93/6177) 12.3-18.1	12.5% (110/880) 10.4-15.0	0.012
HPV31**	16.0% (240/1497) 14.2-18.0	17.8% (110/617) 15.0-21.8	14.8% (130/880) 12.5-17.3	0.003
HPVP2**	26.6% 398/1497 24.5-29.0	28.3% (175/617) 25.0-32.0	25.3% (223/880) 22.5-28.3	0.002
HPV51**	10.7% (160/1497) 9.2-12.5	12.6% (78/617) 10.0-15.5	9.3% (82/880) 7.5-11.4	0.002
HPV52**	12.6% (189/1497) 11.0-14.4	15.2% (94/617) 12.5-18.3	10.8% (95/880) 8.8-13.0	<0.001
HPVP3**	19.6% (290/1497) 17.4-21.4	22.0% (136/617) 19.0-25.5	17.5% (154/880) 15.0-20.2	< 0.001

*All infections observed were counted regardless of whether they were present as single or multiple infections. ** Statistically significant at p-value < 0.05. (P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

The statistical analysis carried out using Fischer's Exact Test showed that the prevalence of all the genotypes was significantly different except HPV45 between <30 years and 30 and over age group (significance was set at ($p<0.05$).

3.5.5 HPV genotypes across cytology grades

As per the current HPV primary screening recommendation, HPV-positive women are triaged with cytology. This is because cellular changes caused by high-risk HPV can develop into cancer if undetected and/or not treated. During this project, Ireland had cytology as a primary screening method. To assess the correlation between those cytology results and HPV genotypes, cytology data was retrieved from the Primary HPV Screening Pilot Study. The cytology was reported using The Bethesda System of reporting, where NILM is negative for intraepithelial lesion of malignancy, ASCUS is atypical squamous cells of undermined significance, LSIL is low-grade squamous intraepithelial lesion, HSIL is high-grade intraepithelial lesion, ASCH is Atypical Cells-High grade Squamous intraepithelial cells cannot be excluded, AGUS is Atypical Glandular Cells of Undermined Significance and AIS is Adenocarcinoma in situ.

Cytology data was available on 1480 of 1497 HPV genotype-positive cases analysed. This included 917 cases NILM (negative for intraepithelial lesion of malignancy) on cytology, 405 cases with ASCUS/LSIL (atypical squamous cells of undermined significance/ LSIL is low-grade squamous intraepithelial lesion) and 158 cases with HSIL (high-grade intraepithelial lesion). Thirteen unsatisfactory and four with no cytology results were excluded from this analysis. Among those with high-grade cytology (HSIL), HPV16 was the most prevalent type, accounting for 52% (82/158) of HPV in HSIL> followed by HPV31 and HPV52, while in the three pooled HPV groups, HPVP1(HPV35/39/68) was the highest (28/158) representative in the HSIL cytology outcome, and HPVP2(HPV56/59/66) was the highest representative HPV group among the ASCUS/LSIL 33% (133/405) and NILM 26% (239/917) cytology outcome (Figure 3.2).

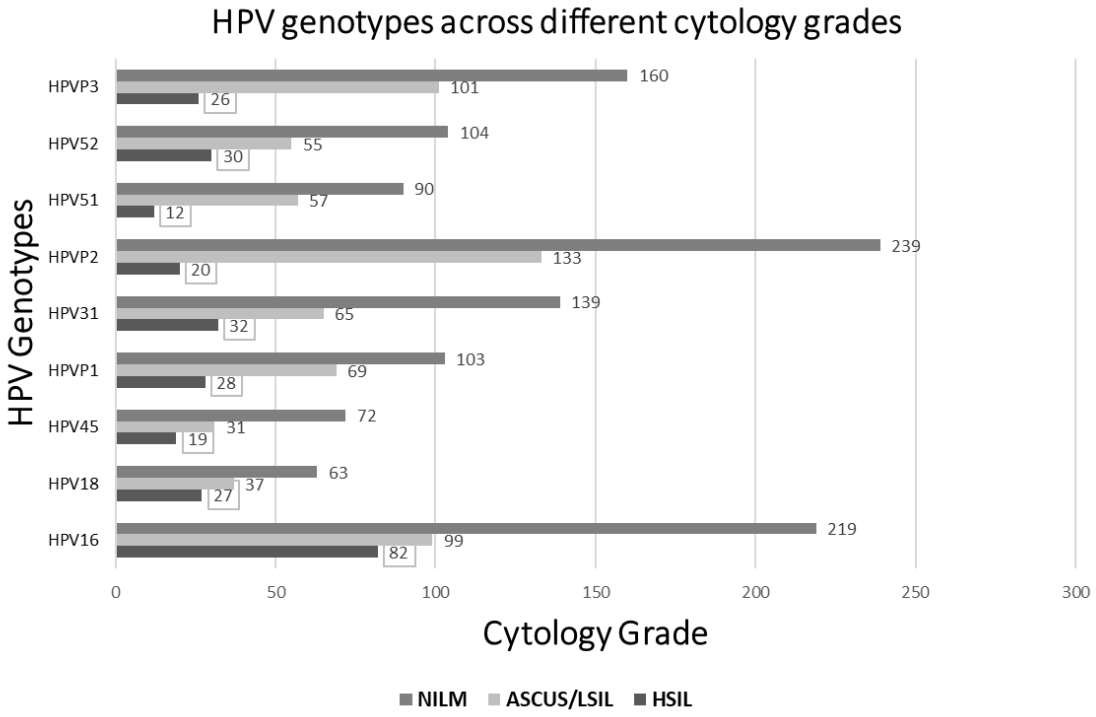

Figure 3.5 HPV genotype distribution number across different cytology grades.

*13 Unsatisfactory, four unavailable excluded. (P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

3.5.6 Clinical performance of extended HPV genotyping

Not all HPV-positive women will have cellular changes, which could lead to cervical cancer and pre-cancers. Positive predictive value (PPV) helps us to understand how many women will actually get high-grade abnormalities. The specificity will help us to know how many of those who tested HPV-negative did not have any cervical cell abnormalities on follow-up. The combination of PPV and specificity is important to assess the utility of the triage method for referring fewer women to colposcopy and detecting more CIN2+. At the same time, absolute risk weighs the possibility of a particular genotype causing cervical cell abnormalities compared to its prevalence in a particular population. This is important because certain genotypes, like HPV18, may have a lower prevalence but carry a higher risk of developing CIN2+ abnormalities. Therefore, HPV genotype performance was analysed using PPV, specificity and absolute risk.

For this analysis, histology and/or cytology follow-up data collected up to 48 months following enrolment in the study was used. Data was available in 869 cases (293 CIN2+ and 576 <CIN2; 194 CIN3+ and 675<CIN3). For analysis purposes, cases where histology was Normal or CIN1 and women with two consecutive NILM cytology under-surveillance groups were categorised as <CIN2. Table 3.4

represents the sensitivity, absolute risk (number of CIN2+/CIN3+ divided by the prevalence), positive predictive value (PPV) and specificity calculated for CIN2+ and CIN3+.

Table 3.4 Clinical performance of extended genotypes when all infections are counted regardless of whether they were present as single infections or multiple infections

HPV Types	CIN2+ n=293, <CIN2 576				CIN3+ n=194, <CIN3 675			
	Sensitivity % (95% C.I.)	Absolute Risk (95% C.I.)	PPV% (95% C.I.)	Specificity % (95% C.I.)	Sensitivity % (95% C.I.)	Absolute Risk (95% C.I.)	PPV% (95% C.I.)	Specificity % (95% C.I.)
HPV16	45.0 (39.3-51.0)	32.7 (28.1-37.5)	49.2 (43.1-55.4)	76.4 (72.7-80.0)	51.6 (44.3-59.0)	25.0 (20.8-29.5)	41.0 (34.6-47.4)	75.5 (72.1-78.7)
HPV18	14.0 (10.2-18.5)	32.0 (24.1-40.8)	47.7 (37.0-58.7)	92.2 (90.0-94.2)	15.6 (10.8-21.5)	23.4 (16.4-31.7)	38.0 (27.3-49.6)	91.7 (89.3-93.6)
HPV45	9.0 (6.0-12.8)	21.3 (14.4-30.0)	33.0 (22.7-44.4)	90.8 (88.1-93.0)	6.8 (3.6-11.3)	10.7 (5.8-17.5)	19.4 (10.7-31.0)	90.2 (87.7-92.3)
HPVP1	17.7 (13.5-22.6)	17.7 (12.7-23.7)	40.0 (31.5-50.0)	86.5 (83.4-89.1)	20.3 (15.0-26.7)	19.2 (14.0-25.3)	33.0 (24.7-42.3)	86.6 (83.8-89.0)
HPV31	19.4 (15.1-24.5)	23.7 (18.5-29.6)	39.0 (31.1-47.4)	84.5 (81.3-87.4)	22.0 (16.2-28.4)	17.5 (13.0-23.0)	34.1 (25.8-43.2)	85.0 (82.0-87.5)
HPVP2	19.8 (15.4-24.8)	14.6 (11.3-18.4)	25.5 (20.1-31.7)	70.7 (66.8-74.3)	16.1 (11.2-22.1)	10.4 (7.2-14.4)	17.0 (12.0-23.3)	71.7 (68.1-75.0)
HPV51	11.5 (8.0-15.4)	20.6 (14.6-28.0)	34.7 (25.5-45.2)	89.2 (86.4-91.6)	10.4 (6.5-15.6)	12.5 (7.8-18.6)	25.0 (16.0-36.0)	89.0 (86.4-91.3)
HPV52	16.7 (12.6-21.5)	26.0 (20.0-33.0)	40.2 (31.4-49.4)	87.3 (84.3-90.0)	18.2 (13.0-24.4)	18.5 (13.2-24.8)	32.4 (23.7-42.1)	87.1 (84.3-89.5)
HPVP3	19.1 (14.8-24.1)	19.3 (15.0-24.3)	35.0 (27.7-42.7)	81.8 (78.4-84.8)	16.1 (11.2-22.1)	10.7 (7.4-14.8)	21.4 (15.0-29.0)	81.2 (78.0-84.0)

*P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68

In 293 CIN2+, all infections observed were counted regardless of whether they were present as single or multiple infections. The sensitivities for CIN2+ with 95% C.I. were HPV16:45% (C.I. 13.9.35.1), HPVP2:19.8%(C.I. 15.4-24.8), HPV31:19.4%(C.I. 15.1-24.5), HPVP3:19.1%(C.I. 14.8-24.1), HPVP1:17.7% (C.I. 13.5-22.6), HPV52:16.7% (C.I. 12.6-21.5), HPV18:14.0%(C.I. 10.2-18.5), HPV51:11.5% (C.I. 8.0-15.4) and HPV45:9% (C.I.6-12.8). HPV16 and 18 genotypes were associated with more than 59% of CIN2+. Non-HPV16/18 were associated with 41% CIN2+. HPV16 was the most prevalent in CIN2+, followed by HPVP2 and HPV31 (Table 3.4).

The PPV for CIN2+ varied from 25.5% (HPVP2) to 49.2% (HPV16). HPV18, HPVP1, and HPV52 had a PPV of more than 40%. The specificity varied from 76.4% (HPV16) to 92.2% (HPV18). HPV18 had the highest specificity (92.2%), followed by HPV45 (90.8%) and HPV51 (89.2%).

The absolute risk was calculated and categorised as low, medium and high by calculating the number of CIN2+ against the prevalence of each genotype in 293 CIN2+ cases. A high absolute risk of 32.7% (C.I. 28.1-37.5) was associated with HPV16 and 32.0% (24.1-40.8) in HPV18. The medium risk associated with CIN2+ was seen in 26.0% (C.I.20.0-33.0) HPV52, 23.7, (95% C.I. 18.5-29.6) HPV31, 21.3% (C.I. 14.4-30.0) HPV45 and 20.6% (C.I.14.6-28.0) HPV51. HPVP1, P2 and P3 showed a low risk of less than 20%. This differs from HPV genotype sensitivities, where HPV16 had the highest sensitivity, followed by HPVP2 and HPV31.

In 194 CIN3+ cases, when all infections observed were counted regardless of whether they were present as single infections or multiple infections, the sensitivities were HPV16:51.6% (C.I. 44.3-59.0), HPV31:22.0% (C.I. 16.2-28.4), HPVP1:20.3% (C.I. 15.0-26.7), HPV52:18.2% (C.I. 13.0-24.4), HPVP2 and HPVP3:16.1% (C.I. 11.2-22.1) and HPV18:15.6% (95%C.I. 10.8-21.5). HPV51 was associated with 10.4% (C.I. 6.5-15.6), and HPV45 was associated 6.8% (C.I. 3.6-11.3) with CIN3+. HPV16 and HPV18 were associated with more than 67.0% of CIN3+ detection in this study. HPV16 was the most prevalent in CIN3+ detection, followed by HPV31 and HPVP1.

The PPV for CIN3+ varied from 17% (HPVP2) to 41% (HPV16). HPV18 showed the highest specificity, followed by HPV45.

HPV16 had the highest absolute risk for CIN3+ (25%), followed by HPV18 and HPVP1. The absolute risk calculated in 194 CIN3+ cases showed a high absolute risk of 25.0% (C.I. 20.8-29.5) associated with HPV16 and 23.4% (95%C.I. 16.4-31.7) for HPV18. The medium risk associated with CIN3+ was seen in HPVP1: 19.2% (C.I.14.0-25.3), HPV52:18.5% (C.I. 13.2-24.8), HPV31:17.5% (C.I.13.0-23.0) and HPV51:12.5% (C.I 7.8-18.6). HPV45, P2 and P3 showed a low risk of less than 11%.

3.5.7 Performance of single infection of individual genotypes and group genotypes in CIN2+ and CIN3+ histology

The extended genotyping data in this study were analysed to see if there is a difference in the prevalence and association with CIN2+ and CIN3+ lesions when HPV is counted as a single infection regardless of whether it was present as a single infection of individual genotype such as HPV16 or multiple infections such as HPV16, HPV18 or more. Six genotypes and three individual groups of genotypes were compared as a single infection with their performance in histology for CIN2+ and CIN3+ (Table 3.5).

Table 3.5 Clinical performance of single infections in extended genotyping using BD Onclarity™ positive cases								
HPV Types	**CIN2+ n=293, <CIN2 576**				**CIN3+ n=194, <CIN3 675**			
	Sensitivity % (95% C.I.)	**Absolute Risk (95% C.I.)**	**PPV% (95% C.I.)**	**Specificity % (95% C.I.)**	**Sensitivity % (95% C.I.)**	**Absolute Risk (95% C.I.)**	**PPV% (95% C.I.)**	**Specificity % (95% C.I.)**
HPV16	19.8% (15.4-25.0)	27.4% (21.5-34.0)	49.1 (39.7-58.6)	86.7% (83.2-89.7)	22.0% (16.2-28.4)	19.8% (14.7-25.8)	36.2 (27.5-45.6)	87.1% (84.3-89.5)
HPV18	3.7% (2.0-6.6)	21.6% (11.3-35.3)	42.3 (23.3-63.1)	96.6% (94.5-98.1)	3.6% (1.5-7.4)	13.7% (95.7-26.3)	27.0 (11.6-47.8)	95.5% (93.7-97.0)
HPV45	2.4% (1.0-5.0)	12.0% (5.0-23.0)	26.0 (11.1-46.3)	95.5% (93.1-97.2)	0	0	0	95.4% (93.5-96.8)
HPVP1	4.8% (2.6-8.0)	14.4% (8.1-23.0)	32.5 (19.1-48.5)	93.5% (90.8-95.6)	5.2% (2.5-9.4)	10.3% (5.1-18.1)	23.2 (11.7-38.6)	93.8% (91.7-95.5)
HPV31	7.2% (4.5-10.7)	17.5% (11.2-25.5)	40.4 (27.0-55.0)	93.0% (90.3-95.2)	8.8% (5.2-13.8)	14.2% (8.5-21.7)	32.1 (20.0-46.3)	92.6% (90.3-94.4)
HPVP2	6.1% (3.7-9.5)	8.6% (5.2-13.2)	23.4 (14.5-34.4)	86.7% (83.2-89.7)	3.6% (1.5-7.4)	3.3% (1.3-6.7)	9.1 (3.7-17.8)	86.4% (83.5-89.0)
HPV51	0.7% (0.08-2.4)	3.0% (0.4-10.4)	9.5 (1.2-30.4)	95.7% (93.4-97.4)	0	0	0	95.2% (93.3-96.7)
HPV52	4.1% (2.1-7.0)	14.0% (7.4-23.1)	30.8 (17.0-47.6)	94% (91.3-96.0)	3.6% (1.5-7.4)	8.1% (3.3-16.0)	18.0 (7.5-33.5)	93.6% (91.5-95.3)
HPVP3	5.5% (3.1-8.7)	11.7% (6.8-18.3)	28.1 (17.0-41.5)	90.8% (87.7-93.3)	4.2% (1.8-8.0)	5.8% (2.5-11.2)	14.0 (6.2-25.8)	92.0% (89.7-94.0)

*P1: HPV33/58, P2

When counted as a single infection per genotype, sensitivity for CIN2+ varied from 2.4% (HPV45) to 19.8% (HPV16). Both individual and group genotypes showed more than 86% specificity when counted as a single infection. The absolute risk was calculated and categorised as high, medium and low using overall HPV prevalence in 1497 and HPV genotype as a single infection by excluding coexisting infections associated with 95% C.I. in 293 CIN2+ cases. A high absolute risk of 27.4% (C.I.21.5-34.0) was associated with HPV16, and 21.6% (C.I.11.3-35.3) was associated with HPV18. Medium risk was associated with CIN2+ 17.5% (C.I. 11.2-25.5) HPV31, 14.4% (C.I. 8.1-23.0) HPVP1, 14.0% (C.I. 7.4-23.1) HPV52, 12.0% (C.I. 5.0-23.0) and HPV45 11.7% (C.I. 6.8-18.3) HPVP3. HPVP2 and 51 showed a low risk of less than 10%. When counted as a single infection, HPV16 had the highest PPV (49%) for CIN2+, followed by HPV18 and HPV31. HPV18 had the highest specificity, followed by HPV51 and HPV45. The top three HPV genotypes for the estimate of CIN2+ were HPV 16, 18 and HPV52 in terms of PPV.

When genotypes were counted as a single infection, sensitivity ranged between 3% and 23% for CIN3+. In the CIN3+ outcome, except for HPV45 and HPV51, these two genotypes were not associated as single infections with CIN3+ in this study. All genotypes showed a specificity of more than 86% for CIN3+.

The absolute risk stratification was calculated and categorised using overall prevalence in 1497 and HPV genotype as a single infection by excluding coexisting infections associated with 194 CIN3+ cases, showing the high absolute risk of 19.8% (C.I. 14.7-25,8) associated with HPV16. Medium risk of CIN3+ was associated with 14.2% (C.I.8.5-21.7) HPV31, 13.7% (C.I. 5.7-26.3) HPV18 and 10.3% (C.I.5.1-18.1) HPVP1. HPVP2 and P3 showed a low risk of less than 10%. HPV51 and HPV45 showed no absolute risk for CIN3+ as a single infection. The top three HPV genotypes for the estimate of CIN3+ were HPV16, HPV 18 and HPV31 in terms of PPV. HPV18 showed the highest specificity (95.5%) for both CIN2+ and CIN3+, followed by HPV45 (95.4%) and HPV51 (95.2%). As a single infection, HPV31 was the second highest after HPV16 in terms of sensitivity, absolute risk and PPV in this study for CIN3+.

In summary, non-HPV16/18 genotypes were associated with more than half of CIN2+ and 40% of CIN3+ (Figure 3.6).

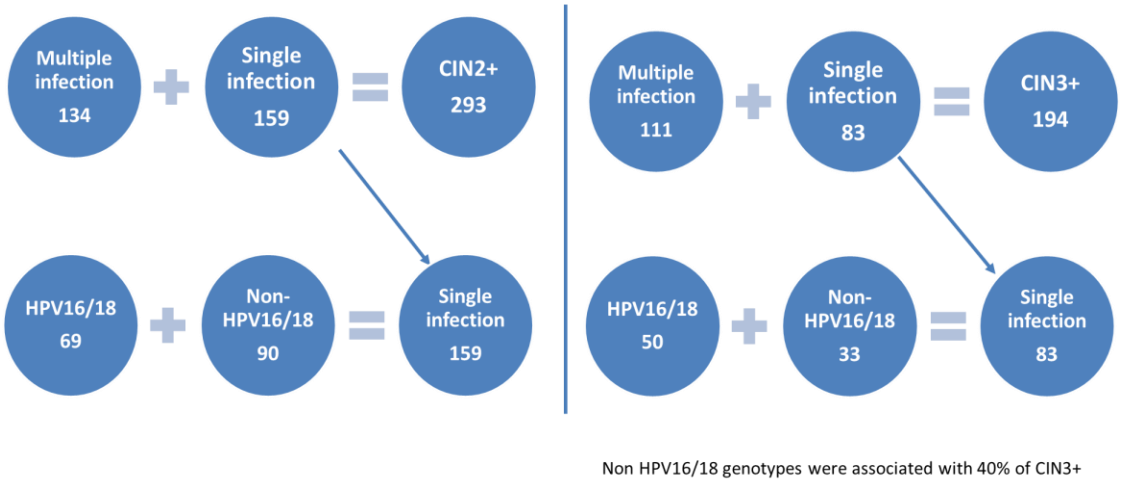

Figure 3.6 HPV genotype prevalence in CIN2+ and CIN3+

3.6 Discussion

Information about HPV genotype and its association with high-grade cervical abnormalities is important to drive the success of cervical screening. Extended genotyping can address this by giving exact information about the prevalence of high-risk HPV genotypes across non-vaccinated and vaccinated populations with or without cytology triage. It is important to highlight that this study was conducted while cytology was still the primary screening method, and the vaccination status of the women participating in this study was not known but it assumed that most women would have been unvaccinated given the national policy at that time. The study focused on high-risk HPV-positive

samples to assess the role of extended genotyping in relation to the detection of CIN2+ cervical abnormalities.

This study assessed the distribution of individual and grouped HPV genotypes using the BD Onclarity™ HPV Assay in HPV-positive cervical screening samples from the CERVIVA HPV Primary Screening Study. HPV genotypes were assessed in two different age groups—less than 30 years and 30 years and above—to see if there is any difference in genotype prevalence among these two age groups. HPV genotype distribution across different cytology and histology outcomes was assessed. The study also included the clinical performance of HPV genotyping for detecting CIN2+ and CIN3+.

Mainly, Cobas 4800 HPV DNA Test positive samples were used in this study. This is important, as different assays have different performance characteristics, use slightly different technologies and detect different gene targets. Both tests showed 81.2% concordance for the detection of HPV (Table 3.1). Of the 20% of discordant results, more than 89% had NILM cytology. Follow-up histology showed that three of these had CIN2+ histology outcomes. The BD Onclarity™ HPV Assay was positive for all CIN2+ cases. Almost 20% discordance can be partly explained due to the nature of the study population, i.e. only Cobas® positive samples were processed by BD Onclarity™. The discordant results may also be explained by 1) Sample variability between aliquots, 2) Between run variability and within run variability, and 3) Intra-laboratory variability. The samples included in this study had been stored at -20C. However, the effect of storage duration and conditions on the discordance rate is unknown. Furthermore, both assays use slightly different technologies and different gene targets and have different detection limits. The Cobas® HPV system has a detection limit of 150 (2.18 log) viral copies, and the limit for β-globin corresponds to that of a single cell (Álvarez-Argüelles et al., 2015). The Onclarity Assay detection limit is not available for comparison. The Onclarity HPV assay is a DNA assay targeting the E6 and E7 genes and has an amplification target range from 79-137 bp (J. H. Bonde et al., 2020), whereas the Cobas® HPV Assay targets the L1 gene and has a target amplification of ∼200 bp (Rao et al., 2013). Taken together, these factors may have influenced the discordance. It is important to note the objective of the study was not to compare the two platforms, as a major limitation of this study is that cases were pre-selected based on Cobas® positive results.

Another aspect, the cycle threshold was also considered. The cycle threshold (Ct value) of the virus copy number, that is, the number of cycles experienced by the fluorescent signal in each tube when it reaches the set threshold (Duan et al., 2020). There is a linear relationship between the Ct value and the logarithmic value of the number of HPV viral copies in the sample. The larger the initial viral copies, the smaller the Ct value (Álvarez-Argüelles et al., 2015). The cut-off values for Ct with the Cobas® HPV Assay are 40.5, 40.0, 40.0 and 40.0 for the four channels HPV16, HPV18, HR-HPV and beta globlin,

respectively (Wright et al., 2012) as compared to the BD Onclarity™ HPV Assay which has a Ct cut-off value for HPV 16: </= 38.3 and HR HPV except HPV 16: </= 34.2 (BD Onclarity™TM HPV Assay, 2018). Detection of HPV is dependent on the number of viral DNA copies present in the sample. A negative test result does not guarantee the absence of HPV DNA in the sample, just that the level is below a defined threshold value based on the clinical characteristics of the test (Rao et al., 2013). As commented by Engesæter, the discrepancy in test results (HPV negative vs HPV positive) may be associated with the inherent stochasticity of the PCR process (Engesæter et al., 2016), i.e., whether the Ct-value will be below or above the clinical Ct-threshold value for samples with low viral load. Whether this had any effect on this study is unknown due to the further inaccessibility of Ct values of one of the assays.

Finally, specimen storage and quality and freeze/thaw effect on the specimens may or may not have affected this discordance. Specimens for the CERVIVA HPV primary screening study were processed as they came from 2016 to 2018. Once the initial HPV testing was performed for the study, the samples were then used for a secondary round of testing after the primary screening was done. The remaining of specimen was separated into a single 15 ml tube and was stored at -20°C for completion of some of the additional triage work on methylation markers and extended genotyping. The reason for this was all testing could not be completed together, and while it is generally accepted that the quality of the samples collected in PreservCyt is stable long-term for downstream analysis, including DNA/mRNA and immunocytochemistry markers, due to space, etc aliquots were frozen for the methylation and genotyping work. The specimens were thawed once for the BD Onclarity analysis. There is no evidence that suggests freezing has an effect on PreservCyt specimens for use with BD Onclarity analysis (Myers et al., 2019).

The Cobas® HPV assays give partial genotyping information on type HPV 16 and HPV 18. Therefore, a comparison was made between the BD Onclarity™ Assay for detection of HPV 16 and 18 with the Cobas® HPV 16/18 genotyping. Excellent concordance for the detection of HPV16 and HPV18 was seen (Table 3.2). Excellent percentage agreement between (Table 3.2) HPV16 and 18 is confirmed in this study, which has been observed in a few studies mentioned above on different populations (J. Bonde et al., 2021; Cuschieri et al., 2015).

Among the worldwide population, there is a difference regarding age-wise prevalence. This is due to dominance or particular genotypes per geographical area, vaccination status, screening number and screening specifications. In this study of HPV-positive women, the older women (>30 years) showed higher HPV positivity for HPV16, 18, 31, P2, 51, 52 and P3 than younger (<30 years) women (Table 3.3, $p<0.05$). There was no statistical difference in the prevalence of HPV45 in both age groups. The

comparison with the existing studies was difficult due to variations in the study protocols, study populations and different HPV technologies. HPV16 was the most prevalent genotype in all age groups in the ATHENA trial (Monsonego et al., 2015) in the U.S. In China, a higher prevalence of HPV 16 was observed among > 60 and ≤ 20 age group (Liu et al., 2023). Another study in the Chinese population found that the proportion of HPV multiple infections among HPV-positive individuals increases with age in people older than 30 years of age (Zhou et al., 2024). In an Ethiopian study, the most HPV-positive group was aged 36 and older (Tesfaye et al., 2024). In eighteen studies including between 897 and 46,900 women from 14, mostly Northern and Western European countries, high-risk (HR) HPV prevalence peaked before age 25 or 30 years with steady declines thereafter and age-adjusted HR HPV prevalence ranged from 2% in Spain to approximately 12% in Belgium and France, where sustained elevated levels were found in women aged ⩾35 years (De Vuyst et al., 2009). This goes against the conventional wisdom that HPV infection rates decline after the age of 30–35 years, which could be related to a number of factors, such as HPV persistence, masking, decline in immunocompetence, or variation in the study groups and numbers.

For more than a decade, most HPV-detecting technologies detected overall high-risk HPV positivity rather than detecting individual genotypes as a single infection. Most HPV detection tests offered HPV16 and HPV18 detection. The development of HPV detection tests has facilitated the detection of specific HPV genotype prevalence in the form of extended genotyping. The HPV genotyping data from this present study show a slightly different genotype distribution than reported in the literature for other cohorts in different geographical regions. Although HPV16 is the most prevalent, HPV31 and HPV52 are more prevalent in the Irish cervical screening population than perhaps other types/ in different screening populations. This may be due to differences in country- or region-specific population differences or the choice of study population, such as screening or enrichment with cytology abnormal specimens. The data analysis of this study showed that HPV 16 is the most predominant type, followed by 31, 52, 51, 18 and 45. Group P2 was the predominant group, followed by groups P3 and P1. A previous study conducted by our group by (Keegan et al., 2007) in the Irish population found HPV16, 18, 66, and 33 as the most prevalent HPV type in a cervical screening population. A high percentage of HPV genotype prevalence ranging from 9 to 28% in this study can be explained by the nature of the study population, mainly consisting of HPV-positive specimens. Individual typing for HPV33 was not possible in our study due to its inclusion in group P1 in the BD Onclarity™ HPV Assay. A study in the Scottish population done in 2015 using BD Onclarity™ Assay reported HPV16, 52, 31, 51, 18 and 45 descending order prevalence (Cuschieri et al., 2015).

HPV genotyping assays carried out using different technologies and different study populations have reported HPV16 and HPV31 as the two most prevalent genotypes in studies such as ATHENA

(Monsonego et al., 2015), NMHPVPR (Wheeler et al., 2013), FUTURE (Insinga et al., 2010)(Insinga et al., 2010), KPNC (Schiffman et al., 2015), Sweden (Smelov et al., 2015), POBASCAM Baseline (Bulkmans et al., 2007) and Denmark (Kirschner et al., 2013). A cross-sectional study performed (Campos-Romero et al., 2019) in women attending a routine cervical screening in 20 states of Mexico detected HPV16, 31 and 51 as the first three and HPV18 as less prevalent, showing similarity with the first two HPV genotypes and HPV18 in our study. The most prevalent genotypes were HPV 16, 31, 51, and 53 in cervical samples among unvaccinated women from Vojvodina province, Serbia (Kovacevic et al., 2021). In the recent Canadian study, the most frequently detected genotypes were HPV56/59/66, followed by HPV16, HPV35/39/68, and then HPV31 using a similar assay and HPV-positive specimen. (Volesky et al., 2022).

Due to the historical status of cytology as the primary screening test for cervical cancer, most of the early studies on HPV detection focused on the correlation between the two. Findings shared by (Andrews, 2019) in his systematic review of 36 research articles which examined the use of genotyping with cytology concluded that reporting genotyping provided discrimination of current and future CIN2+ and CIN3+ risk. It is important to understand how genotyping can contribute to the management of positive cases, especially in persistent HPV-positive women and ASCUS cytology outcomes. In the study, Pan et al., 2020, confirmed that HP16, 18, 31, 33, 52 and 58 is an alternative strategy for ASCUS triage and can effectively reduce the high burden of colposcopy in China (Pan et al., 2020). Also, cytology is still one of the triage tests for HPV-positive women. The baseline phase of the Onclarity trial conducted to determine the screening performance of the Onclarity HPV assay for detecting cervical cancer and pre-cancer (≥CIN2) during triage of women ≥21 years with ASC-US cytology by (Stoler et al., 2018) showed HPV16 18 and 31 were more significant for ≥CIN3. More than 60% of HPV-positive women in this study were associated with NILM cytology, compared to 50-54% in the English cervical screening pilot (Rebolj et al., 2022). HPV16, HPV31 and HPV52 were associated with HSIL cytology in 10.5% of HPV-positive women. This was slightly different than in the Belgian population, reporting the most common high-risk HPV types in women with HSIL as HPV16, 31, 51,52 (Schmitt et al., 2013).

Extended genotype analysis using the BD Onclarity™ HPV Assay at a population level in different geographical regions shows the prevalence of the “Other'' high-risk HPV genotypes, i.e., non-HPV16 or HPV18, and their usefulness in disease risk stratification. As expected, both HPV16 and HPV18 were more prevalent in cases with subsequent CIN2+ and CIN3+ diagnoses. However, non-HPV16 and HPV18 genotypes carried a considerable amount of absolute risk for developing CIN2+ (> than 40%) and CIN3+ (> 50%) as demonstrated in Table 3.4. HPV16, HPV18 and HPV31 were the most common individual HPV types associated with CIN3+ in our study, similar to that observed in the Scottish

screening population (Cuschieri et al., 2019). In the U.S., a stratified study of the risk of high-grade cervical disease in women with NILM cytology recommended the addition of HPV31 due to the identification of its increased risk to HPV16 and HPV18 genotyping. (Stoler, Wright, Parvu, Yanson, Eckert, et al., 2019).

As the circulating genotypes are changing worldwide due to the success of HPV vaccination programmes, some of these genotypes are now reported to have higher positive predictive values (PPVs) than HPV18 for high-grade lesions. This phenomenon is covered extensively in an editorial by (Cuzick & Wheeler, 2016). The prevalence of HPV16, 18, 31 and 33 and their positive predictive value or relative risk for CIN2+ and CIN3+ were compared (Cuzick & Wheeler, 2016). HPV16 was number 1 in 9 out of 10 studies. The interesting fact was HPV33 was number 2 in 6/10 studies, and HPV31 was number 3 or 4 in 8/10 studies. In our study, based on CIN2+outcome, HPV16 was number one, followed by HPV18 and HPV52 in terms of PPV and absolute risk. Based on the CIN3+ outcome, HPV16 was number one, followed by HPV 18 and HPV31 in terms of PPV. Extended genotyping may help us understand the potential impact of HPV vaccination on HPV distribution and, ultimately, HPV-based screening in the future. According to J. Bonde (J. Bonde et al., 2019), genotype information can be used with advanced screening algorithms where individual follow-up recommendations are issued for HPV16 and 18.

Extended genotype absolute risk was calculated as the proportion of CIN2+/CIN3+ against the prevalence of each genotype using similar analysis in other studies (Kjær et al., 2010; Naucler et al., 2007). In this study, HPV16, HPV18 and HPVP1(33,58) were the first three genotypes/groups for the absolute risk for CIN2+ and HPV16, HPV31, and HPV18 were the first three genotypes/groups for the absolute risk for CIN3+. This analysis semphasises the importance of genotyping. HPV16 conferred the greatest absolute risk of ≥CIN3 both in women aged 25-29 years and ≥30 years, followed by HPV31, HPV52 and HPV18 in the ATHENA trial (Monsonego et al., 2015). A population-based Swedish study during a four-year follow-up reported HPV 16, 31 and 33 as the first three carrying genotype-specific risk for CIN2+ (Naucler et al., 2007). In a Danish population study, HPV16, HPV18, HPV31, and HPV33 infection were associated with high absolute risks for CIN3+ (Kjær et al., 2010). Prior to this, a multicentre U.S. clinical trial on archival specimens using BD Onclarity™ found the relative risk of CIN3 disease in women positive for HPV31 or HPV33/58 (combined) was comparable to that of HPV18 (Wright et al., 2014).

Identification of HPV genotype as a single infection can be advantageous to choose the most appropriate HPV detection test, HPV vaccination type and management strategies for triaged HPV-positive women. Although more and more HPV tests are offering some kind of genotyping, it is mainly

focused on nine high-risk HPV types. Most HPV tests detect HPV16 and HPV18 and others as a group of different groups. Limitations of widely used PCR based HPV tests in detecting single HPV infection and effects in the Japanese population have been discussed recently (Sakamoto et al., 2018). Single infection and its association with clinical performance was carried out in individual and grouped HPV genotypes in BD OnclarityTM positive cases. As a single infection, HPV31 outperformed HPV18 in this study. Another interesting finding was that HPV45 and HPV51 were not associated with CIN3+ detection as a single infection, which was different from a study that reported that HPV45 was the third most common genotype involved in invasive cervical carcinoma and was associated with 6% of cervical cancers (de Sanjose et al., 2010). Another study suggested that HPV45 was the third most carcinogenic type after HPV16 and HPV18 (Guan et al., 2012).

It must be acknowledged that the sensitivity of HPV testing will be affected by changing patterns of HPV genotypes due to HPV vaccination and depending on different geographical regions, differences in populations and migration of the populations. This will influence the identification of specific genotypes to evaluate their frequency in epidemiological studies (Pesic et al., 2019). Multiple HPV subtype infections can have an effect on the sensitivity of HPV tests and their influence on risk stratification (Trottier et al., 2006)(Trottier et al., 2006). As mentioned above, it is important to consider the fact that there will be mixed populations of women vaccinated and unvaccinated, requiring screening, and screening approaches need to be tailored differently for different groups.

3.7 Limitations

The study aimed to evaluate extended genotyping in HPV-positive individuals and provide further detail of the extended genotypes identified within the 14 high-risk types for which the samples tested positive. Therefore, it is not possible to comment on population level-based HPV type-specific prevalence, as we only focused on samples that screened positive for one or more of the 14 high-risk HPV types. The genotype grouping with the BD Onclarity™ does not allow for calculating the individual prevalence of particular HPV genotypes in those groups, e.g., HPV33 in group P1, as stated above.

3.8 Conclusion

The study highlights the distribution of high-risk HPV genotypes in an HPV-positive cervical screening cohort from the Irish cervical screening population. High-risk HPV genotype prevalence ranged from 8.1% (HPV45) to 27.0% (HPV16). The most frequent individual genotypes are HPV16 and 31, and HPVP2 and P3 are group genotypes. Non HPV16 genotypes were associated with more than half of CIN2+. More than 40% absolute risk estimate for CIN2+ and more than 50% absolute risk for CIN3+ associated with non-HPV16 and HPV18 suggests that extended genotyping may be useful in risk stratification, especially in non-vaccinated and partially vaccinated women. Extended genotype may

improve the specificity of detection of CIN2+. HPV genotyping offers a useful triage strategy in HPV-positive women (see Chapter 4).

3.9 Future

In the future, HPV extended genotyping using HPV31, 33, 52,51, 18, and 45 could be the key to managing young, sexually active women who may be susceptible to recurrent infection. This is potentially beneficial to clinicians to track true genotype persistence during follow-up (Stoler, et al., 2019). Genotypes other than HPV 16/18 should not be underestimated in organised HPV primary screening (Kares et al., 2019), in risk stratification (Stoler et al., 2019), post-treatment follow-up and in persistent high-risk HPV-positive women(Wong et al., 2019). Extended genotyping may also be used to monitor persistent infection with high-risk genotypes over time.

3.10 References

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Chapter 4 Extended Genotyping for HPV as a Potential Triage Strategy for HPV-positive Women from Primary Screening Study

4.1 Introduction

A positive primary cervical screening test (e.g. HPV testing) increases the risk of cervical pre-cancer (Wentzensen et al., 2016) and a triage test aids in distinguishing these HPV-positive cases requiring continued surveillance or referral to colposcopy while safely balancing lower colposcopy referral and unnecessary treatments (Benevolo et al., 2024). Triage strategies are influenced by the risk of CIN progression and clinical management, and the risk thresholds may vary between health systems and populations (Wentzensen et al., 2016). The triage test positive referral rate is determined by the threshold used for the referral. When cytology is used as a triage, ASCUS and above leads to over-referral of HPV-positive women, especially in young women. Negative cytology tests can be a cause of anxiety in women due to the knowledge of HPV positivity.

These imbalances create a need for an improved triage test. Risk-based management of HPV-negative women varies from minimal risk, where HPV-negative women are screened at minimal intervals. HPV-positive and cytology negative women with low risk are subjected to repeat testing, and HPV-positive and ASCUS plus cytology women with medium and high risk are referred to colposcopy, some of whom undergo the treatment for CIN (Figure 4.1).

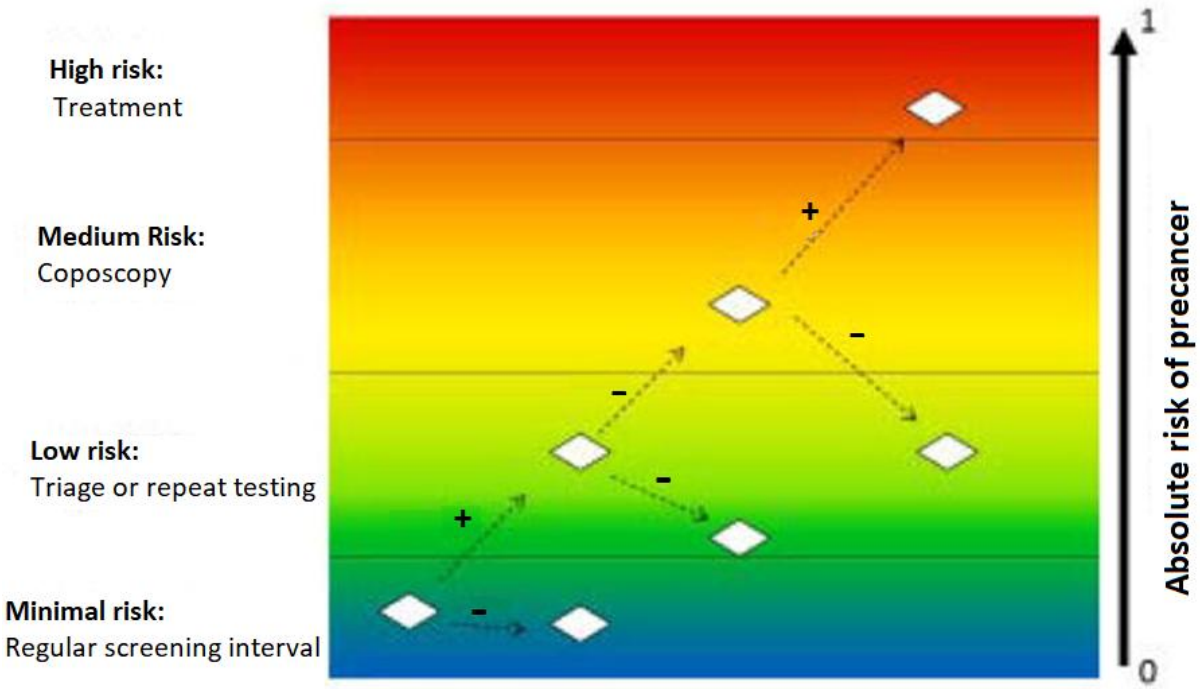

Figure 4.1 Risk-based management in cervical screening. The absolute risk of pre-cancer from 0 to 1 is shown on the y-axis. Clinically relevant risk strata are shown, ranging from minimal to high risk of pre-cancer (Wentzensen et al., 2016).

Currently, used triage methods vary depending on country-specific cervical programme policies based on the availability of resources. These include HPV genotyping, cytology testing, visual inspection with acetic acid (VIA) or colposcopy. Partial genotyping using HPV16 and HPV18 has been evaluated in some studies (El-Zein et al., 2023; Gori et al., 2021; Stanczuk et al., 2017; Wright et al., 2011) and is currently implemented in Australia and Sweden. As per 2015 European guidelines, reflex cytology followed by repeat cytology or by hrHPV retesting if reflex cytology is negative are recommended as triage methods (von Karsa et al., 2015). In Ireland, the Health Information and Quality Authority recommended HPV testing as the first screening test and cytology testing as the follow-up (triage) test every five years for women aged 25 to 60 years in 2017 (HTA of HPV Testing for Cervical Cancer Screening | HIQA, 2017). CERVIVA, in partnership with the Irish Cervical Screening Programme-CervicalCheck, has undertaken an HPV primary screening study, which evaluates and compares different strategies for triaging women with an HPV-positive primary screening test. HPV primary screening was implemented in Ireland in March 2020. Recent studies on extended genotyping in HPV-positive women show promising results (Pruski et al., 2024; Stoler et al., 2023; Volesky et al., 2022). In this chapter, extended HPV genotyping is evaluated as a potential triage strategy using the above approach. Extended genotyping is compared to other triage test datasets available within the CERVIVA Primary Screening Study, including cytology and CINTec® PLUS.

4.2 Hypothesis

Extended genotyping, on its own or in combination with other triage tests of HPV-positive samples, may improve the performance of the cervical screening process in terms of detection of high-grade cervical abnormalities and reducing the number of women unnecessarily referred to colposcopy.

4.3 Aim

- To compare the clinical performance of extended genotyping to other triage methods as a triage method for colposcopy referral and detecting high-grade cervical abnormalities.
- To assess the impact of extended genotyping as an alternative to current triage methods on colposcopy referral rates and detection of CIN2+ disease.
- To explore the impact of multi-stage triage incorporating extended genotyping and other triage methods on the clinical performance for detection of CIN2+ and referral rates to colposcopy

4.4 Methods

4.4.1 Data Analysis

Follow-up data was available for the first 4 years on all women enrolled in the study. The primary disease endpoint was histologically confirmed CIN2 or greater [CIN2+]. Histologically confirmed CIN1/Normal and women who remained under surveillance with no histology or did not require treatment were categorised as having no high-grade disease (<CIN2) at 4 years. Women with two consecutive NAD on cytology were considered to have no detectable disease and classified, for analysis, as <CIN2. CIN3+ was considered a secondary disease endpoint. CIN3 or greater was classified as CIN3+; the category <CIN3 included women with two consecutive NAD cytology tests during the 4 year follow-ups, women under surveillance with no histology and histology Normal, CIN1 and CIN2. Clinical performance, over 4 years follow-up, for extended genotyping, cytology (current standard approach) and CINtec® PLUS staining (data obtained from the larger CERVIVA HPV primary Screening dataset) was assessed by calculating sensitivity for detection of CIN2+ and CIN3+ and specificity for detection of <CIN2.

The potential real-world impact of each extended genotyping as a triage method compared to cytology (the current standard approach) and CINTec® PLUS staining (data obtained from the larger CERVIVA HPV primary Screening dataset) was established by applying the study data to the proposed cervical screening algorithms. The estimated number of triage tests required, colposcopy referrals and proportion of CIN2+ and CIN3+ detected for extended genotyping were compared to other triage methods (cytology and CINtec® PLUS).

4.5 Results

4.5.1 Clinical performance of extended Genotyping using the BD OnclarityTM HPV Assay compared to other triage approaches

The clinical performance of extended HPV genotyping using the BD Onclarity HPV Assay was compared to that of cytology (current standard approach) and CINtec® PLUS staining (data obtained from the larger CERVIVA HPV primary Screening dataset) in terms of sensitivity, specificity, PPV (positive predictive value) and NPV (negative predictive value). Over 48 months, histology and/or cytology follow-up was available in 869 cases. Histology follow up confirmed 293 CIN2+ and 576 <CIN2, 194 CIN3+ and 675 <CIN3 (Table 4.1).

Table 4.1 Clinical performance of Extended Genotyping using the BD Onclarity™ HPV Assay compared to other triage approaches

Triage strategies	CIN2+ n=293, <CIN2 576				CIN3+ n=194, <CIN3 675			
	Sensitivity% (95% C.I)	Specificity% (95% C.I)	PPV% (95% C.I)	NPV% (95% C.I)	Sensitivity% (95% C.I)	Specificity% (95% C.I)	PPV% (95% C.I)	NPV% (95% C.I)
Cytology (N=1480)	81.6 (76.6-85.8)	59.7 (55.6-63.7)	50.7 (46.0-55.3)	86.6 (83.0-90.0)	80.7 (74.4-86.0)	53.2 (49.3-57.0)	33.0 (29.0-37.3)	90.6 (87.3-93.3)
CINTec (N=1096)	75.6 (69.0-81.4)	70.0 (63.1-72.5)	53.8 (47.7-59.7)	85.0 (81.0-89.0)	73.0 (64.7-80.2)	62.2 (57.7-66.6)	36.0 (30.4-42.0)	91.5 (88.5-94.6)
HPV16	45.0 (39.3-51.0)	76.4 (72.7-80.0)	49.2 (43.1-55.4)	73.2 (70.0-76.7)	51.6 (44.3-59.0)	75.5 (72.1-78.7)	41.0 (34.6-47.4)	84.5 (81.4-87.3)
HPV18	14.0 (10.2-18.5)	92.2 (90.0-94.2)	47.7 (37.0-58.7)	67.8 (64.4-71.0)	15.6 (10.8-21.5)	91.7 (89.3-93.6)	38.0 (27.3-49.6)	79.0 (76.-81.8)
HPV45	9.0 (6.0-12.8)	90.8 (88.1-93.0)	33.0 (22.7-44.4)	66.2 (62.8-69.5)	6.8 (3.6-11.3)	90.2 (87.7-92.3)	19.4 (10.7-31.0)	77.0 (74.0-80)
HPVP1	17.7 (13.5-22.6)	86.5 (83.4-89.1)	40.0 (31.5-50.0)	67.4 (64.0-70.7)	20.3 (15.0-26.7)	86.6 (83.8-89.0)	33.0 (24.7-42.3)	79.0 (76.0-82.0)
HPV31	19.4 (15.1-24.5)	84.5 (81.3-87.4)	39.0 (31.1-47.4)	67.4 (63.8-70.8)	22.0 (16.2-28.4)	85.0 (82.0-87.5)	34.1 (25.8-43.2)	79.0 (75.8-82.0)
HPVP2	19.8 (15.4-24.8)	70.7 (66.8-74.3)	25.5 (20.1-31.7)	63.4 (59.5-67.0)	16.1 (11.2-22.1)	71.7 (68.1-75.0)	17.0 (12.0-23.3)	74.8 (71.2-78.0)
HPV51	11.5 (8.0-15.4)	89.2 (86.4-91.6)	34.7 (25.5-45.2)	66.4 (63.0-69.7)	10.4 (6.5-15.6)	89.0 (86.4-91.3)	25.0 (16.0-36.0)	77.5 (74.4-80.4)
HPV52	16.7 (12.6-21.5)	87.3 (84.3-90.0)	40.2 (31.4-49.4)	67.3 (63.8-70.7)	18.2 (13.0-24.4)	87.1 (84.3-89.5)	32.4 (23.7-42.1)	78.7 (75.6-81.6)
HPVP3	19.1 (14.8-24.1)	81.8 (78.4-84.8)	35.0 (27.7-42.7)	66.5 (63.0-70.0)	16.1 (11.2-22.1)	81.2 (78.0-84.0)	21.4 (15.0-29.0)	77.0 (73.7-80.0)

*P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68

In terms of sensitivity (81.6%) and NPV (86.6%), cytology outperformed in comparison to CINtec PLUS and individual and grouped HPV genotypes. This was not unexpected as the study design was biased towards cytology as cytology was the management test at the time of study and was focused mainly on HPV-positive women, leading to all ASCUS+ cytology cases being referred to colposcopy. This was also accompanied by the fact that ASCUS reported on cytology could be due to a few different conditions such as inflammation, infection, hormone-related, any other benign processes or due to pre-cancer onset. CINtec PLUS showed relatively high sensitivity compared to each genotype. Combined HPV16 and/or HPV18 sensitivity for CIN2+ (53%) and CIN3+ (60%) was lower than Cytology and CINtec PLUS (Supplementary data). In terms of specificity, extended genotyping for either individual or grouped HPV types was better than either cytology or CINtecPLUS triage. For detecting CIN2+ and CIN3, specificity was the highest for HPV18 (92.2%). All genotypes were more than 80.0% specific except HPVP2 (70%). Extended genotype outperformed CINtec PLUS and cytology in terms of specificity.

For cytology triage, the PPV was 50.7% and 33.0% for CIN2+ and CIN3, respectively. Triage with CINtec® PLUS showed a PPV of 53.8% for CIN2+ and a PPV of 36% for CIN3+. For CIN2+, HPV16 showed a PPV of 49.2% for CIN2+ and 41.0% for CIN3+. PPV for CIN2+ for other genotypes was as follows: HPV52 40.2%, HPVP1 40.0%, HPV31 39.0%. PPV for CIN3+ varied from 17% (HPVP2) to 41% (HPV16). PPV of HPV16 was the highest, followed by HPV18, compared to cytology and CINtec® PLUS plus for CIN3+ detection. HPVP2 showed the lowest PPV for CIN2+ and CIN3+ compared to cytology, CINtec® PLUS Plus and all other genotypes. In terms of PPV, triage with CINtec PLUS was better than cytology and extended genotyping with individual or grouped HPV genotypes for CIN2+ and in detecting CIN3+, HPV16 and HPV18 outperformed PPV compared to Cytology and CINtec PLUS.

4.5.2 Clinical performance of HPV16 combined with other genotypes, cytology and CINtec PLUS in CIN2+

The reported rate for HPV16 and HPV18 associated with cervical cancer is 60-70% (Guan 2012), which has led to the recommendation of immediate colposcopy referral as per ASCCP guidelines (Castle, 2011). HPV16, combined with other genotypes, cytology and CINtec plus, was analysed in terms of sensitivity, specificity, PPV and NPV (Table 4.2).

Table 4.2 Clinical performance of HPV16 combined with other genotypes, cytology and CINtec PLUS

HPV Types	CIN2+ n=293, <CIN2 576											
	Sensitivity% (95% C.I.)			Specificity (95% C.I.)			PPV% (95% C.I.)			NPV% (95% C.I.)		
	With other genotypes	With cytology	With CINtec PLUS	With other genotypes	With cytology	With CINtec PLUS	With other genotypes	With cytology	With CINtec PLUS	With other genotypes	With cytology	With CINtec PLUS
HPV 16/18	53.0 (47.0-58.7)	45.0 (39.3-51.0)	28.7 (23.6-34.2)	69.6 (65.7-73.3)	88.0 (85.1-90.6)	88.0 (85.1-90.6)	47.0 (41.5-52.5)	65.7 (58.7-72.2)	55.0 (46.7-63.0)	74.4 (70.5-78.0)	76.0 (72.5-79.1)	70.8 (67.3-74.1)
HPV 16/45	52.2 (46.3-58.1)	44.0 (38.3-50.0)	29.7 (24.5-35.3)	68.2 (64.2-72.0)	87.1 (84.1-89.8)	87.7 (84.7-90.2)	45.5 (40.1-51.0)	63.5 (56.5-70.2)	55.1 (47.0-63.0)	73.7 (69.8-77.4)	75.4 (72.0-78.6)	71.0 (67.5-74.3)
HPV 16/P1	57.3 (51.4-63.1)	48.8 (43.0-54.7)	33.1 (27.7-38.8)	64.4 (60.3-68.3)	85.1 (82.0-88.0)	85.4 (82.3-88.2)	45.0 (40.0-50.2)	62.4 (55.8-68.7)	53.6 (46.0-61.0)	74.8 (70.7-78.6)	76.7 (73.1-79.8)	71.5 (68.0-75.0)
HPV 16/31	57.3 (51.4-63.1)	46.8 (41.0-52.6)	31.1 (25.8-36.7)	62.0 (58.0-66.0)	85.1 (82.0-88.0)	86.8 (83.8-89.5)	43.4 (38.4-48.5)	61.4 (54.7-68.0)	54.5 (46.6-62.2)	74.1 (70.0-78.0)	75.8 (72.4-79.1)	71.2 (67.7-74.5)
HPV 16/P2	59.0 (53.2-64.7)	49.1 (43.3-55.0)	29.7 (24.5-35.3)	51.2 (47.0-55.4)	79.7 (76.2-83.0)	83.8 (80.6-86.8)	38.1 (33.6-42.7)	55.2 (49.0-61.3)	48.3 (40.8-56.0)	71.1 (66.5-75.4)	75.5 (72.0-79.0)	70.8 (67.2-74.2)
HPV 16/51	49.8 (44.0-55.7)	42.0 (36.3-48.0)	27.3 (22.3-32.8)	66.8 (62.8-70.7)	87.0 (84.0-89.6)	88.5 (85.6-91.0)	43.3 (38.0-48.8)	62.1 (55.0-69.0)	54.8 (46.3-63.0)	72.4 (68.4-76.1)	74.7 (71.2-78.0)	70.5 (67.1-73.8)
HPV 16/52	54.0 (48.0-59.7)	45.4 (39.6-51.3)	28.7 (23.6-34.2)	65.3 (61.2-69.2)	86.6 83.6-89.3)	86.3 (83.2-89.0)	44.1 (39.0-49.4)	63.3 (56.4-70.0)	51.5 (43.6-59.4)	70.8 (66.7-74.6)	75.7 (72.3-79.0)	70.4 (67.0-73.7)
HPV 16/P3	58.7 (52.8-64.4)	49.1 (43.3-55.0)	32.1 (26.8-37.8)	61.1 (57.0-65.1)	83.0 (79.6-86.0)	85.1 (82.0-88.0)	43.4 (38.5-48.5)	59.5 (53.0-65.7)	52.2 (44.7-59.7)	74.4 (70.2-78.3)	76.2 (72.7-79.5)	71.1 (67.6-74.5)

*P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68

HPV16 combined with one of the other genotypes (HPV18 or 45 or P1 or 31 or 51 or 52) and cytology outperformed in terms of PPV and specificity compared to all other combinations, cytology alone and CINtec PLUS alone strategies. Individual All genotypes combined with HPV16 had PPV between 43 to 47% compared to 50.71% of cytology alone and 53.84% of CINtec PLUS alone strategies. Individual genotypes with HPV16 and combined cytology had PPV of more than 60%, except HPV P2 and P3. Individual genotypes with HPV 16 and CINtec PLUS had PPV of more than 50%, except for HPVP2.

HPV16 combined with other individual and grouped genotypes had a sensitivity between 49-59%,42-49% when combined with cytology, and 27-34% when combined with CINtec PLUS for the detection of CIN2+. These were considerably low compared to cytology alone and CINtec PLUS alone strategies. Individual genotypes with HPV16 had specificity above 60% except for HPVP2 and above 80% when combined with cytology except for HPV with P2. Specificity above 80% was seen with the combination of the same genotypes when combined with CINtec PLUS.

4.5.3 Comparison of Cytology, CINtec® PLUS staining and genotype combination sensitivity and specificity for CIN2+ detection

Using sensitivity and specificity, a comparison was made between cytology, CINtec® PLUS plus staining, and genotype combination with these two (figure 4.8). The sensitivity for CIN2+ detection was 81.6% in cytology and HPV positive, 75.6% in CINtec® PLUS and HPV positive. The specificity was 59.7% in cytology and HPV positive and 77.0% for CINtec® PLUS and HPV positive.

The sensitivity for CIN2+ detection in genotype combinations was HPV16/18/31:64.5%, HPV16/18/P1:63.8%, HPV16/18/52:61.0% and HPV16/18/45: 60.0%. Genotype combinations HPVP1/P2/P3 and HPVP1/31/P2 had a sensitivity of 47.8% and 46.4% for CIN2+ detection. Combining these HPV genotype combinations with cytology or CIntec Plus did not improve sensitivity (Figure 4.2).

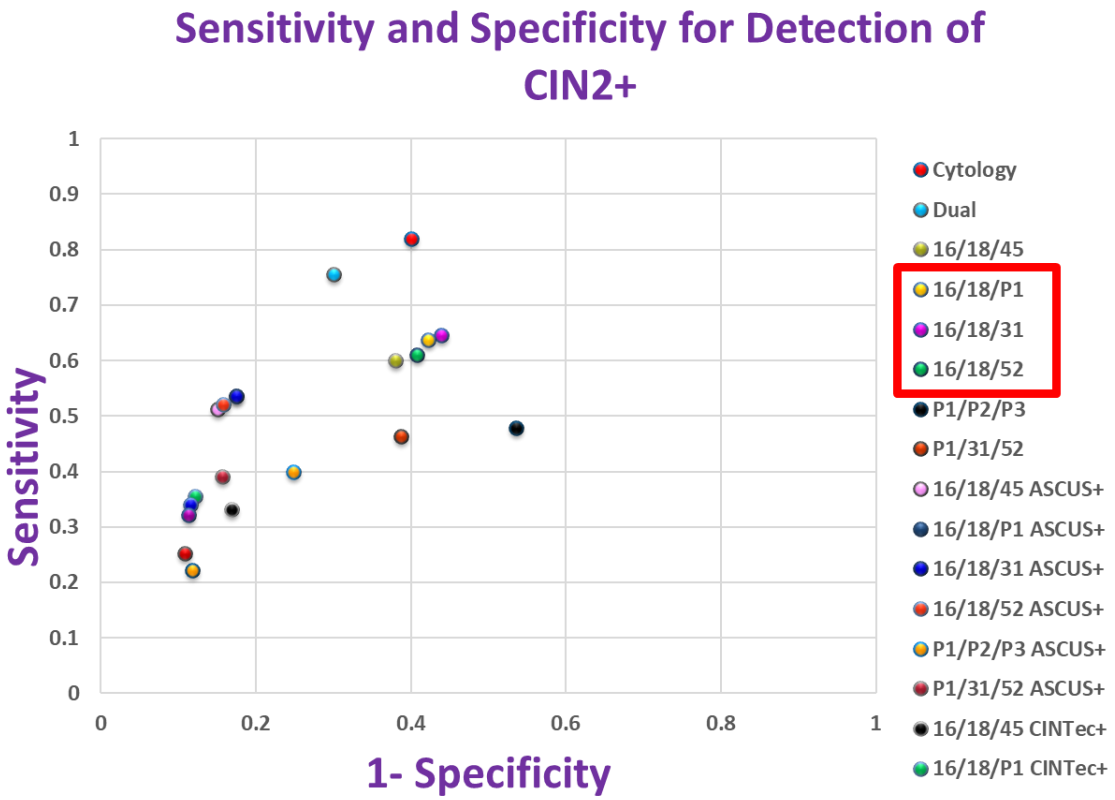

Figure 4.2 Sensitivity and specificity of triage strategies for detection of CIN2+. Cytology had lower specificity than HPV16/18 combined with HPV31, HPVP1 and HPV52. In terms of sensitivity and specificity, these three HPV combinations outperformed the strategies of cytology alone and CINtec PLUS alone. (P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

4.6 The impact of extended HPV genotyping as a triage strategy on colposcopy referrals compared with other triage methods

To assess the impact of extended genotyping as a triage test on the number of triage tests and colposcopy referral rates for the detection of CIN2+ and CIN3, we modelled the data using the different proposed screening /triage algorithms to estimate the differences in number of triage tests and colposcopy referral rates between different algorithms and their impact on the detection of CIN2+ disease. The potential real-world impact of each extended genotyping as a triage method method compared to cytology (the current standard approach) and CINtec® PLUS Plus staining (data obtained from the larger CERVIVA HPV primary Screening dataset) was established by by applying the study data to the proposed cervical screening algorithms. The estimated number of triage tests required, colposcopy referrals and proportion of CIN2+ and CIN3+ detected for extended genotyping were compared to other triage methods (cytology and CINtec® PLUS- Figure 4.3).

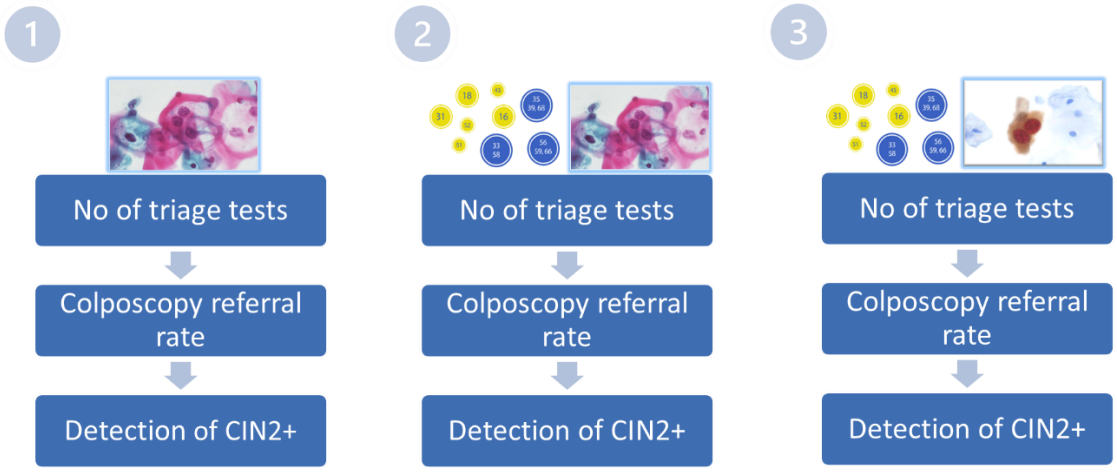

Figure 4.3: Metrics assessed for different triage strategies. 1: Cytology as a triage strategy, 2: Extended genotype with cytology as a triage strategy, 3: Extended genotype with CINTec PLUS as a triage strategy

4.6.1 HPV primary screening with cytology triage

Cytology is the current gold standard triage test for HPV primary screening. In this model, as all cases evaluated in the study were HPV positive, they would be subjected to a cytology test. Any woman with ASCUS+ on cytology would be referred for colposcopy assessment. The follow-up was available in 730 cases in this triage scenario. Using our CERVIVA dataset; this means that 38% (95%C.I. 35.6-40.6) of

women with HPV-positive screening tests would be referred to colposcopy, and high-grade disease was detected in 16% (Figure 4.4).

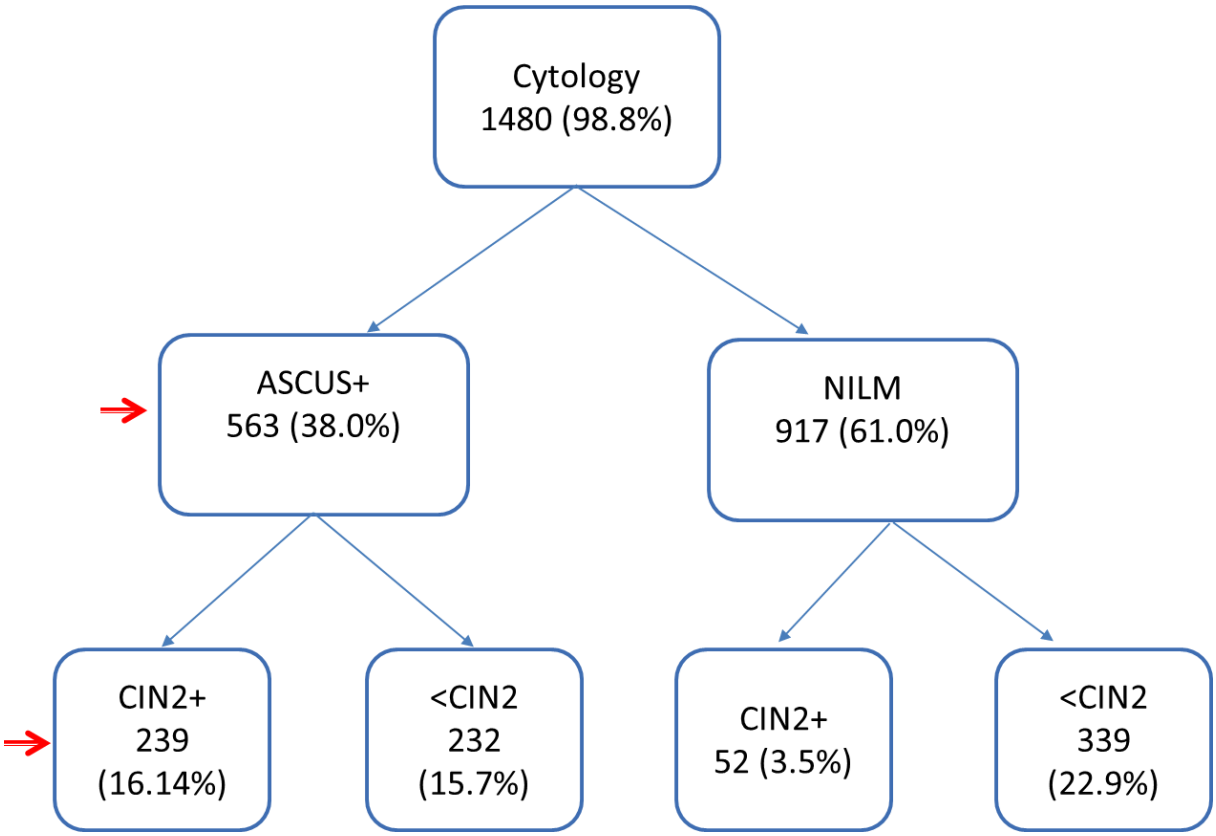

Figure 4.4 Flow chart showing screening algorithm with cytology as triage test.

The data is biased, as cytology was the standard of care test used to screen women when the study was conducted, and histology/cytology follow-up was available in 862 cases under this category.

A comparable number of those referred to colposcopy had < CIN2 and CIN2+, highlighting the potential for over-referral. In 61% (917/1480) of NILM cytology cases, follow-up confirmed 3.5% CIN2+. All 61% with negative cytology will have follow-ups after one year due to NILM cytology with the possibility of detecting those 3.5% CIN2+cases.

4.6.2 HPV primary screening with CINtec® PLUS Plus staining as a triage strategy

Data was available within the CERVIVA primary screening study to assess CINtec® PLUS as a triage strategy. Here, we model the impact of the CINtec® PLUS plus triage algorithm on colposcopy referral rates and disease detection. CINtec® PLUS PLus data was available on 1096 (73.2%) BD on clarity HPV-positive cases, and this data was used for the model below (Figure 4.5).

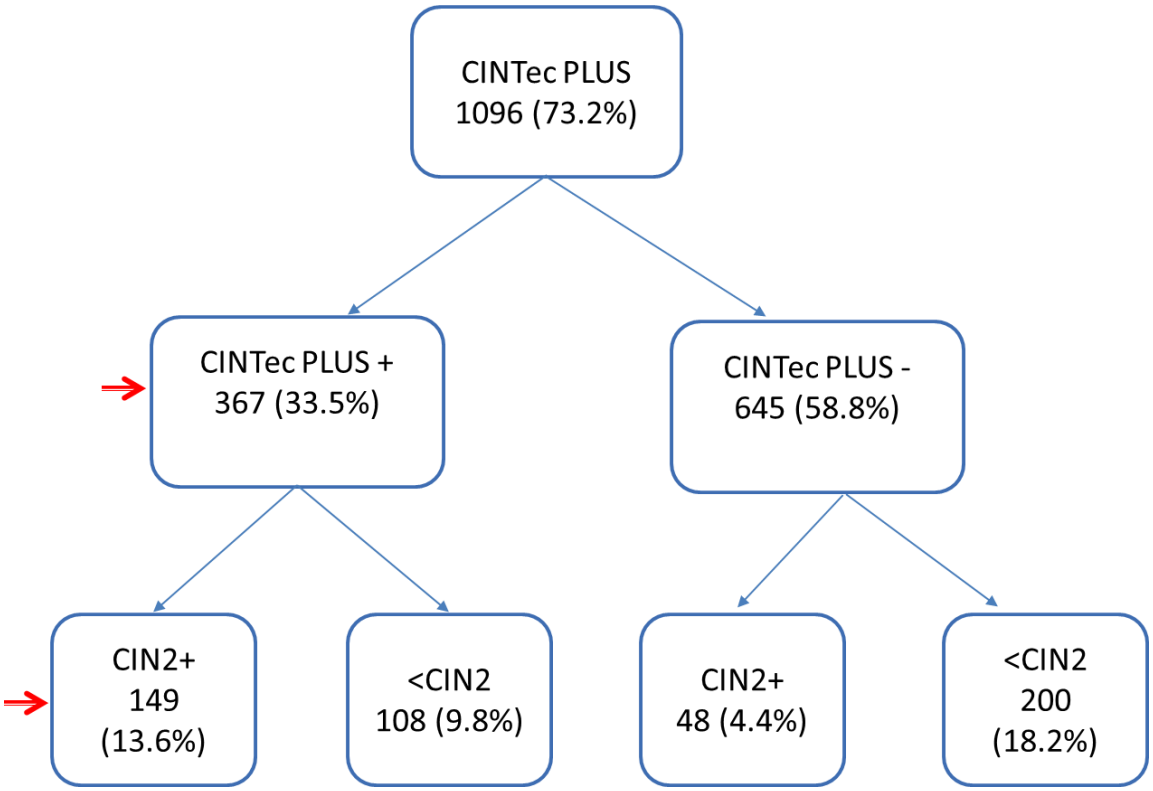

Figure 4.5 Flow chart showing screening algorithm with CINtec® PLUS plus as triage test.

CINTec positive- 367, CINTec negative- 645, insufficient and no results and others- 84. Histology/Cytology follow-up was available in 505 cases in the triage scenario

367/1096 (33.5%) of HPV-positive women were CINtec® PLUS positive, while 645/1096 (58.8%) were CINtec® PLUS negative. Adopting this as a triage test would result in 367 women being referred to colposcopy and disease detected in 149/1096 (13.6%) in 705 cases with follow-up. The percentage of HPV-positive women referred using CINtec® PLUS Plus is slightly lower than that shown for cytology triage (34% vs 38% respectively). At the same time, the proportion of disease detected by cytology triage was 2% more than CINTec PLUS triage. In 58.8% (645/1096) CINtec® PLUS negative cases, follow-up confirmed 4.4% CIN2+ and 18.2% <CIN2. Any proposed algorithm would include HPV testing at designated intervals (not defined yet) for those HPV Positive CIntec Plus negative cases.

4.6.3 HPV primary screening with extended HPV genotyping as a triage strategy

An alternative approach to triage is to incorporate HPV genotyping into triage. In this scenario, HPV-positive women could potentially be further stratified based on specific HPV types or combinations of HPV types. Using the data generated in this project, we modelled the impact of HPV extended genotyping as a potential triage strategy for HPV primary screening on colposcopy referral rates and detection of high-grade disease. Partial genotyping with HPV 16 alone resulted in the highest referral

rate of all HPV types, with 27% of HPV positive cases positive for types 16 with 9% of disease detected. Using HPV 16 genotyping alone to refer a woman for colposcopy is 11% less than cytology and 6% less than CINtec PLUS, and the CIN2+ detection rate is almost 40% less. BD Onclarity™ positive cases (Figure 4.6) had the following referral rate and disease detection if HPV genotyping was considered as a triage strategy:

HPVP2(56/59/66) group: referral rate 26.6% and CIN2+ detection 4.0%, HPVP3(35/39/68) group: referral rate 19.4% and CIN2+ detection 3.8%, HPV31: referral rate 16.0% and CIN2+ detection 3.8%, HPVP1(33/58): referral rate 13.6% and CIN2+ detedtion 3.5%, HPV52: referral rate 12.6% and CIN2+ detection 3.3%, HPV51: referral rate 10.7% and CIN2+ detection 2.2% and HPV18: referral rate 8.6% and CIN2+ detection 2.8%. HPV genotype will have a lower referral rate and detection rate compared to cytology and CINtec PLUS triage. Partial genotyping with HPV45 alone will have the lowest % referral rate of 8.6%, detecting the lowest CIN2+ (1.8%).

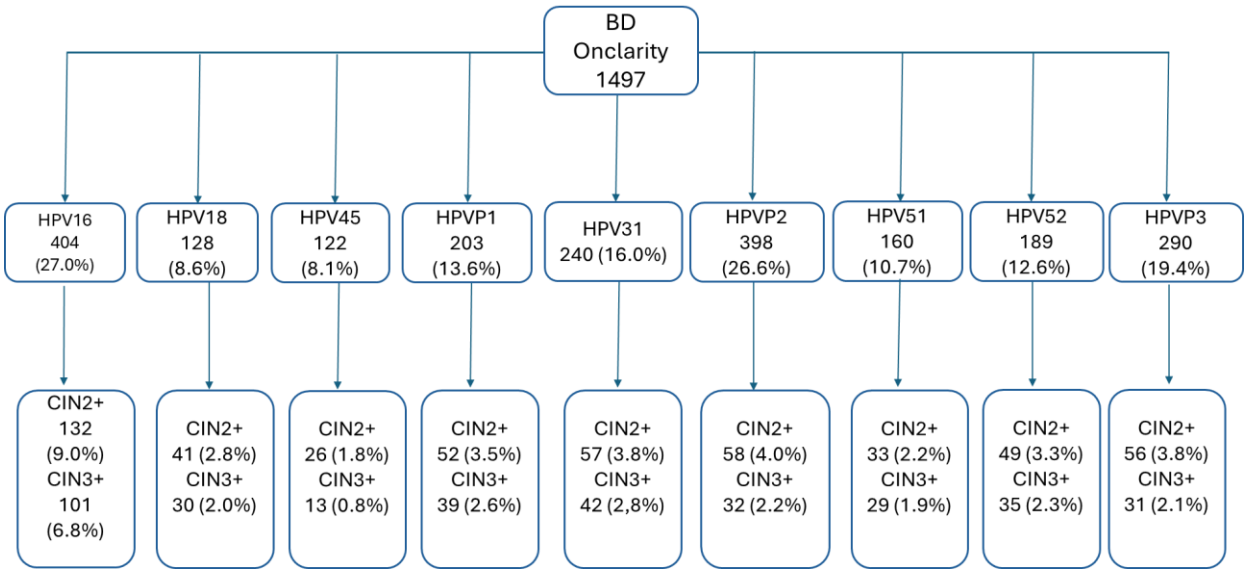

Figure 4.6 Flow chart showing HPV genotyping as a triage strategy. (The percentages are calculated from all 1497 BD positive cases.) (P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

Partial genotyping using HPV16 and other genotypes was assessed to calculate referral rate and high-grade disease. HPV16 prevalence, combined with other genotypes (Figure 4.7), varied from 33.5% (HPV16/18) to 49.9% (HPV16/P2). The referral rate increased from 6 to 23% when HPV16 was combined with other genotypes, detecting 1-2% more high-grade disease compared to HPV16 alone.

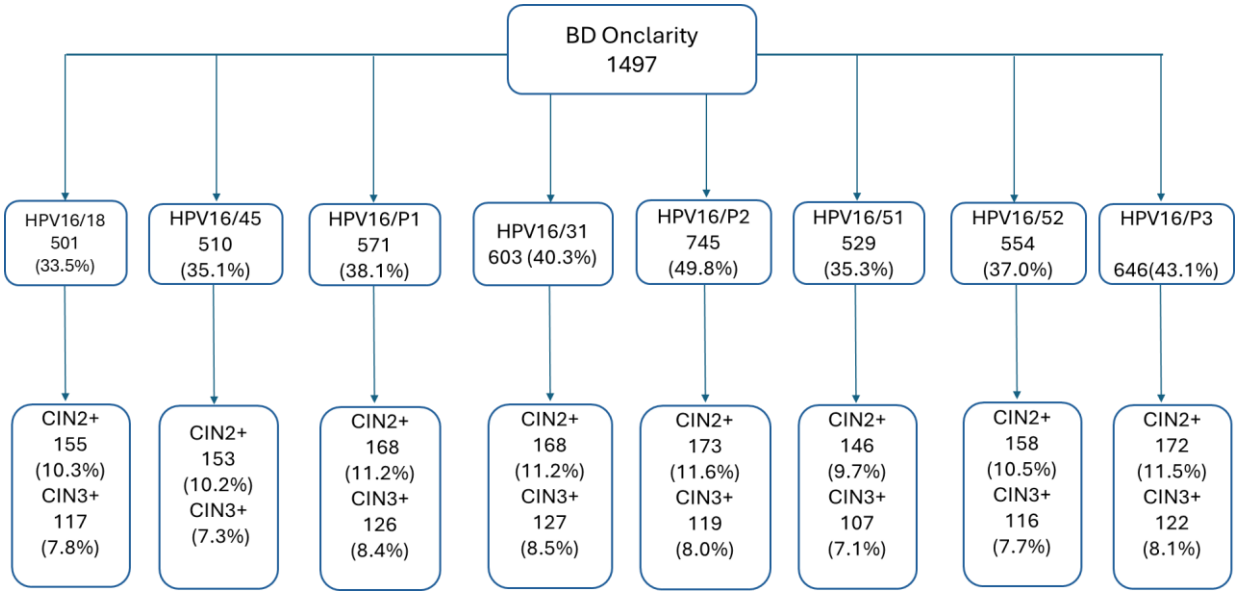

Figure 4.7 Flow chart showing HPV16 with individual and grouped genotypes genotyping as a triage strategy. (P1: HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

There was not much difference in the detection of CIN2+ when HPV16 was combined with one of the other genotypes. The detection rate varied from 10.2-11.6%, except HPVP2 with HPV16 detected 9.7% CIN2+. The detection rate of CIN3+ was 7 to 8% for all genotypes combined with HPV16. Using only genotypes or combining HPV16 with one of the other genotypes will miss 6-7% CIN2+ compared to cytology and CINtec PLUS as a triage strategy. The referral rate will vary depending on the choice of genotypes in combination with HPV16.

4.6.4 Screening algorithm with HPV genotyping combined cytology or CINtec® PLUS as a triage strategy

The introduction already alluded to the need to improve the specificity of triage options for HPV primary screening. One approach would be to combine extended HPV genotyping with the current gold standard cytology or another triage test, such as CINtec® PLUS staining. Analysis of individual HPV genotypes with cytology and CINtec PLUS decreased the referral rate compared to the partial genotype referral. However, the detection of CIN2+ cases did not improve (supplementary data). Similarly, an analysis of HPV16 combined with one of the other genotypes and cytology or CINtec PLUS was carried out, and a similar conclusion was reached (supplimentaty data). Further analysis was done by combining more than two HPV genotypes with CINtec® PLUS staining to see if the referral rate would decrease and if the detection of high-grade diseases would increase.

HPV16/18 combined with HPV45, HPVP1, HPV31 and HPV52 would result in a 40-47% referral rate which was considerably higher than all other triage strategies. Combined HPVP1/P2/P3 had a referral rate of 52.7% (Appendix IV).

4.6.5 Genotype combination with CINtec® PLUS as a triage strategy

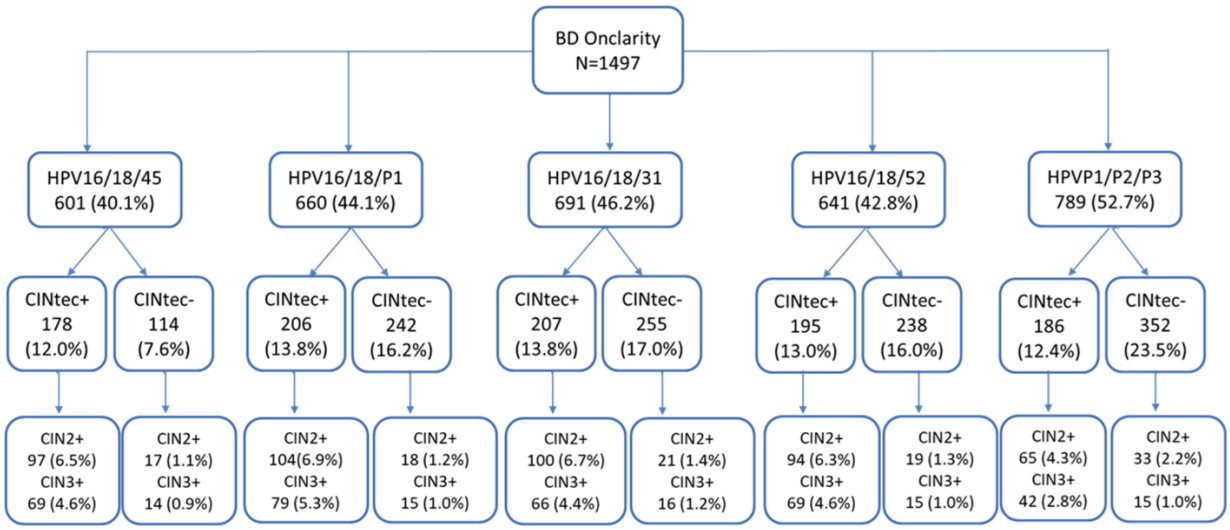

Figure 4.8 Flow chart showing three Genotype combinations with CINtec® PLUS as a triage strategy. (P1 : HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

HPV16 with one or more genotypes and combined with CINtec® PLUS plus staining were analysed for referral rate and histology outcome in 1497 Onclarity positive cases (Figure 4.8). When HPV genotypes were combined with the CINtec® PLUS plus staining test, the referral rate varied between 12-14% for the CINtec® PLUS positive group. There were 7.6% to 23.5% CINtec® PLUS negative cases in all these five groups. CIN2+ detection rate varied between 6.0% and 7.0% in the CINtec® PLUS plus positive group, which was associated with the combination of HPV16/18 combined with HPV45, HPVP1, HPV31, and HPV52. HPVP1/P2/P3 combination was associated with 4.3% CIN2+ detection. HPV16/18/P1 combination with CINtec® PLUS positive detected 5.3% CIN3+ followed by HPV16/18/45, HPV16/18/52 and HPV16/18/45. HPVP1/P2/P3 with CINtec® PLUS plus staining detected 2.8% CIN3. Compared to cytology alone (referral rate 38%) and CINtec PLUS alone (referral rate 34%) triage strategies, this strategy will have a considerably low referral rate (1/3rd), but the detection of CIN2+ will be almost half. Therefore, it is crucial to look at the different triage methods with a focus on detecting high-grade disease.

4.6.6 Genotype combination with cytology as a triage test

HPV16 with two genotypes and combined with cytology were analysed for referral rate and histology outcome in 1497 BD Onclarity positive cases (Figure 4.9).

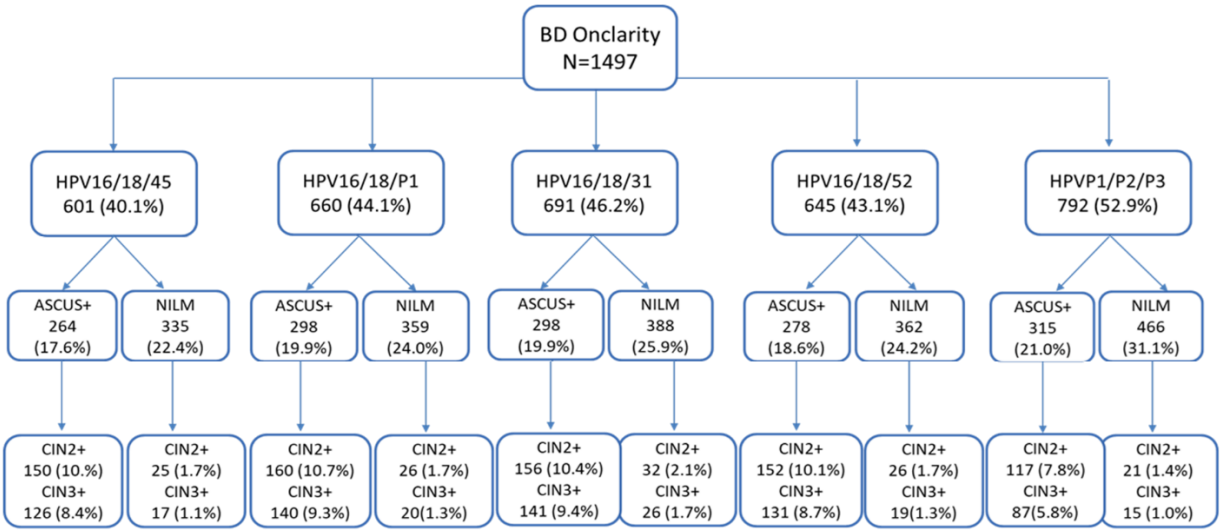

Figure 4.9 Flow chart for three genotype combinations with cytology as a triage strategy. (P1 : HPV33/58, P2: HPV59/56/66, and P3: 35/39/68)

Referral rate in HPV16/18 combined with HPV45 or HPVP1 or HPV31 or HPV52 and ASCUS+cytology varied between 16.0-19.0%. There was 22-26.0% NILM cytology for the same HPV genotype combinations, which will get HPV testing on yearly recall. HPVP1/P2/P3 combination had a referral rate of 21% in the ASCUS+ group. The same combination showed 31.1% NILM cytology. CIN2+ detection rate varied between 10.0- 11.0% in HPV16/18 combined with HPV45 (the highest-10.7%) or HPVP1 or HPV31 or HPV52 in the ASCUS+ cytology group. HPVP1/P2/P3 had detected 7.8% CIN2+. CIN3+ detection rate was the highest of 9.4% associated with HPV16/18/31, followed by HPV16/18/P1, HPV16/18/52 and HPV16/18/45 in ASCUS+ cytology group. In the NILM cytology group, the CIN2+ detection rate was less than 2.0%, except for HPV16/18/31 (2.1%), and the CIN3+ detection rate was less than 2.0% for all HPV genotype combinations. Three genotype combinations with ASCUS+ showed better detection of CIN2+ than with CINtec® PLUS by referring to 6% more cases. Compared to cytology and CINtec PLUS triage, this strategy will refer almost half (19%) to colposcopy and detect 60% of CIN2+.

4.7 Comparison of triage strategies

The data in this study was analysed to find the optimal triage strategy to reduce the number of colposcopy referrals while maintaining the corresponding PPV and specificity for detecting CIN2+.

4.7.1 Clinical performance of cytology as a triage strategy

In this first scenario, the data was modelled to reflect the current standard of care approach where cytology is used as the triage test for HPV-positive cases. In this case, 38% of women triaged with cytology would be referred to colposcopy, with a detection rate of approximately 16% for CIN2+ disease with a PPV of 50.7% and specificity of 59.7% (Table 4.3).

Table 4.3 Clinical performance of cytology as a triage strategy in CIN2+

Triage strategy	Referral rate	PPV (95% C.I.)	Specificity (95% C.I.)
Cytology	38%	50.7% (46.0-55.3)	59.7% (55.6-63.7)

4.7.2 Clinical performance of CINTec PLUS as a triage strategy

In this first scenario, the data was modelled to reflect the current standard of care approach where CINTec PLUS is used as the triage test for HPV-positive cases. In this case, 34% of women triaged with CINTec PLUS would be referred to colposcopy, with a detection rate of 13.6% for CIN2+ disease with a PPV of 53.8% and specificity of 70.0% (Table 4.4). CINTec PLUS showed improved specificity than cytology triage.

Table 4.4 Clinical performance of cytology as a triage strategy in CIN2+

Triage strategy	**Referral rate**	**PPV (95% C.I.)**	**Specificity (95% C.I.)**
CINTec PLUS	**34%**	**53.8%** **(47.7- 59.7)**	**70.0%** **(63.1- 72.5)**

4.7.3 Clinical performance of HPV16 combined with other genotypes and cytology as a triage strategy

The second scenario is whereby we use extended genotyping as part of the triage algorithm combined with cytology. In this scenario, applying extended genotyping for HPV16 along with one or more of the other HPV types reduces the number of cytology triage tests by between 50-60%, reducing the number of women referred to colposcopy from 38% if you were using cytology alone to anything from

18-20% when incorporating extended genotyping, Resulting in a detection rate of 10% for CIN2+ (Table 4.5).

Table 4.5 HPV16 Combined with Other Genotypes and Cytology

HPV Types	Referral rate (95% C.I.)	PPV (95% C.I.)	Specificity (95% C.I.)
HPV16/18	15.1 (13.3-17.0)	65.7 (58.7-72.2)	88.0 (85.1-90.6)
HPV16/45	14.8 (13.1-16.7)	63.5 (56.5-70.2)	87.1 (84.1-89.8)
HPV16/P1	17.2 (15.3-19.2)	62.4 (55.8-68.7)	85.1 (82.0-88.0)
HPV16/31	17.1(15.2-19.1)	61.4 (54.7-68.0)	85.1 (82.0-88.0)
HPV16/51	15.1(13.3-17.0)	62.1 (55.0-69.0)	87.0 (84.0-89.6)
HPV16/52	17.2(15.3-19.2)	63.3 (56.4-70.0)	86.6 83.6-89.3)
HPV16/18/45	17.6 (15.7-19.7)	63.3 (56.4-86.9)	84.9 (81.7-87.7)
HPV16/18/P1	20 (18.0-22.0)	61.3 (55.1-67.2)	82.5 (79.1-85.5)
HPV16/18/31	20 (18.0-22.0)	60.7 (54.4-66.7)	82.5 (79.1-85.5)
HPV16/18/52	18.6 (16.6-20.6)	62.5 (56.1-68.7)	84.2 (81.0-87.1)

*HPVP1: HPV33/58

4.7.4 Clinical performance of HPV16 combined with other genotypes and CINTec PLUS as a triage strategy

Finally, the third scenario uses extended genotyping as part of the triage algorithm combined with CINTec PLus.In this case, you can see applying extended genotyping for HPV16 along with one or more of the other HPV types reduces the number of triage tests by between 50-60%, reduces the number of women referred to colposcopy from 38% if you were using cytology alone to from 12-24% using extended genotyping combined with CINTec Plus (Table 4.6). Extended genotyping combination HPV16/18/45 combined with CINTec Plus showed a comparable PPV and improved specificity compared to HPV16 combined with other genotypes and cytology.

Table 4.6 HPV16 combined with other genotypes and CINTec PLUS			
	Referral rate (95% C.I.)	**PPV (95% C.I.)**	**Specificity (95% C.I.)**
HPV16/18	17.1 (15.0-19.5)	55.0 (46.7-63.0)	88.0 (85.1-90.6)
HPV16/45	17.2 (15.0-19.6)	55.1 (47.0-63.0)	87.7 (84.7-90.2)
HPV16/P1	17.9 (15.7-20.3)	53.6 (46.0-61.0)	85.4 (82.3-88.2)
HPV16/31	14.0 (12.0-16.1)	54.5 (46.6-62.2)	86.8 (83.8-89.5)
HPV16/51	17.2 (15.0-19.6)	54.8 (46.3-63.0)	88.5 (85.6-91.0)
HPV16/52	16.0 (13.8-18.3)	51.5 (43.6-59.4)	86 .3(83.2-89.0)
Hpv16/18/45	12 (10.3-13.6)	63.8 (55.6-71.4)	90.4 (87.7.-92.7)
HPV16/18/P1	13.8 (12.0-15.6)	59.8 (52.1-67.1)	87.8 (85.0-90.4)
HPV16/18/31	13.8 (12.1-15.7)	60.2 (52.4-67.7)	88.5 (85.6-91.0)
HPV16/18/52	13 (11.4-14.8)	59.1 (51.0-66.8)	88.7 85.8-91.2)

*HPVP1: HPV33/58

As depicted in Table 4.3, HPV16 combined with HPV18 and one of the other genotypes (HPV18 or HPV45 or HPVP1 or HPV31 or HPV51 or HPV52) and cytology outperformed all other triage methods in terms of PPV and specificity. Genotype combination HPV16/18/45 combined with CINtec PLUS showed PPV and specificity as good as HPV16 combined with other genotypes and cytology Table 4.4). Detecting HPVP2 and P3 on its own, in combination with HPV16 and /or cytology and CINtec PLUS, revealed lower PPV and specificity (Appendix IV).

4.8 Discussion

This chapter assesses triage strategies using extended HPV genotyping and its combination with cytology and CINtec® PLUS staining for the first time in an Irish HPV primary screening population of women enrolled in the CERVIVA HPV Primary Screening Study. The triage of HPV-positive women for the best outcome has been widely discussed and debated over a decade. There is no international consensus regarding the best triage method among various cervical screening programmes (Benevolo et al., 2024). Triage tests vary depending on country-specific national policies, available resources, expertise and vaccination status. In addition to this, societal perception of risk, established clinical practice, different weight on benefits and harms, health economic considerations, and health infrastructure play an important role in determining a choice of triage test (Wentzensen et al., 2016). Risk stratification of high-risk HPV-positive women is vital since less than 10% of women who are

persistently infected with high-risk HPV infection will develop cervical cancer (Bodily & Laimins, 2011). In Ireland, the progression risk of the individual HPV genotypes is not yet known.

In this study, when the impact of extended genotyping was looked at on colposcopy referral rates and detection of disease, extended genotyping on its own – single or in combination would result in a lower number of women referred to colposcopy and fewer disease-detected. Therefore, extended genotyping on its own is not adequate to pick up diseases and will miss a high proportion of high-grade disease. However, when combined with cytology, HPV16 with other genotypes (HPV 18 or HPVP1 or HPV31 or HPV51 or HPV52) performed the best in this study compared to another triage strategy.

Based on testing positive for HPV on a primary screen and subsequent ASCUS+ cytology on triage test, the referral rate for colposcopy was 38.0% (563/1480), leading to the detection of 13.6% CIN2+ and 9.1% CIN3+. The risk HPV positive and ASCUS+ cytology group is reported as ≥10% for CIN3+ in this group (Castle et al., 2007; Walker, 2003). A cytology-based triage test based on ASCUS+ will refer three times more HPV-positive women to colposcopy compared to any genotype or their combinations with cytology and CINtec PLUS as per the existing model. Existing cytology as a triage strategy showed the highest sensitivity for the detection of CIN2+ (81.6%) with a referral rate of 38%, which is broadly similar to the earlier Italian triage study with a referral rate of 31.2% ((Ronco et al., 2006). Cytology in HPV-positive women is expected to be much higher, in some cases 85.6% (Bergeron et al., 2015), because it is only done in HPV-positive women and also affected by the prior knowledge of HPV positivity by the screening cytologist (Ebisch et al., 2016). Cytology triage had a PPV of 33% compared to the reported PPV for CIN3+ of 38% in the Norwegian study (Arbyn et al., 2020). The specificity of the cytology as the triage strategy was the lowest for CIN2+ compared to genotypes and their combinations combined with cytology and CINtec plus.

NILM cytology in HPV-positive women remains a question. A study done on 27,037 women with normal cytology in ≥25 years women by Stoler et al. showed HPV 16/31 values exceeded the risk threshold for colposcopy referral (Stoler et al., 2019). In this study, out of 61% of HPV-positive and NILM cytology, 3.5% had CIN2+ follow-up similar to an Italian study in HPV-positive and Cytology negative on triage (Benevolo et al., 2024), and 2.5% had CIN3+ follow-up. Although the percentage is small for pre-cancer detection, it is difficult to know which of those 61% of women will have pre-cancer and will be detected on regular screening intervals at 3 or 5 years.

Cytology showed the highest sensitivity for CIN2+, but the specificity was low compared to genotypes and CINtec® PLUS. This was an expected finding, as reported in the literature (Bergeron et al., 2015; Richardson et al., 2015). To sustain cytology as a triage test will require a sufficient number of cytology

personnel. Recruiting and retaining highly trained cytology personnel is becoming a day-to-day issue in existing cytology laboratories. The performance of cytology as a triage test in self-sampling is yet to be proved (Brink et al., 2006). Although cytology outperformed in terms of sensitivity, PPV and NPV, the high referral rate, the subjectivity of the analysis, the inability to perform on self-sampled material, and, most importantly, current issues regarding recruiting and retaining cytology personnel question its survival as a triage method in the future.

The referral rate to colposcopy of CINtec PLUS was more or less similar (34% vs 38%) to cytology, detecting more or less the same number of cases (14% vs 16%). In terms of PPV and specificity CINtec PLUS triage strategy outperformed cytology alone and any genotype on its own strategies. However, combined with genotypes and their combinations, it has a considerably low PPV. CINtec PLUS dual study showed a sensitivity of 75.6% for CIN2+, more than the study done by Wright et al. (63.1%) using a similar strategy (Wright et al., 2017). Limited studies have been performed with immediate p16/Ki-67 triage of hrHPV-positive women (Ebisch et al., 2016). CINtec, as a triage method, will need more clinical trials and validation within HPV primary screening programmes to estimate cost versus identification of the high-grade disease compared to the established cytology triage. It will also have the same issues as cytology, such as the requirement of trained and experienced personnel to interpret the results, the subjectivity of the examination, the inability for high-throughput testing, and its inability to be used on self-sampled material shortcomings. Finally, negative cytology and dual-stain results still need follow-up, as well as a positive cytology dual-stain result without colposcopic or histological abnormalities (Ebisch et al., 2016).

The availability of various HPV-detecting tests, high throughput, qualified and experienced molecular scientists and options of different combinations of genotypes to be identified makes extended genotype an attractive triage strategy in HPV-positive women compared to cytology and CINtec PLUS. An Italian study published this year has suggested adopting partial genotyping in women who are cytology-negative and positive for non-16/18 HPV types and adopting extended genotyping in women cytology-negative and infected with the low oncogenic types or Onclarity negative(Benevolo et al., 2024). However, each population will have different high-risk non-16/18 HPV genotypes. Delayed triage with hrHPV testing at 12 months has been proposed in a Norwegian study for women with HPV16/18+/NILM and other hrHPV+/ASC-US-LSIL, and at 24 months for women with other hrHPV+/NILM (Arbyn et al., 2020).

Recently, extended genotyping has been explored as a triage strategy in a few studies in HPV-positive women (Benevolo et al., 2024; Stoler et al., 2023). The referral rate varies from 8-27% in this study when extended genotyping was considered with individual genotypes and grouped genotypes. CIN2+

detection rate varied from 1.8% (HPV45) to 9% (HPV16). So, genotype on its own strategy refers almost 11% less to colposcopy by detecting 9-14% less CIN2+ compared to cytology alone strategy, depending on the choice of the genotype. Extended genotyping using HPV16/18, HPV16/31, HPV16/P1 and HPV16/52 detects an additional 1-2% of CIN2+ cases with the same referral rate as cytology. As reported by other studies (Schiffman et al., 2011), HPV16 was the most associated with the detection of CIN2+ (9.0%) and CIN3+ (6.8%) singly or with ASCUS+ (7.5% and 5.7%) and CINtec® PLUS positive (6.7% and 5.2%) combinations. HPV16/18 triage in HPV-positive women has reported a sensitivity of 49.7% (Schreiberhuber, L., Barrett, J.E., Wang, 2024). Although the FDA-approved algorithm for primary HPV screening, women testing positive for HPV16 or HPV18 are referred to immediate colposcopy (Wright et al., 2015), non-HPV16 or HPV18 genotypes constituted 1.8% (HPV45) to 4.0% (HPVP2) CIN2+ histology outcome in this study. The percentage for non-HVP16 or HPV18 varied from 0.8% (HPV45) to 2.8% (HPV31) in CIN3+ outcomes compared to 6.8% of HPV16. Although the risk is reduced in non-HPV16/18 groups, it still constitutes 1/3rd of the HPV-positive cases in this study. These non-HPV16 or HPV18 do possess a risk also in HVP16/18 vaccinated cases.

HPV16 with HPV 18, HPVP1, HPV31, HPV51 and HPV52 and cytology had more than 60% PPV and more than 80% specificity (Table 4.5). This clinical performance was the highest, with the lowest referral rate compared to other triage strategies. In this study, HPV16/18 combined with ASCUS+ had a PPV of 65.7% compared to 71% reported in a Norwegian study (Arbyn et al., 2020). Combining HPV16/18 with HPV45, HPV31, HPVP1 and HPV52 had the second-best performance with a low referral rate. In terms of PPV, the same genotype combination with CINtec PLUS showed lower PPV but similar specificity.

Based on the result of this study, it is evident that more referral leads to more detection of high-grade abnormalities. However, depending on the desired referral rate, resources and achieving maximum NPV, the extended genotype can be used for risk stratification (Stoler et al., 2023) in a reliable and flexible manner. It will also help provide information for colposcopy thresholds and enhance cytology-based triage testing or contesting, as well as the utilisation of CINtec® PLUS staining in a given setup. To identify women with a low enough risk of CIN3+ that they can be referred to screening after 2 or 3 years, 3-level strategies have been suggested in one of the recent studies based on the risk level (Benevolo et al., 2024). These include the highest risk level requiring immediate colposcopy, the intermediate risk level requiring short-term HPV retesting, and the lowest risk level with sufficiently low risk to be safely referred to a new HPV testing at a longer interval. Extended genotyping can be useful for detecting these different levels. Non-HPV16 and HPV18 genotypes have become important as demonstrated in Chapter 3, especially in the post-vaccination era and risk stratification using these

can be more accurate to determine the best course of clinical action in the treatment and management of the HPV positive women (Stoler et al., 2023)).

The study shows real value of incorporating some form of extended genotyping with HPV 16 and one or more other hr HPV types. The referral rate is similar; however, the performance of the test increases with improved specificity. Therefore, it would appear that incorporating some form of partial genotyping for HPV 16 along with one or more of the other high-risk HPV types alongside cytology triage reduces the number of cytology triage tests while also reducing the number of women referred to colposcopy and increasing the specificity of the approach. This is important for two reasons. First, there are significant challenges in recruiting and retaining staff with cytology expertise and second, too often, with HPV-based screening, there is a risk of over-referring women to colposcopy, causing unnecessary anxiety for women and increasing the workload on already overburdened colposcopy services.

4.9 Conclusion

The study concludes that extended genotypes HPV16 combined with HPV18 and one of the other genotypes (HPV18 or HPVP1 or HPV31 or HPV51 or HPV52) and cytology offer the optimal triage model for HPV-positive women. HPV16/18 and one of the other genotypes (HPVP1 or HPV31 or HPV51 or HPV 52) combined with CINTec PLUS is the second-best model for improved PPV and specificity. HPV16/18/45 combined with CINTec PLUS also showed comparable PPV and specificity with HPV16 combined with HPV18 and one of the other genotypes and cytology. Genotype groups P2 (HPV types 56/59/66) and P3 (HPV types 35/39/68) can be considered lower risk based on the above data and could be the candidates for routine recall management strategy (Appendix IV). The study recommends that incorporating some kind of genotyping, followed by cytology, would reduce the colposcopy referral rate, detect more CIN2+ cases, and improve specificity. The data can inform screening practices in Ireland and internationally tailor specific triage strategies with genotyping and cytology or CINTec PLUS.

4.10 Limitations

The data is biased, as cytology was the standard of care test used to screen women when the study was conducted. Also, CINtec PLUS results were available only in 1096 cases compared to 1480 cytology cases. The study is an observational study and should be carried out as a pilot in population-based study.

4.11 References

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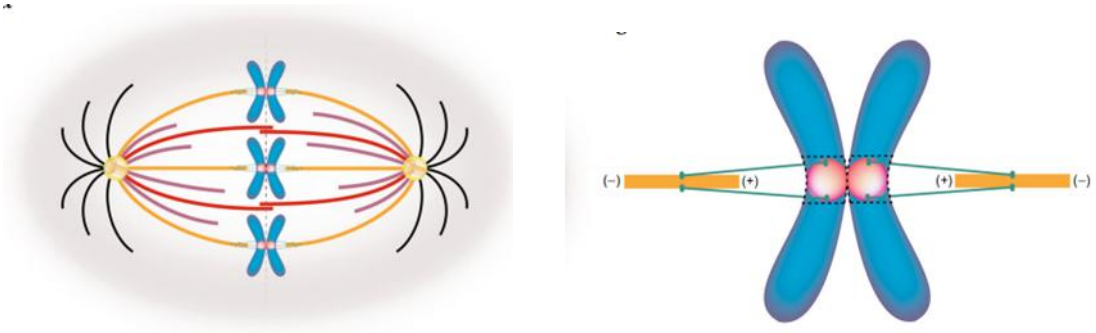
Chapter 5 Clinical Evaluation of the Utility of Biomarkers Nuf2 and Hec1 for Detecting Cervical Pre-cancer

5.1 Introduction

During mitotic cell division, chromosomes are equally distributed between the daughter cells due to the action of the mitotic spindle (Titus & Wadsworth, 2012). The mitotic spindle (Figure 5.1A) is formed by the microtubules that represent dynamic polymers of tubulin protein. Spindle microtubules are attached (Figure 5.1B) to the end of the kinetochores (large multi-protein complexes on chromosomes known as Ndc80) (Ustinov et al., 2020).



A. Mitotic spindle B. Chromosome attachment to Kinetochore microtubules

Figure 5.1 The role of the Ndc80 complex in mitosis. The Ndc80 complex mediates interactions between the microtubules and chromosomes during mitosis: A. mitotic spindle; B. bidirectional attachment of replicated chromosomes to the kinetochore microtubules (kinetochores are shown as circles on the chromosomes). The (–) and (+) ends of a pair of kinetochore microtubules attached to the sister chromatids are shown; the dashed line represents the metaphase plate, and the dashed boxes represent the centromere sections where the kinetochores are located (Ustinov et al., 2020).

The Ndc80 complex, which forms part of the outer kinetochore, comprises two heterodimers, Hec1(Ndc80)/Nuf2 and Spc24-Spc25 (Figure 5.2) and is essential for metaphase chromosome alignment and anaphase chromosome segregation (DeLuca et al., 2005). The Ndc80 complex plays a crucial role in kinetochore-microtubule interactions due to its involvement in the dynamic interface between centromeres and spindle microtubules (Ciferri et al., 2007; Tooley & Stukenberg, 2011). In addition to its role in microtubule binding by the kinetochore, the Ndc80 complex is essential for recruiting the mitotic checkpoint proteins Mad1, Mad2, and Mps1 to the kinetochore (Martin-Lluesma et al., 2002; Martin et al., 2009).

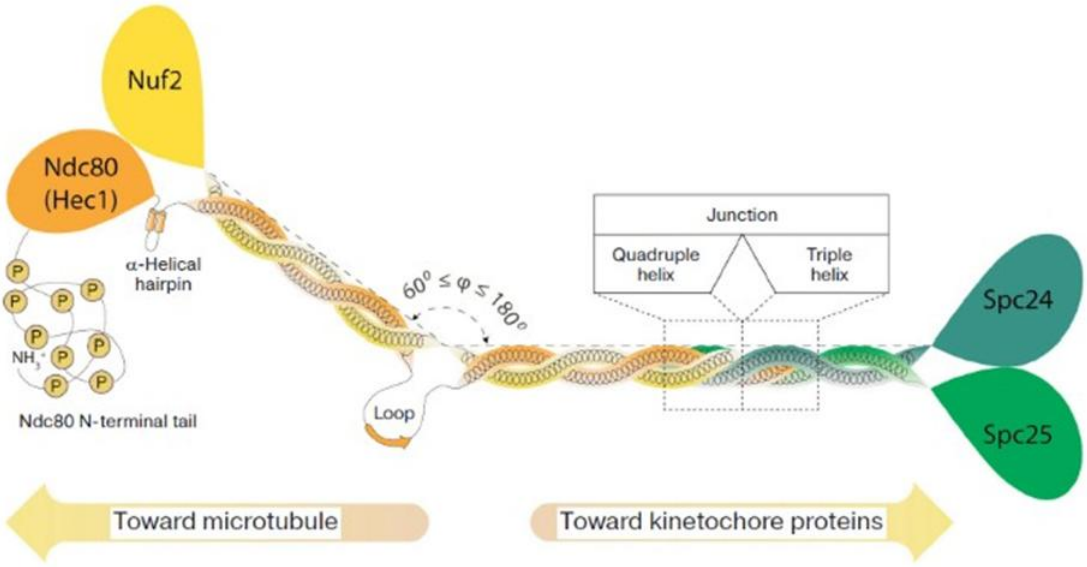


Figure 5.2 The subunit/domain architecture of the Ndc80 complex with the key structural elements and conformational flexibility. The NDC80 complex is a heterotetramer consisting of four subunits: Hec1, Nuf2, Spc24, and Spc25. Hec1 and Nuf2 form a heterodimer via their C-terminal coiled-coil regions, while Spc24 and Spc25 interact through the N-terminal fragments. The C-terminus of Hec1 (Ndc80) extends ~80 amino acids residues beyond the C terminus of Nuf2, and the N-terminus of Spc24 extends ~20 amino acids beyond the N-terminus of Spc25, which allows the Hec1 (Ndc80)/Nuf2 and Spc24/Spc25 subcomplexes to connect and form fully functional NDC80 complex. Phosphorylation sites (P) in the N-terminal tail of the Ndc80 subunit are shown by circles. The range of the angle ϕ values demonstrates the ability of the NDC80 fibrillar rod to bend in the plane of the figure (Ustinov et al., 2020).

Accurate chromosome segregation during mitosis relies on kinetochore-microtubule interactions at the mitotic spindle (Bloom, 2005). Cell cycle progression is controlled via the spindle assembly checkpoint, which monitors local tension and microtubule attachment and delays the separation of sister chromatids until they are correctly attached to microtubules from opposite poles (Ciferri et al., 2005; Santaguida & Musacchio, 2009). The Hec1-Nuf2 complex is crucial for recruiting mitotic checkpoint proteins to the kinetochore (Martin et al., 2009). Nuf2 plays a vital role in the correct attachment of kinetochore-microtubules and the normal separation of chromosomes during cell mitosis (Ren et al., 2023). It has been proposed that a prolonged pre-cancerous tetraploid (cells that

have double the number of chromosomes compared to normal cells) state facilitates a transient intermediate on the road to aneuploidy and tumorigenesis (Ganem et al., 2007) and may precede the development of an abnormal number of chromosomes, other known as aneuploidy (Margolis, 2005). Previous studies on cervical carcinogenesis have confirmed that tetraploidy and chromosomal instability are related events occurring during the early stages of cervical carcinogenesis that predispose cervical cells to the formation of aneuploidy, frequently involving the loss of chromosome 17 (Olaharski et al., 2006). The tumour suppressor proteins p53 and pRb are known to prevent the further proliferation of tetraploid cells (Ganem et al., 2007) and are inhibited by the E6 and E7 proteins of high-risk HPV types, responsible for the majority of cervical carcinomas, thus facilitating the progression from tetraploidy to aneuploidy (Münger et al., 2004).

Overexpression of Nuf2 and Hec1 genes causes hyperactivation of the mitotic checkpoint (Diaz-Rodríguez et al., leading to prolonged tetraploidy. According to Ustinove et al., this complex mechanism involves redundant or "extra" molecules of the Ndc80 complex, which do not localise at the kinetochore but concentrate in the cytoplasm, where they bind and sequester confirmed and putative loop ligands together with their binding partners. This process prevents these proteins from regulating microtubule–kinetochore attachments and provokes chromosomal defects in daughter cells. As a result, the dysregulated expression of these sequestered proteins promotes further proliferation of aneuploid cells, some of which eventually become malignant.

Nuf2 and Hec1 over-expression, at gene and protein levels, have been identified in non-small cell lung cancers, with high levels of over-expression associated with poor prognosis (Chen et al., 2021; Jiang et al., 2022). Overexpression of Hec1 and Nuf2 is reported in gastric, colorectal and pancreatic cancer (P. Hu et al., 2015; Qu et al., 2014; Sugimasa et al., 2015).

High-risk HPV bypasses cellular regulatory controls, allowing HPV oncoproteins E6 and E7 transcriptional deregulation, leading to chromosomal instability and eventually aneuploidy (Mallick et al., 2024). These authors describe micronuclei as a common consequence of chromosomal instability elicited by HPV infection via mechanisms such as Bloom syndrome protein (BLM) reduction and telomere erosion DNA damage. Other mechanisms, such as Polo Like Kinase 4 (PLK4) upregulation, lead to multipolar spindles, and centrosome-associated protein E (CENP-E) degradation leads to polar chromosomes. (Figure 5.3).

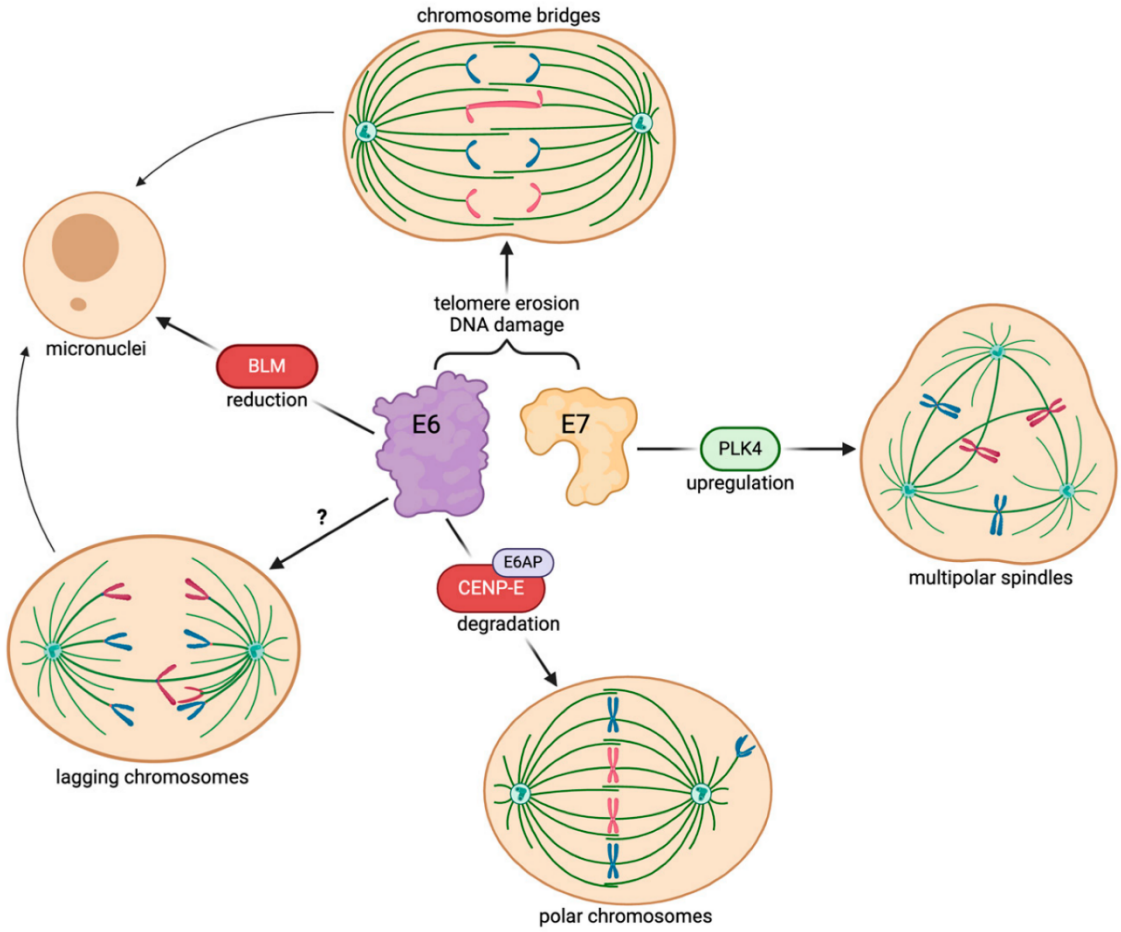


Figure 5.3 HPV E6 and E7 induced chromosomal instability via unique mechanisms. These include Bloom syndrome protein (BLM) reduction, telomerase erosion DNA damage, centrosome-associated protein E (CENP-E) degradation and PLK4 upregulation and Polo Like Kinase 4 (PLK4) upregulation (Mallick et al., 2024).

The Nuf2-Hec1 heterodimer plays a crucial role in kinetochore function and overexpression of these results in mitotic checkpoint hyper-activation (Diaz-Rodríguez et al., 2008) and the resultant development of aneuploidy. Previous gene expression work in our laboratory has shown that Nuf2 and Hec1 were among the most significantly overexpressed genes in cervical cancer cell lines compared to normal cervical tissue. Immunohistochemistry demonstrated that focal basal epithelial cell staining for Hec1 was seen in the normal cervix, while strong Hec1 positivity was seen in cervical intraepithelial neoplasia. Nuf2 staining varied from moderate to strong in CIN 1, with more variable staining patterns in high-grade cervical intraepithelial neoplasia (CM Martin Personal communication). This pattern of Nuf2 and Hec1 over-expression in cervical intraepithelial neoplasia (CIN) and cervical cancer suggested its potential use as biomarkers and is the basis for the work included in this thesis chapter.

5.2 Hypothesis

I hypothesised that the mitotic spindle complex biomarkers Nuf2 and Hec1 are dysregulated in cervical intraepithelial neoplasia (CIN), and their dysregulated expression relates to chromosomal aneuploidy in CIN.

5.3 Aim

- To technically validate and evaluate the performance of Nuf2 and Hec1 biomarkers in cervical cancer cell lines and cervical tissue samples.
- To evaluate the expression of Nuf2 and Hec1 biomarkers in cervical pre-cancer cases
- To establish the ploidy status in cervical pre-cancer to confirm that mitotic spindle formation is dysregulated in cervical pre-cancer.

5.4 Method

5.4.1 Study Cohort

Patient recruitment was conducted as described in Chapter 2 (section 2.1) through the HISTOCERV enrolment project.

Clinical recruitment resulted in the enrolment of 239 women in the HISTOCERV biomarker project. Of these 239 women, 205 had LLETZ procedures performed and were eligible for biomarker study. The age range varied from 23- 64y, of which 54 were less than 30 years, and the rest, 151, were 30 years and above. Histology breakdown consisted of 123 CIN1, 35 CIN2, 42 CIN3. The remaining five consisted of no CIN, no results available, or no slides available for review.

This was a “proof of concept” study to determine the expression of the biomarkers in cases of disease compared with normal. Analysis of the 205 eligible cases recruited for biomarker studies revealed that almost 60% had CIN1 diagnosis, consisting mainly of small biopsies in a single block. In some cases, there was insufficient tissue available for the study; in others, the representative disease tissue was not present, or the blocks or slides were not available. Due to the subjective nature of CIN2 category reporting, it was not included in this study; however, it is fully recognised this is an important category where biomarkers may be useful to aid in diagnosis. Nonetheless, for the purpose of the proof of concept evaluation, the extreme end of the disease spectrum was selected from the normal, CIN1 and CIN3 categories. Following the pathologist's review, 51 cervical tissue blocks from 33 LLETZ were chosen for immunohistochemistry for this initial clinical proof of concept study. These included 13 normal tissues from 9 LLETZ, 15 CIN1 tissues from 9 LLETZ and 23 CIN3 tissues from 15 LLETZ (Table 5.1).

Tabel 5.1 Cervical tissues selected for proof of concept study	
Histology grade	No of tissues
Normal	13
CIN1	15
CIN3	23
Total	51

Cervical tissues for the normal tissue category were selected from the LLETZ block with no CIN, CIN1, CIN2, or CIN3 categories, showing normal tissue on the review due to the lack of availability of normal LLETZ. For the CIN1 tissue category, LLETZ blocks with CIN1 and CIN2 showing CIN1 were selected on review, as agreed by the reviewing pathologists. For CIN3 tissue category, LLETZ with CIN3 block showing CIN3 were selected as agreed by the reviewing pathologist.

Processing and H&E staining of these selected cervical tissues was done as per the protocol described in Chapter 2 (Section 2.8).

5.4.2 Immunohistochemistry

Antibodies were optimised, and tissue samples were processed using the Benchmark ULTRA immunostaining system (Benchmark LT, Ventana, Tucson, Arizona, USA) and the OptiView DAB detection Kit (Ventana, USA) as detailed in Chapter 2 (section 2.11). Rabbit polyclonal Nuf2 antibody (Abcam ab230313) and Rabbit polyclonal Hec1 antibody (Thermo-Fischer Scientific PA5-102765) were optimised using an FFPE HeLa cell block. Normal and CIN3 cervical tissue served as negative and positive controls, respectively. Following optimisation, the Hec1 antibody was utilised at a dilution of 1:35, and the Nuf2 antibody was utilised at a dilution of 1:75. Antigen retrieval was performed using heat-induced epitope retrieval (HIER) using CC1 buffer (Ventana, USA) for 32 minutes. Antibody detection was performed using the DAB detection Kit.

5.4.3 D-DISH

Chromosomal instability resulting from various mechanisms, such as hyperactivation of the mitotic spindle, is known to be the pathway to aneuploidy (Thompson et al., 2010). Detection of aneuploidy can be performed by a method that quantifies chromosome numbers, such as in situ hybridisation (ISH). The VENTANA HER2 dual ISH assay is a closed two-probe system used to determine HER2 gene amplification status in breast and gastric carcinoma. This assay uses two-colour chromogenic ISH to enable the enumeration of the ratio of HER2 to chromosome 17 nuclear signals. We adapted this method for ploidy analysis by using the chromosome 17 enumeration probe to help classify the ploidy

status of these CIN cases. The VENTANA HER2 Dual ISH DNA Probe Cocktail determines HER2 gene status by enumeration of the ratio of the HER2 gene to Chromosome 17 by light microscopy (Roche, 2020). The HER2 and Chromosome 17 probes were detected using two-colour chromogenic in situ hybridisation (ISH) in formalin-fixed, FFPE cervical tissue specimens, following staining on BenchMark ULTRA IHC/ISH instruments as described in Chapter 2 (section 2.9).

5.4.4 IHC and D-DISH analysis

Two pathologists reviewed all cases. Pre and post-H&E review was performed before and after sections were taken for IHC to confirm the presence of representative cervical tissue. IHC staining pattern/localisation and staining intensity were noted for positive and negative control. Scoring of Nuf2 and Hec1 biomarkers was based on H-scoring, which includes a combination of scores for immunostaining intensity of positive cells and percentage of tumour cells displaying positivity. The pathologist scored the intensity of Nuf2 and Hec1 biomarkers on an ordinal score of 0-3. The percentage of tumour cells displaying immunostaining was estimated within a 10% increment. The H score was derived from the cross-product of the intensity score (0 to 3) and the percentage of tumour cell staining (0 to 100%), as described in Chapter 2 (section 2.11). The pathologist reviewed and scored immunostained slides for staining intensity as 0=Negative, 1=Weak, 2=Moderate and 3=Strong and percentage positivity as 0=0-10%, 1=10-40%, 2=40-70% and 4=70-100%. H scores for different grades of disease were aggregated and compared to determine any differences. HER2 D-DISH staining was evaluated for the presence of diploid and aneuploidy nuclei in HeLa tissue, negative cervical tissue and CIN3 cervical tissue. No scoring was involved in the D-DISH analysis since the purpose was to confirm aneuploidy.

5.5 Results

5.5.1 Hec1 and Nuf2 antibody optimisation

Nuf2 and Hec1 optimisation was carried out using cervical cancer cell line blocks as described in Chapter 2 (section 2B.6). Initial optimisation was performed using SiHa, HeLa and C33-A cells embedded in FFPE blocks. Additional optimisation was carried out with negative and positive cervical tissue. Initially, a titration of 1:300 was chosen for Nuf2 staining, and a titration of 1:200 was chosen for Hec1 with CC1:56 and CC1:32, based on the product data sheets. On review by the pathologist, the HeLa cell block was chosen for the purpose of further optimisation along with the negative and positive cervical tissue FFPE blocks. Sections of HeLa, negative control and positive control cervical tissues showed no staining when no antibodies were added. Nuf2 and Hec1 antibodies showed good staining in the HeLa tissue section. Tissue sections from the HeLa cell block showed strong positivity depending on the stage of cell division. The nuclear staining intensity varied in cells stained by Hec1. Nuf2 positivity was expressed as dot-like positivity in the nuclei, with accentuation of the nuclear

membrane and intense staining in most cells, both in the nucleus and cytoplasm (Figure 5.4). Some nuclei had a peripheral accentuation around the Nuclear Organising Region.

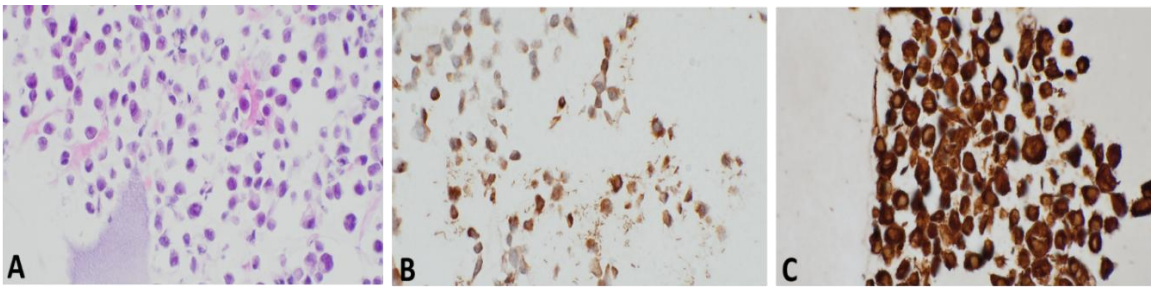


Figure 5.4 Optimisation of Nuf2 and Hec 1 in FFPE HeLa cells. A: HeLa cells H&E 400X magnification. B: HeLa cells with Hec1 optimised staining (1:35 antibody dilution and CC1:32) 400X magnification. C: HeLa cells with Nuf2 optimised staining (1:75 antibody dilution and CC1:32)) 400X magnification

Nuf2 titration varied from 1:300, 1:200, 1:100, 1:50 and 1:75 using CC1:32. On review of immunohistochemistry slides with the pathologist, 1:75 using CC1:32 was selected to stain all tissue sections. Hec1 titration varied from 1:200, 1:100, 1:50, 1:25 and 1:35 using CC1:32. On review of immunohistochemistry slides with the pathologist, 1:35 using CC1:32 was chosen to stain all tissue sections. Optimised protocol using titration of 1:75 for Nuf2 staining and 1:35 for Hec1 staining was applied to 51 LLETZ tissue sections from 33 LLETZ. These were consisted of 13 normal, 15 CIN1 and 23 CIN3 cervical tissues.

5.5.2 Hec1 and Nuf2 expression in cervical tissue

Hec1 and Nuf2 staining in 13 normal, 15 CIN1 and 23 CIN3 cervical tissue specimens were assessed for the intensity of positive cells and percentage of dyskaryotic epithelium displaying positivity. Hec1 and Nuf2 staining was identified in basal and parabasal layers of normal cervical epithelium along with endothelial, inflammatory, and stromal cells in connective tissue (Figure 5.5 A and B). It is difficult to say that the diffuse cytoplasmic staining for Nuf2 is genuine due to the polyclonal nature of the antibody or possibly a thermal artefact.

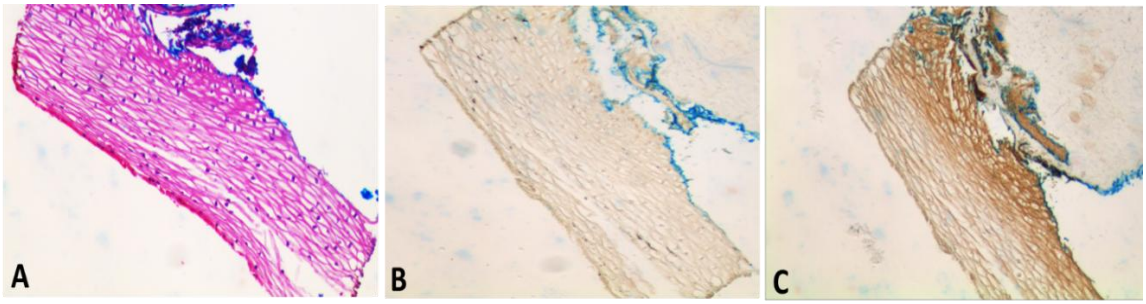


Figure 5.5 (A) Hec1 and Nuf2 expression in normal cervical tissue under optimised conditions.

A: Normal cervical tissue H&E 100X magnification. B: Normal cervical tissue with Hec1 staining 100X magnification. C: Normal cervical tissue Nuf2 staining 100X magnification.

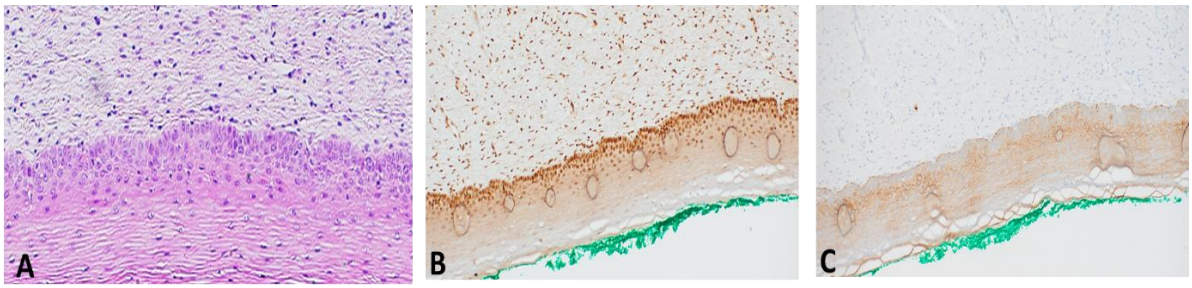


Figure 5.5 (B) Hec1 and Nuf2 expression in normal cervical tissue under optimised conditions.

A: Normal cervical tissue H&E 100X magnification. B: Normal cervical tissue with Hec1 staining 100X magnification. C: Normal cervical tissue Nuf2 staining 100X magnification.

In CIN1 cases, Hec1 and Nuf2 staining was seen with enhanced intensity in abnormal nuclei. In Nuf2 positive cases, dot-like positivity in the nuclei with accentuation of the nuclear membrane was identified (Figure 5.6). Stromal staining for HEC1 can be explained by dividing fibroblasts and dividing lymphocytes (where replisome protein-associated markers can be seen). Nuf2 did show sporadic marking of these cells, but not with the same intensity.

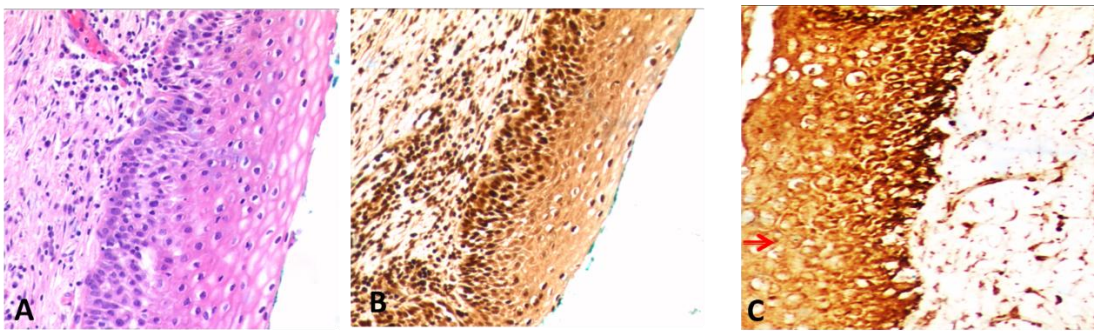


Figure 5.6 Hec1 and Nuf2 expression in CIN1 cervical tissue under optimised conditions.

A: CIN1 cervical tissue H&E 200X magnification. B: CIN1 cervical tissue and fibroblasts in non-epithelial tissue with Hec1 staining 200X magnification. C: CIN1 cervical tissue and fibroblasts in non-epithelial tissue with Nuf2 staining and dot-like positivity in the nuclei with accentuation of the nuclear membrane pointed by the arrow 200X magnification.

In CIN3 cases, the entire CIN3 section corresponding to H&E was stained with enhanced intensity in abnormal nuclei. Dot-like positivity in the nuclei with accentuation of the nuclear membrane was more prominent in CIN3 cases (Figure 5.7).

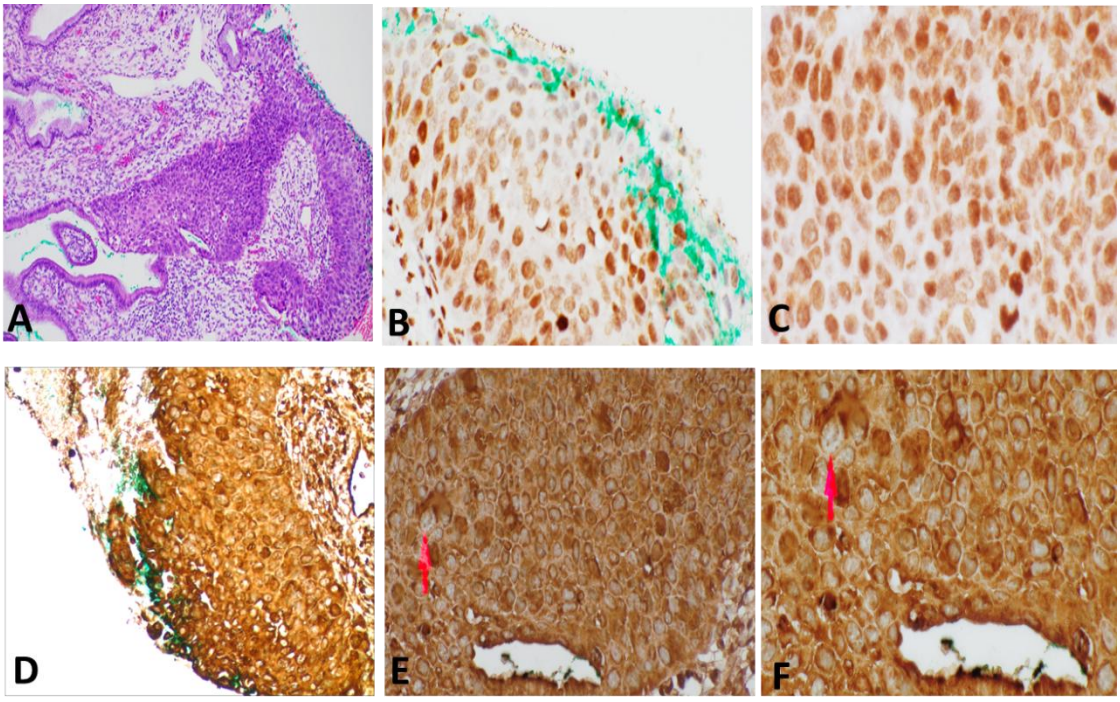


Figure 5.7 Hec1 and Nuf2 expression in CIN3 cervical tissue under optimised conditions. A: CIN3 cervical tissue H&E 100X magnification. B: CIN3 cervical tissue with Hec1 staining 400X magnification. C: CIN3 cervical tissue Hec1 staining 600X magnification. D: CIN3 cervical tissue Nuf2 staining 400X magnification. E: CIN3 cervical tissue Nuf2 staining with red arrow showing nuclear dots 400X magnification. F: CIN3 cervical tissue Nuf2 staining with red arrow showing nuclear dots 600X magnification.

In 13 normal cervical tissues, both Hec1 and Nuf2 showed basal and parabasal staining with positivity in inflammatory and endothelial cells. This was expected due to the presence of dividing cells and was not considered while scoring. As described in Chapter 2 (section 2.11), the sections were given H scores for Hec1 and Nuf2 staining in normal, CIN1 and CIN3 cervical tissues. A score of

4 or more was considered an overexpression of Hec1 and Nuf2. Table 5.1 shows Hec1 and Nuf2 scoring in 51 cervical tissues.

Table 5.2 Immunohistochemistry score for Hec1 and Nuf2

Histology		Hec1 H Score							Nuf2 H Score			
	0	1	3	4	6	9	0	1	3	4	6	9
Normal N= 13	13	-	-	-	-	-	13	-	-	-	-	-
CIN1 N=15	-	-	-	2	-	13	-	-	-		-	15
CIN3 N=23*	-	1	-	5	1	14	-	1	1	1	-	20

*Two of CIN3 tissue in Hec1 had a technical failure.

All 13 normal cervical tissue sections had H scores of 0 for Hec1 and Nuf2. Out of 15 CIN1 cervical tissue sections, two cases had an H score of 4 and 13 cervical tissue sections had an H score of 9 for Hec1 staining. Nuf2 staining had an H score of 9 in all 15 CIN1 cervical tissue sections,

Two CIN3 cervical tissues were excluded from the HEC1 analysis as they failed to pass QC on IHC. The remaining 21 CIN3 cervical tissues had H scores between 1-9, with the majority 14/21 having a H score 9. Similarly, for Nuf2, the majority of CIN3 cervical tissue had an H score of 9 (20/23), as illustrated in Table 5.2. Hec1 was overexpressed in 13 CIN1 cervical tissues, and NUF2 was overexpressed in all 15 CIN1 cervical tissues. Hec1 was overexpressed in 19 out of 21 CIN3 cervical tissue, and Nuf2 was overexpressed in 21 out of 23 CIN3 cervical tissue.

5.5.3 D-DISH staining in three CIN3 cervical cases

Overexpression of Hec1 and Nuf2 is associated with hyperactivation of the mitotic spindle, leading to chromosome instability and creating a pathway for aneuploidy. One of the currently used techniques to detect the aneuploidy is *in situ* hybridisation. The VENTANA HER2 Dual ISH DNA Probe Cocktail used in this study contains HER2 probes (labelled with the hapten dinitrophenyl or DNP) and Chromosome 17 probes (labelled with the hapten digoxigenin or DIG) formulated in a formamide-based buffer. The probes are designed to detect amplification of the HER2 gene in invasive breast carcinoma. The HER2 DNAProbe is a mixture of oligo probes that spans approximately 300,000 base pairs along the genomic region containing the HER2 gene (also known as ERBB2 and NEU), which is located on human Chromosome 17 (17q12). The Chromosome 17 probe is a mixture of oligo probes that target

sequences within the centromeric region and serves as a reference for aneusomy (cells containing different numbers of chromosomes). This study used the D-DISH technique to confirm the presence of aneuploidy in cervical tissue, which also showed over-expression of Hec1 and Nuf2. D-DISH evaluation of C17 and HER2 in three selected CIN3 cervical tissues showed multiple copies of Chromosome 17 (C17)(denoted by red dots) and HER2 (denoted by silver black dots). HIgh-grade abnormal cells showed aneuploidy (Figure 5.8 red arrow) compared to diploid cells in the adjacent normal epithelial (Figure 5.8 black arrow) and fibroblasts.

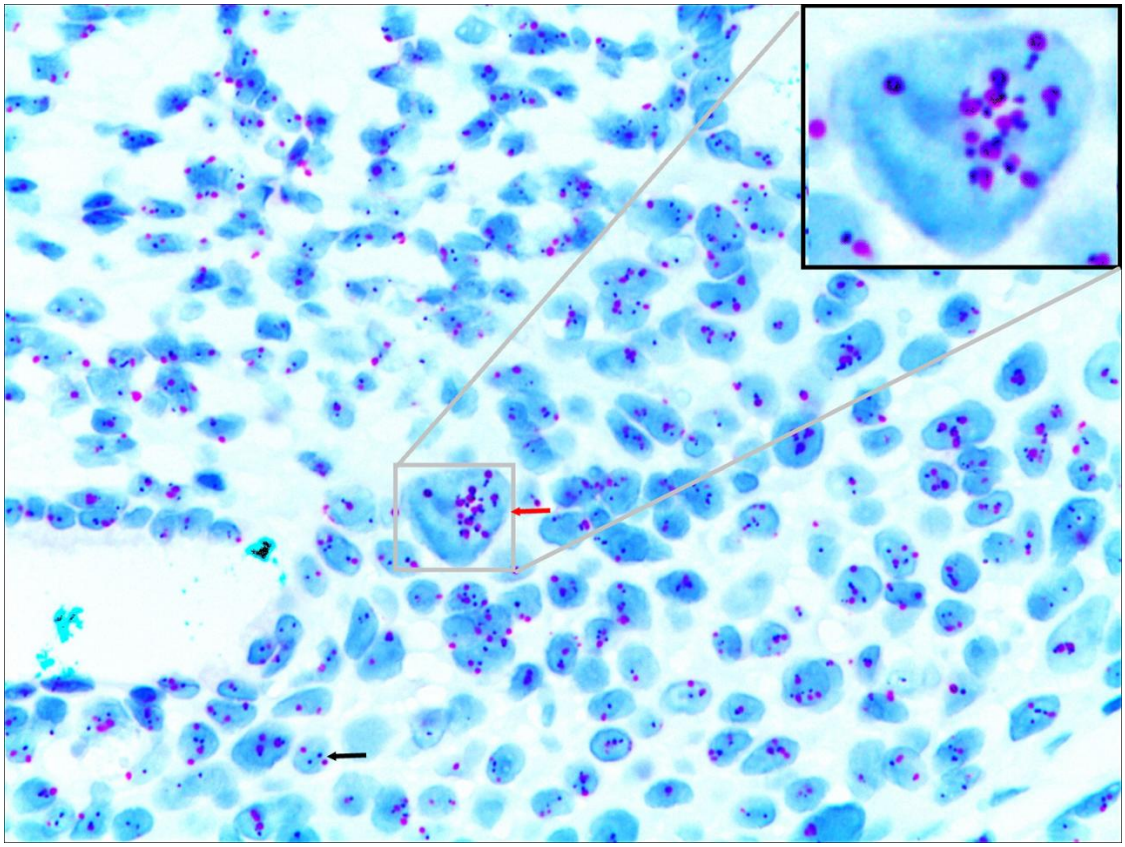


Figure 5.8 HER2/C17 copy number using D-DISH staining in cervical epithelium.

The cervical epithelium shows C17 staining as red dots and HER2 staining as black dots. The black arrow shows the diploid ploidy status, and the red arrow shows aneuploid ploidy status 500X magnification.

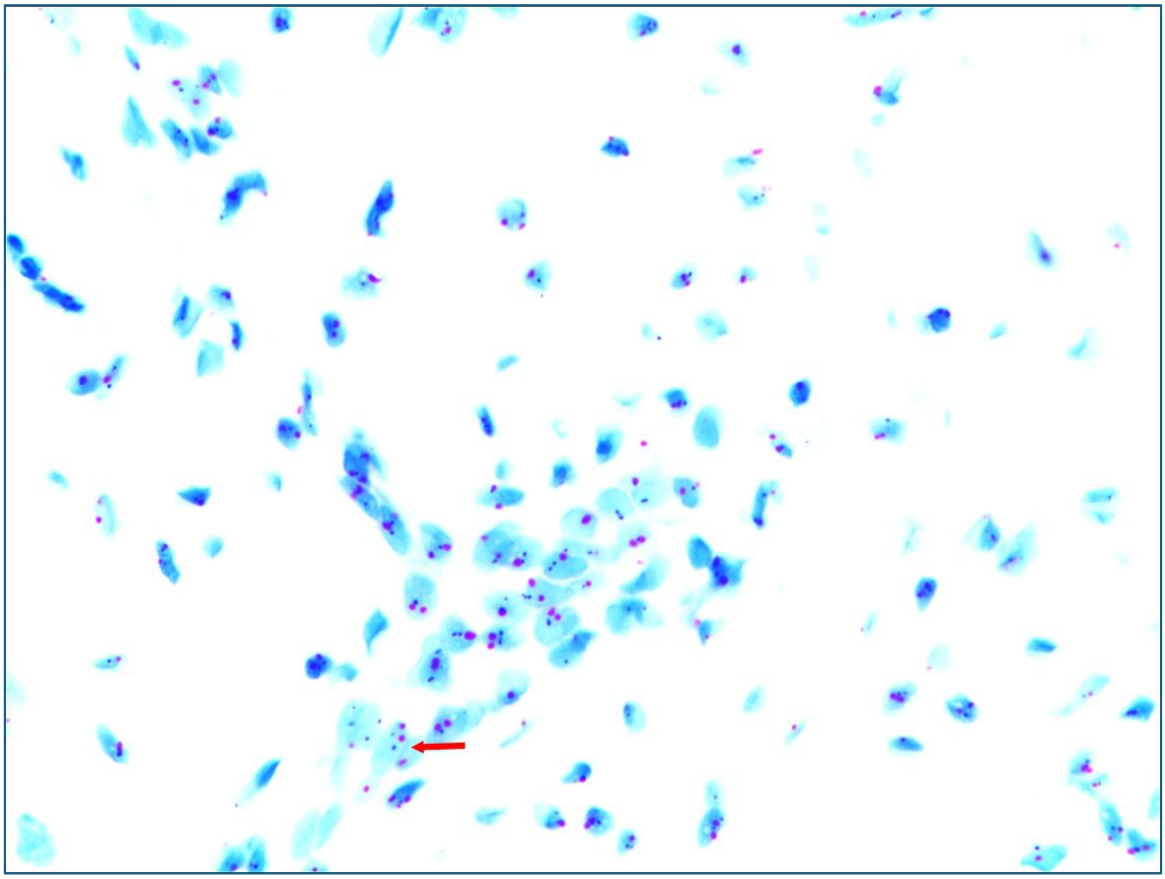


Figure 5.9 HER2/C17 copy number using D-DISH staining in cervical epithelium.

The cervical epithelium shows red C17 staining and black HER2 staining. The red arrow shows the diploid ploidy status in fibroblasts 500X magnification.

5.6 Discussion

This chapter aimed at exploring the potential of Hec1 and Nuf2 as biomarkers of progression in cervical cancer and pre-cancer. The findings of this study demonstrate that both Hec1 and Nuf2 are overexpressed in CIN1 and CIN3 cervical tissues compared to the normal cervical tissue. Although staining was seen in the basal and parabasal layers of the epithelium, the staining intensity was more remarkable in abnormal cells than in normal cells. In the case of Nuf2, the abnormal tissue corresponding to H&E showed dot-like positivity in the nuclei with accentuation of the nuclear membrane. Hec1 showed enhanced staining intensity from normal to CIN3 tissue. Hec1 and Nuf2 exhibiting staining in normal tissue have been reported in an ovarian cancer study (Leng et al., 2022; Mo et al., 2013). In addition, this study demonstrates that overexpression of these two proteins is associated with hyperactivation of the mitotic spindle, causing chromosomal instability and leading to aneuploidy in CIN3 cervical tissues.

There are few review papers on the potential use of Nuf2 and Hec1 as a biomarker in various cancers, but very few clinical studies using immunohistochemistry have been published. Recent experimental studies on endometrial carcinoma (Zhang et al., 2020), melanoma tumours (Tokuzumi et al., 2016), breast cancer (Deng et al., 2023), lung cancer (Jiang et al., 2022), hepatocellular cancer (Xie et al., 2021) osteosarcoma (Fu & Shao, 2016) and ovarian cancer (Ren et al., 2023) discussed the tumorigenesis of Nuf and its association with poor prognosis. These studies have also suggested the importance of Nuf2 in cancer therapeutic strategies. Overexpression of HEC1 is associated with poor prognosis in ovarian cancer (Mo et al., 2013), prostate cancer (Wang et al., 2015), renal cell carcinoma (C. Hu et al., 2023) and in cell lines of breast, colon and liver cancer (Huang et al., 2014) have been reported. For the first time, this study explored the Hec1 and Nuf2 expression in cervical pre-cancer and cancer cell line tissues.

Researchers have explored reasons for chromosomal instability and aneuploidy for over two decades. Lagging chromosomes can occur when a single kinetochore is attached to microtubules emanating from both spindle poles (DeLuca et al., 2003). One of the theories regarding chromosomal instability relates to lagging or otherwise missegregated chromosomes or chromosome fragments that appear as small, distinct membrane-bound structures called micronuclei (Harding et al., 2017) in keratinocytes expressing HPV16 E6 oncoprotein (Cosper et al., 2023). Lagging chromosomes can occur when a single kinetochore is attached to microtubules emanating from both spindle poles (DeLuca et al., 2006). Micronuclei are a common consequence of chromosomal instability elicited by HPV infection facilitated by HPV E6 and E7 oncoproteins via various mechanisms described in Figure 5.3 of section 5.1 of this chapter. Since Hec1 and Nuf2 play an essential role in kinetochore-microtubule attachment (Sundin et al., 2011), hyperactivation or dysregulation of these facilitates chromosomal lagging, leading to chromosomal instability followed by the formation of micronuclei.

Dysregulation of Nuf2/Hec1 is not entirely unexpected, given that aneuploidy is common in cervical cancer, and Hec1 over-expression has been shown to result in aneuploidy in an inducible mouse model. (Diaz-Rodríguez et al., 2008). Our experience in the previous study showed that Nuf2 and Hec1 were among the most significantly overexpressed genes in cervical cancer cell lines, cervical intraepithelial lesions and cervical cancer compared to normal cervical tissue. Dysregulation of Kinetochore proteins Hec1 and Nuf2 by HPV oncoproteins E6 and E7 causes hyperactive kinetochore-microtubule attachments and defective correction of kinetochore-microtubule attachments, leading to chromosomal instability (Thompson et al., 2010). Chromosomal instability (CIN) represents a rate of continuous chromosome missegregation and always causes aneuploidy (Mallick et al., 2024). Aneuploidy is a state of abnormal chromosome content that differs from a multiple of the haploid.

The use of HER2 staining for the detection of aneuploidy in various cancers, such as breast cancer (Gajaria et al., 2020), gastric cancer (Gravalos & Jimeno, 2008), and lung cancer (Lashkarizadeh et al., 2023), has been well established. A few studies have explored HER2 expression in cervical cancer (Chavez-Blanco et al., 2004; Kunos et al., 2023; Varshney et al., 2020). Whole-exome sequencing studies provided evidence of HER2 activation by somatic mutation, amplification, and human papillomavirus (HPV) integration in cervical cancer, and its incidence ranges from 1-12% (Oh et al., 2015). Dual probe ISH includes a probe for the ERBB2(HER2) gene and a probe for the centromere of Chromosome 17 (CEP17), so copy number changes can be found for both locations (Halilovic et al., 2019). Chromosomal copy number gain (more than two copies per nucleus) is called polysomy (Lewontin et al., 2000). In this study, HER2 D-DISH staining was done in CIN3 cervical tissue to confirm aneuploidy. All three CIN3 cervical tissues showed aneuploidy status, which confirms that over-expression of Hec1 and Nuf2 could have been the cause of mitotic spindle hyperactivation leading to chromosomal instability.

5.7 Conclusion

The study demonstrated that Hec1 and Nuf2 are over-expressed in cervical pre-cancer and cancer. The overexpression of Hec1 and Nuf2, accompanied by the demonstration of aneuploidy with D-DISH staining in CIN3 cervical, indicates that Hec1 and Nuf2 dysregulation is associated with chromosomal instability leading to aneuploidy. The results of this study create a strong possibility for Hec1 and Nuf2 as potential prognostic and/or diagnostic biomarkers in cervical pre-cancer and cancer and warrant further exploration in chromosomal instability-based therapies for cervical cancer.

5.8 Limitation

Out of 200 specimens, 51 cervical tissues from 33 LLETZ were selected for the proof of concept. The small number of cases was limited due to (a) funding/resources available for this part of the work (b), in many cases, sufficient material was not available because the majority of cases of LLETZ biopsy with limited material and with only block, In some cases, sufficient tissue was not available. In others, there was a minor difference of opinion on review. In very few cases, blocks were not available for unknown reasons. None of the cases were truly normal in the Normal cervical tissue group because all the cases had CIN diagnoses before and after review. It is difficult to say that the no CIN blocks chosen had already hyperactivated mitotic spindle, which was expressed in basal and parabasal layers.

It is hard to say if the polyclonal nature of the Rabbit Nuf2 antibody (Abcam ab230313) and Rabbit Hec1 antibody (Thermo-Fischer Scientific PA5-102765) used in this study contributed to non-epithelial and endothelial background staining. Two of the 23 CIN3 cervical tissues stained with HEC1 had a technical failure, which could be due to the pre-analytical stage because the controls and other

cervical tissues in the same batch showed appropriate staining. The exact cause of this pre-analytical stage remains unknown since there are 60 variables reported in the literature in the pre-analytical stage, which can affect the immunohistochemistry staining (Engel & Moore, 2011). Some listed are proper sample collection and handling, including multiple aspects of fixation, processing, embedding, slide drying, and storage (Meyerholz & Beck, 2018).

5.9 Future directions for research

Further evaluation of Hec1 and Nuf2 staining on larger sample cohorts is needed. Development of monoclonal antibodies, if warranted, that these markers are showing potential might improve specificity in histology diagnosis of cervical intraepithelial neoplasia.

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Chapter 6 Discussion, Conclusion and Future Direction

Overview

The work in this PhD focused on three main areas vital to supporting cervical cancer screening and diagnostics in Ireland.

While work has accelerated in recent years in the area of HPV primary screening and triage of HPV-positive cases, there continues to be a need to improve the performance of cervical screening and cervical cancer diagnostics in the changing world of cervical cancer, where countries are rapidly moving toward cervical cancer elimination.

Chapter 3 highlights the different high-risk HPV genotype prevalence among an HPV-positive screening population and the risks for developing cervical intraepithelial neoplasia with each of those genotypes. Chapter 4 evaluates the performance of extended genotyping for HPV as a triage tool to stratify women who may be at higher risk of developing cervical intraepithelial neoplasia. It models the impact extended genotyping would have on colposcopy referral rates and disease detection. Chapter 5 looks more at diagnostic "tests of disease", specifically two novel biomarkers, Nuf2 and Hec1, to identify the risk of disease progression in women diagnosed with high-grade CIN.

This work is vital to inform future screening and diagnostic protocols as we advance and move into an era of significant change in cervical cancer prevention.

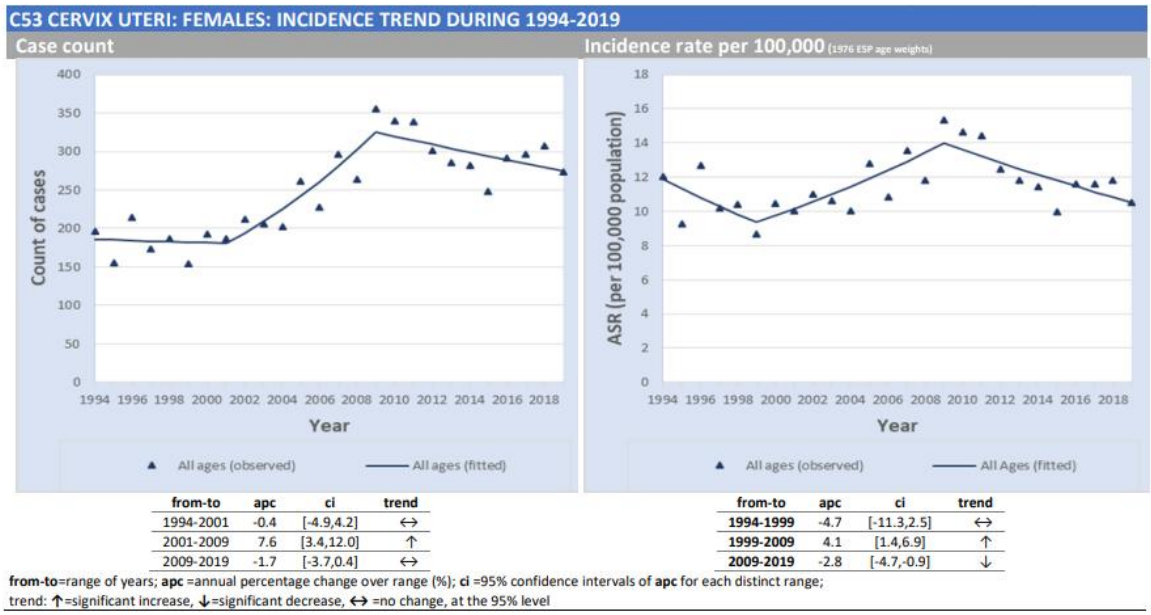
6.1 Cervical Cancer Screening -the past, the present and the future

The Past

The history of cervical screening goes back as far as 1928, when The Papanicolaou smear, a routine screening test for cancer of the uterine cervix, was reported (Vilos, 1998). Thirteen years later, in 1941, the efficacy of this test was proven, and for more than 40 years, cervical screening using cytology has helped save thousands of lives across the world. Around the same time, the first description of HPV was provided in 1949 by Strauss et al., followed by the description of physical properties of HPV DNA by Crawford and Crawford and finally, the role of HPV in cervical cancer was postulated for the first time by Professor Harald zur Hausen in 1970s (Mammas et al., 2014). Thirty-three years later, in 2003, The Pan-Canadian Forum on Cervical Cancer Prevention and Control first recommended HPV testing for triaging ASCUS lesions in Canada in 2003 (Saraiya et al., 2013). Cervical screening has come a long way in these 80 years. Observational studies in Europe show a 41%–92% reduction in cervical cancer-associated mortality in populations with access to cervical screening (Jansen et al., 2020). It is estimated in England that screening currently prevents 70% of cases (Landy et al., 2016).

In Ireland, the National Cervical Screening Programme commenced in 2008. Cervical screening was offered to those aged 25 to 44 every three years and to those aged from 45 years to 60 years every

five years. CervicalCheck screening coverage is around 80% (CervicalCheck, 2022). Overall, the age-standardised rates of cervical cancer have shown a significant decreasing trend of 2.8% per year since 2009 (NCRI, 2021) following the introduction of screening (Figure 6.1).



from-to	apc	ci	trend
1994-2001	-0.4	[-4.9,4.2]	↔
2001-2009	7.6	[3.4,12.0]	↑
2009-2019	-1.7	[-3.7,0.4]	↔

from-to	apc	ci	trend
1994-1999	-4.7	[-11.3,2.5]	↔
1999-2009	4.1	[1.4,6.9]	↑
2009-2019	-2.8	[-4.7,-0.9]	↓

from-to=range of years; **apc** =annual percentage change over range (%); **ci** =95% confidence intervals of **apc** for each distinct range;
trend: ↑=significant increase, ↓=significant decrease, ↔ =no change, at the 95% level

Figure 6.1 Trends in cervical cancer case counts and age-standardised rates (ASR), 1994-2019, based on joinpoint analysis. The age-standardised rates of cervical cancer have shown a significant decreasing trend of 2.8% per year since 2009 following the introduction of screening (NCRI, 2021).

In the screening age group (25-60y), the proportion of cases detected was 48%, and the proportion of women diagnosed with cervical cancer at stage I or II was 70%. This indicates that 88% of early-stage cancers diagnosed were in screened women compared to 52% of cancers in non-screened women. An increase of 13% in survival was seen in women in the screening age group, and age-standardised mortality rates have shown a significant decreasing trend by, on average, 1.1% per year from 1994 to 2019 (NCRI, 2021).

The Present

Cervical cancer prevention in Ireland and across the world has made massive strides towards reducing the burden of cervical cancer.

Ireland is one country that has overcome many challenges with respect to cervical cancer prevention measures, ranging from huge declines in HPV vaccination rates to controversies in cervical screening to the impact of COVID-19 on cancer detection rates (Figure 6.1). Despite these challenges, Ireland remains on track to achieve the WHO target of cervical cancer elimination by 2040 through vaccination, screening and early treatment.

Currently, HPV primary screening has been adopted or is in the process of adoption in many European countries and worldwide. Following the implementation of the National Cervical Screening Program, the Health Service Executive, Ireland has launched a number of measures to control HPV-related cancers, which included HPV vaccination, HPV testing in ASCUS+ cytology cases and finally, the implementation of HPV primary screening in 2020 (Figure 6.2).

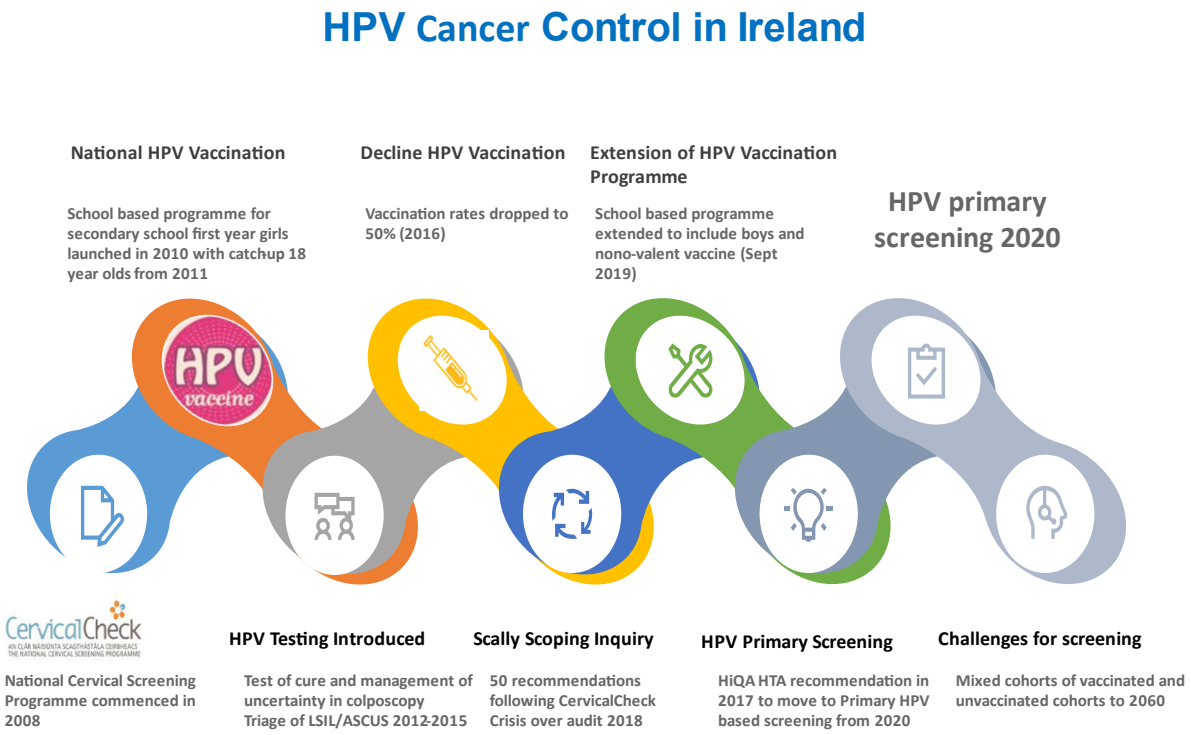


Figure 6.2 HPV Cancer Control in Ireland. Measures taken by the Health Service Executive from 2008 to 2020.

Currently, Ireland has four types of women in its screening population- screened and vaccinated, screened and non-vaccinated, unscreened and vaccinated, and unscreened and unvaccinated. Until 2060, the population will have a mixed cohort of vaccinated and unvaccinated.

An HPV test is a 'test of risk' with a very high negative predictive value and sensitivity but poor specificity. Although European guidelines for quality assurance in cervical cancer screening have recommended that women testing positive for oncogenic HPV at primary screening should be tested without delay for cervical cytology, there is no consensus yet for the best triage test. Optimal triage is really important to avoid over-referring women for unnecessary follow-up, over-treating women and causing unnecessary anxiety and costs.

Current challenges that need to be addressed in cervical cancer screening

Cervical screening in today's world has both vaccinated and non-vaccinated populations. In vaccinated cohorts, the effect of vaccination on primary HPV testing is unclear and will result in a reduction in HPV16/18 infections and cross-protection against certain non-16/18 high-risk genotypes (Rebolj, M. et al., 2024). The increase in the HPV testing specificity and decrease in its positive predictive value (PPV) for CIN2+ has been suggested by Rebolj M et al., especially in girls vaccinated at younger ages (Lei et al., 2020). Cytology is currently the recommended triage test and, as an established test, has a higher PPV than other triage tests explored. In many instances, it also has the advantage of indicating to the colposcopists what to expect during the examination. However, it has limitations- more than 50% of women will have no abnormalities; cytology as a triage test lacks sensitivity, having a high false negative rate; cytology as a profession is struggling to survive, with recruitment and retention of trained and experienced cytology personnel becoming increasingly difficult. At present, cervical screening is at a crossroads, and the decision to choose the correct triage test remains crucial. To improve the specificity and PPV, there is an urgent need to develop newer, better triage tests to minimise unnecessary colposcopy follow-up and detect more cervical high-grade abnormalities. (O'Leary et al., 2018). A triage test is a 'test of disease' to detect a woman who needs a colposcopy referral to locate and biopsy the cervical lesions. The diagnosis is performed using histology, the 'test of diagnosis'. Our research group, CERVIVA, has done extensive work to explore new triage methods, such as extended HPV genotyping and cellular and viral biomarkers as a 'test of disease' in HPV-positive women (Figure 6.2).

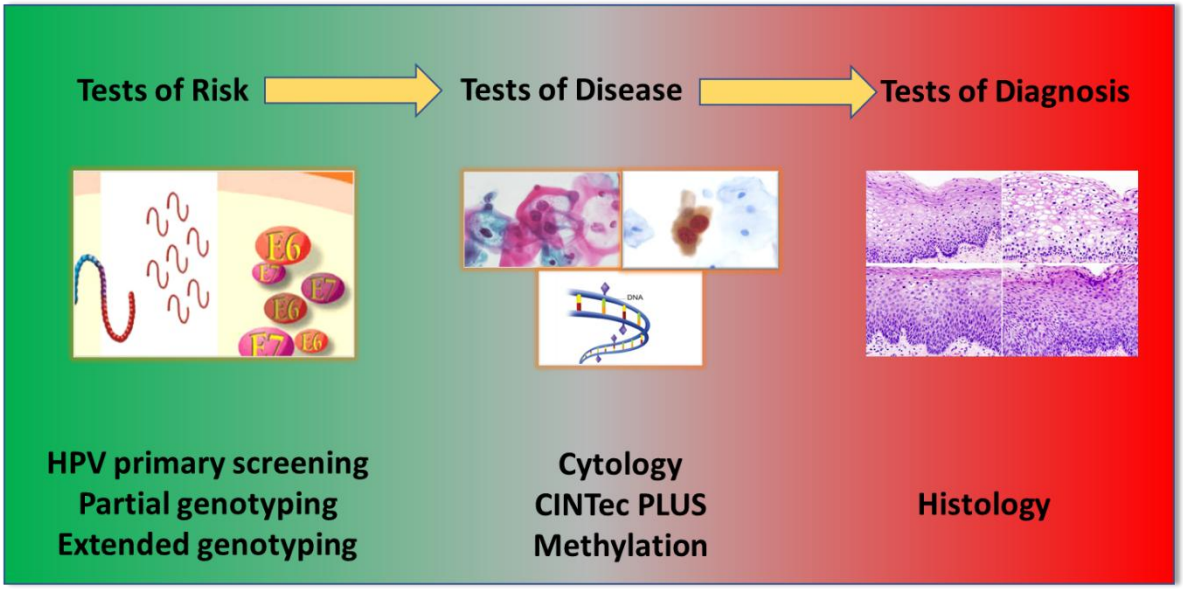


Figure 6.3 "Tests of risk" and "tests of disease" for cervical cancer screening and diagnosis. HPV primary screening is the test of risk to detect HPV-positive women, the triage test is the test of disease to find the women for a colposcopy referral, and histology is the test of diagnosis to diagnose women with high-grade abnormalities.

One of the challenges will be choosing the appropriate HPV assay to detect the most prevalent genotypes specific to the particular population due to the availability of the vast number of assays and efficiently carrying out the risk assessment where partial or extended genotyping is the triage method. Where the triage involves a combination of more than one triage method, it will be crucial to recruit the best trained and experienced staff and make a provision for high-end cutting-edge HPV assay along with an efficient management system. Triage methods are expected to maintain the PPV and NPV ratios, which can be challenging.

Study findings and benefits to women

This PhD study focused on two different biomarker approaches to improve the performance of screening and diagnostics for cervical pre-cancer and cancer.

Chapters 3 and 4 focused on the first approach (part of the CERVIVA project) of extended genotyping as a biomarker for triage in HPV-positive women from primary cervical screening. Extended genotyping provides the knowledge of high-risk HPV genotypes specific to a particular population. For more than a decade, major HPV trials have been taking place on different continents of the world. So, the first part of my study explored the prevalence of specific high-risk HPV genotypes in the Irish primary screening population. The most frequent individual genotypes found were HPV16 and 31, and HPVP2 and P3 are group genotypes. The study also found that non-HPV16 genotypes were associated

with more than half of CIN2+, similar to that of the Scottish study (Cuschieri et al., 2023). More than 40% of the absolute risk estimate for CIN2+ and more than 50% absolute risk for CIN3+ were found to be associated with non-HPV16 and HPV18. These findings suggest that extended genotyping has an important role in triaging HPV-positive women in future cervical screening, in vaccinated cohorts to find the genotypes not covered by the vaccination and in non-vaccinated cohorts to find the prevalence and risk stratification due to the growing evidence which shows a significant increase in the prevalence of non-vaccine-covered HR types following vaccination(Cuschieri et al., 2023; Hampson IN & AW, 2024).

My study highlights the message that genotypes other than HPV 16/18 should not be underestimated in organised HPV primary screening, in risk stratification, in post-treatment follow-up and in persistent high-risk HPV-positive women, confirming what other first international studies have shown (Kares et al., 2019; Stoler et al., 2019; Wong et al., 2019). Extended genotyping is needed to define the risk in vaccinated and herd-protected women with HPV positivity. The predictor study mentioned earlier showed that different grades of CIN and cancer had different genotype profiles. Further, genotype persistence is a strong predictor of increased risk of high-grade disease. Extended genotyping will benefit those screening programmes where cytology services are limited for reasons such as the unavailability of cytology personnel, resources, and the need for high throughput. Other benefits of carrying out extended genotyping are to measure the effectiveness of the vaccines in vaccinated cohorts, monitor the persistence of high-risk HPV, potential to be set up as a point-of-care test or self-sampling test, ability of immediate and combined genotyping (Renée MF Ebisch, 2016).

The study also compared alternative triage options to cytology for HPV-positive women for reduced referral rates and more detection of high-grade abnormalities. The WHO has suggested partial genotyping, colposcopy, VIA, cytology or dual stain cytology to triage women after positive HPV results (Ramírez et al., 2023). Results of previous studies, combined with the ability of immediate triage of both clinician-taken (Avian et al., 2022; Inturrisi et al., 2021) and self-sampled specimens (Aranda Flores et al., 2021; Solnæs et al., 2023)), make hrHPV genotyping an interesting triage option for hrHPV-positive women. HPV16/18 genotyping has been explored in a good few studies (Arbyn et al., 2020) and may improve the triage of hrHPV-positive women (Huh WK, 2015); for the management of hrHPV-positive women with infections with non-HPV16 and HPV18, other triage strategies need an exploration. When hrHPV testing is used alone for treatment decisions, a proportion of women of childbearing age receive unnecessary treatments (Katayoun Taghavi, 2023). The utility of extended HPV genotyping to detect cervical intraepithelial neoplasia grade 2 or more (CIN2+) in a 'screen-and-treat' strategy for HPV-positive women in low-resource settings has been explored by (Broquet et al., 2022). Portnoy et al., 2023 suggested using age to guide primary HPV triage, paired with selective

genotype and follow-up time for retesting, as cost-effective strategies which can improve the effectiveness and efficiency of the cervical screening programme (Portnoy et al., 2021).

My study findings show that extended genotyping combined with HPV16 and one of the other genotypes (HPV18 or HPVP1 or HPV31 or HPV51 or HPV52) and cytology offer the best triage model for HPV-positive women with the highest PPV and specificity and a relatively low colposcopy referral rate. Notwithstanding the inherent limitation and bias with the study design, patients were primarily screened using cytology and managed on that result when the study commenced.

In a scenario where cytology may not be available/viable as a triage method, CINTec PLUS combined with HPV16 and the same combinations of genotypes could be a good alternative. Major trials like IMPACT in the US and NTCC in Italy have explored dual staining using p16/Ki-67. Although recent studies show that extended genotyping combined with p16/ki-67, a proven marker 'test of disease' had the same performance as cytology, it was the second-best option in this study. This could be due to the fact that cytology was the primary screening test at the time of this study, and the study design included mainly HPV-positive samples. Genotype groups P2 and P3 carry less risk and could be the candidates for routine recall management strategy. Caution must be maintained to include the genotypes specific to the particular population. Also, there is very little information available regarding genotypes not adequately covered by the nanovalent vaccine and their performance with p16/ki-67. The longitudinal performance of three options: HPV16/18 genotyping (HPV16/18), cytology (LBC), and p16/Ki-67 dual stain cytology (DS) for the triage of high-risk Human Papillomavirus-positive (Hr-HPV+) women within the cervical screening program in Scotland was published recently. The study concluded that Two-step triage with HPV 16/18 genotyping before LBC (or DS) for Hr-HPV other+ women was associated with a lower risk of significant disease at follow-up compared with single triage approaches (Stanczuk et al., 2022).

HPV positive and triage test positive women are referred to colposcopy. Diagnosis of cervical pre-cancer and cancer is performed on biopsies taken during the colposcopy. Like cytology, histology also has its challenges. These include getting the correct sample where the disease is located post colposcopy procedure, the influence of technical issues during the processing of these specimens, morphology dilemmas created by the look-alikes and benign process in the tissue and finally, inter-pathologist variability in reporting the histology diagnosis such as challenging CIN2 (Stoler et al., 2015) leading to 8-28% discrepancy in cytology and histology reporting (Alanbay et al., 2017; Parker et al., 2002). Tissue biomarkers are used to assess the exact type and subtype of cancer- to assess the prognosis in patients with cervical cancer Wu 2024. So far, p16 immunohistochemistry has been a good surrogate for the presence of transforming or HPV infection in head and neck cancers and has

been found useful in cervical cancer and pre-cancer (Galgano et al., 2010; Holmes et al., 2015; Stoler et al., 2018). In recent years, the use of p16/Ki67 dual staining such as CINTec PLUS has been gaining momentum after the United States Food and Drug Administration (US FDA) approval in March 2020 for the triage of HPV-positive women, either in primary HPV screening or HPV/Pap cytology co-testing (FDA, 2020)

The second part of my thesis (Chapter 5) focused on novel kinetochore proteins (Hec1 and Nuf2) biomarkers of chromosomal abnormalities and cell cycle dysregulation in cervical pre-cancer to explore these kinetochore marker's potential utility in cervical cancer diagnostics and prognostics. Numerical chromosome abnormalities, mainly including aneuploidy and chromosome instability, are caused by chromosome segregation errors in mitosis (Kou et al., 2020), which is governed by the mitotic spindle activity and its kinetochore proteins Hec1 and Nuf2. For more than two decades, overexpression of Hec 1 and Nuf 2 has been investigated in a variety of human tumours and was correlated with the tumour grade and prognosis (Diaz-Rodríguez et al., 2008; Jiang et al., 2022; Mo et al., 2013; Shan et al., 2021; Sugimasa et al., 2015).

The overexpression of kinetochore markers Hec1 and Nuf2 and These biomarkers have the potential as markers of disease progression since the presence of aneuploidy means the potential for developing cervical intraepithelial neoplasia. They might become more valuable in the vaccinated cohorts of both HPV-dependent and HPV-independent cervical cancer and pre-cancer.

Study limitations/ challenges

This genotype study was derived from ≈ 10% of the Irish primary screening population and included only HPV-positive samples. Histology and cytology follow-up was available in 60% of cases and is ongoing. The BD Onclarity^{TM} HPV DNA Assay detects six specific individuals and three groups of genotypes. Other genotypes that were not included in the assay and could be prevalent in the Irish population may not have been detected. The group genotype positivity does not allow the detection of the specific genotypes in that combination and thus the attribution of the risk of the particular genotype in that group (HPV33 in the HPVP1 group). Finally, the ≈20% BD Onclarity HPV negative, which was positive in Cobas HPV DNA Test, poses a caution regarding the specificity of various HPV detecting assays and their impact on the follow-up of HPV-positive women. The highest PPV of cytology as triage and HPV16 combined with HPV18 and one of the other genotypes and cytology as triage are affected by the inherent bias of the study design which was based on cytology as the primary screening method at the time of this study.

There were a few challenges for the biomarker part of the study. Delayed clinical recruitment due to changes in European GDPR regulations in 2018 and COVID-19 in 2019 affected the technical validation timeline. The biomarker study had the issue of background staining of non-epithelial, endothelial and stromal cells due to the polyclonal nature of the Hec1 and Nuf2 antibodies. There were very few normal histology cases in the biomarker study due to HPV-positive and ASCUS+ cytology cases, resulting in limited normal cervical tissue availability. Insufficient tissues in some cases and unavailability of blocks in others for unknown reasons also had an impact on the small sample size for the validation.

6.2 A vision for the future of cervical cancer prevention

Despite current challenges, the future of cervical cancer prevention looks bright.

In 2020, the WHO issued a call to arms for all countries across the world to eliminate cervical cancer by vaccinating 90% of girls, screening 70% of women and treating 90% of cervical pre-cancer cancer by 2030 (Harlfinger et al., 2023). This year, Ireland will be mapping a path to cervical cancer elimination by 2040 (HSE, 2024). On the prevention strategy side, established prophylactic vaccines Cervarix (GlaxoSmithKline, Middlesex, UK), a bivalent vaccine against HPV 16 and 18; Gardasil (Merck Inc., Rahway, NJ, USA), a quadrivalent vaccine against HPV 6, 11, 16, and 18, and Gardasil 9 (Merck Inc.), a nonavalent vaccine against HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58 will start showing its benefits worldwide soon. A Scottish study demonstrated that the bivalent vaccine confers worthwhile cross-protection against HPV 31, 33 and 45, and the nonavalent vaccine does not offer much more protection than the Cervarix (Palmer et al., 2019). In young women, prior to sexual debut, the current vaccines have demonstrated very significant efficacy against vaccine-covered HPV types and associated disease (Hampson IN & Oliver AW, 2024). In a recent publication, early evidence of HPV vaccination impact in 25 year old women in Ireland showed a significant reduction in HG cytology proportions (Rourke et al., 2024). There are currently over 20 therapeutic HPV vaccine candidates at different stages of development and getting reviewed by the WHO (WHO, 2024).

Advances in screening technologies

The use of artificial intelligence at every step of the screening process is just at the doorstep. This includes using artificial intelligence to inform and educate women regarding cervical vaccination and screening and providing guidance for self-sampling techniques using online platforms such as the Medicaid Cancer Foundation and the Global Initiative Against HPV and Cervical Cancer, reaching a vast audience. Digital data analysis of the population screening and survival rates using upcoming

technologies, such as currently under pilot Roche's navify® Cervical Screening software, will help to understand the effectiveness of cervical screening programmes (https://medically.roche.com/content/dam/pdmahub/restricted/oncology/eurogin-2024). Recently developed, a deep learning-based method for automated visual evaluation of the cervix to help recognise the pre-cancerous changes among HPV-positive individuals is an essential tool in low-income countries (Parham et al., 2023). A few studies with CERVIVA, have explored the potential of Raman Spectroscopy in cervical screening (Duraipandian et al., 2018; Shaikh et al., 2023).

The usefulness of extended genotyping in screening and AI-assisted visual evaluation is currently under investigation in the PAVE project international effort involving nine low- and middle-income sites worldwide (Inturrisi F et al., 2024). Partial and/or extended genotyping is gaining popularity as a triage method, and major HPV trials are assessing its potential in routine risk stratification. HPV genotyping will be crucial in early and better risk stratification in conjunction with digital pathology tools (Xue et al., 2023). DNA methylation as a triage tool for cervical cancer screening is being explored in some countries, including the CERVIVA consortium in Ireland (Burdier et al., 2024; Reynolds, 2020).

Finally, validation for the use of artificial intelligence to report cytology slides coming into effect in the US, UK and other European countries (Ikenberg et al., 2023). The Hologic Genius™ Digital Diagnostics System with the Genius™ Cervical AI algorithm with clearance from the US Food and Drug Administration (FDA) uses artificial intelligence (AI) to facilitate screening on scanned ThinPrep glass slides (Yao et al., 2022). A validation study done using this system demonstrated that AI-based digital Pap test evaluation had improved diagnostic accuracy and reduced screening time compared to light microscopy, and participants in this study reported a positive experience using this system (Cantley RL et al., 2024). The American Society of Cytopathology, in conjunction with the International Academy of Cytology and the Digital Pathology Association, established a special task force comprising 20 members with expertise and/or interest in digital cytology (Kim et al., 2024). This group investigated the feasibility of incorporating digital cytology, specifically cytology whole slide scanning and AI applications, into the laboratory workflow and recently published a two-part review. It has best practice recommendations for incorporating digital cytology into practice, a comprehensive review of AI in cytology practice, and best practice recommendations and legal considerations.

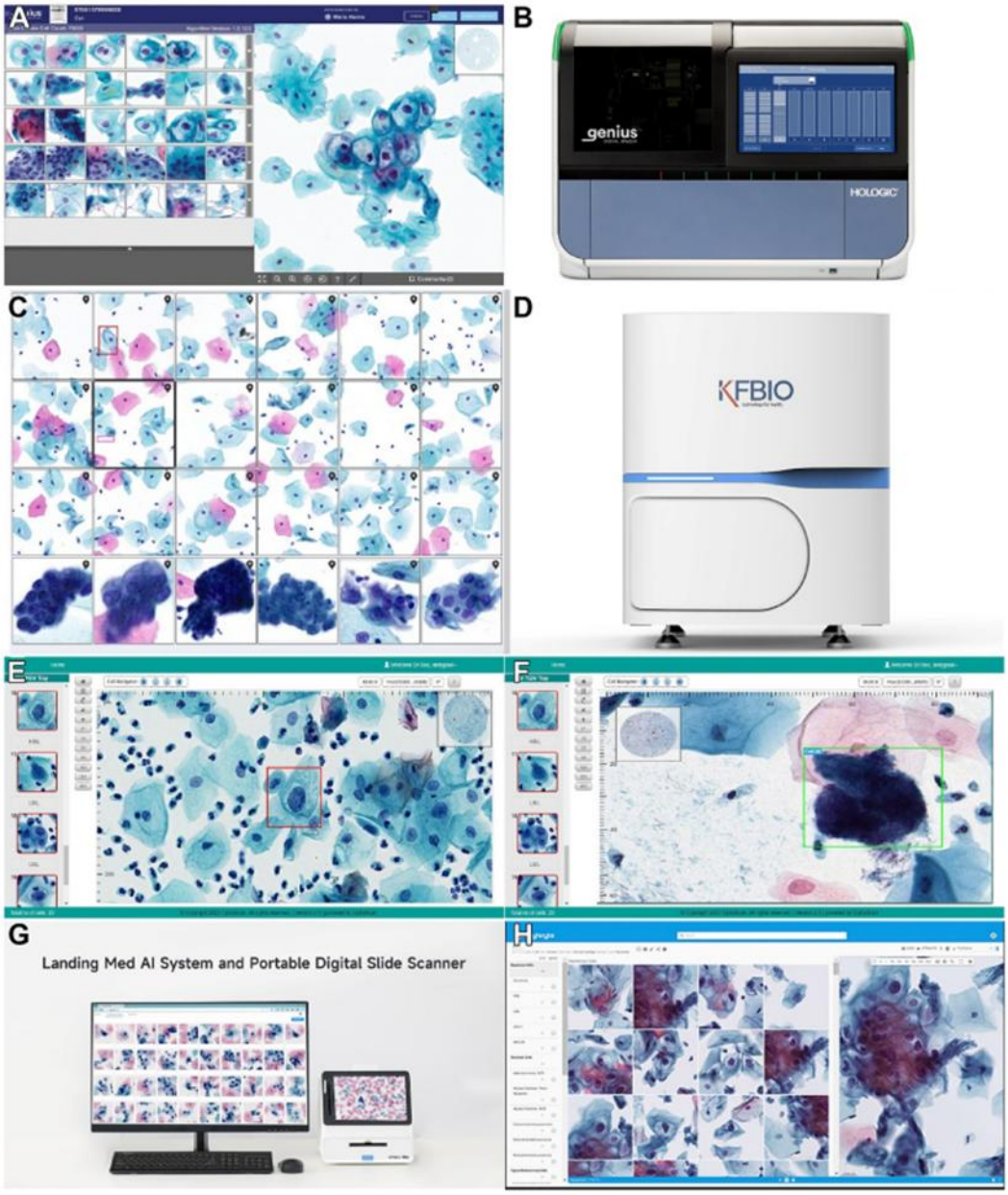


Figure 6.4 Representative images from several whole slide image (WSI) based artificial intelligence (AI) platforms for screening Pap tests. (A, B) Hologic Genius AI platform and WSI scanner; (C, D) KFBIO AI-assisted system and WSI scanner; (E, F) OptraSCAN CytoSIAplatform with abnormal cells (E) and microorganism (F); (G) Landing Med AI platform and its WSI scanner; (H) Techcyte AI platform (Kim et al., 2024).

Targeted next-generation sequencing for screening and genotyping of human papillomaviruses has already begun (Andersen et al., 2022; Lee et al., 2024). Development, validation and implementation of various tissue biomarkers related to chromosomal instability and aneuploidy, such as Hec1 and Nuf2, will be the bridge to the future of reporting cervical cancer and pre-cancer.

In summary, the future of cervical screening looks very promising due to the availability of AI-based technologies, advanced molecular-based tests and the commitment of international organisations to eliminate cervical cancer, illustrated in Figure 6.5 (Arip et al., 2022; Chandrani et al., 2015; Ouh et al., 2024).

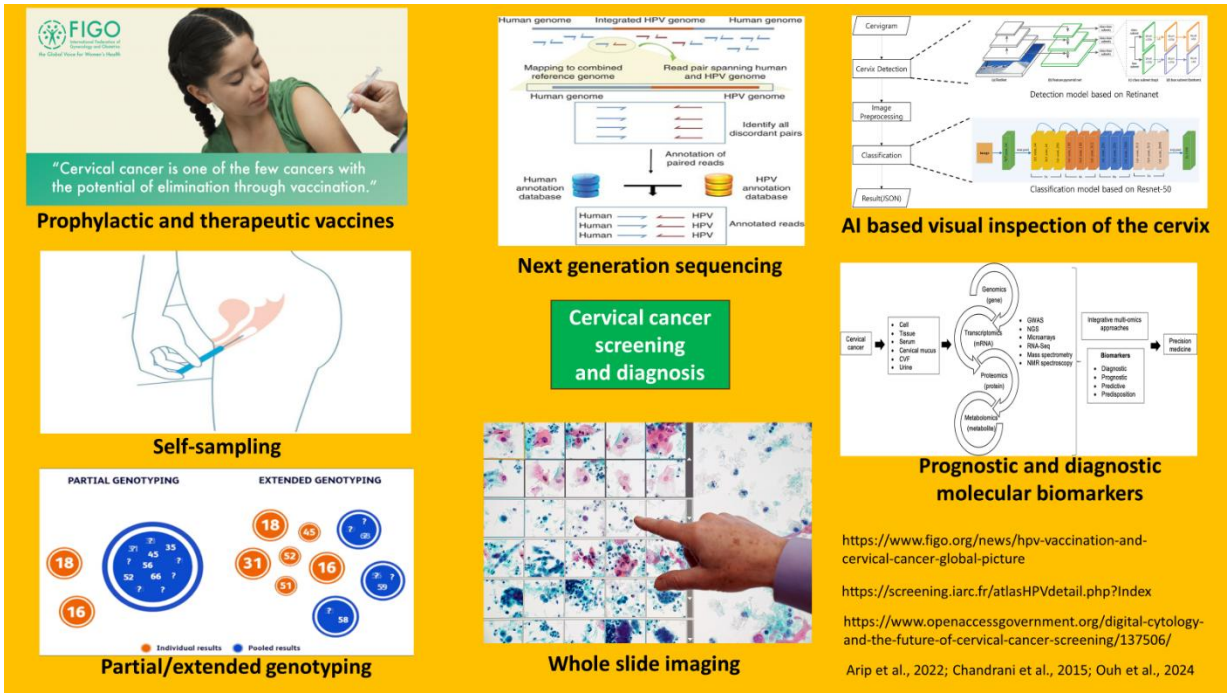


Figure 6.5 Future of cervical screening. The future of cervical screening will include prophylactic and therapeutic vaccination, self-sampling, partial and extended genotyping, followed by partial or extended genotype, whole slide imaging, AI-based visual inspection of the cervix, next-generation sequencing and new prognostic and diagnostic molecular biomarkers.

6.3 Future direction for this work

Future research should extend the validation work on Nuf2 and Hec1 biomarkers into a larger validation/test cohort, working out how Nuf2 and Hec1 could be used in cervical screening, examining the use of biomarkers and extended genotyping in specimens that are not amenable to cytology and understanding the role of HPV in Nuf2/Hec1 dysregulation. Validate work on extended genotyping, explore HPV vaccination's effect on the role of extended genotyping in screening., and examine the possibility of combining extended genotyping with digital cytology.

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Appendix I

Private and Confidential

Invitation to participate in "HistoCerv" Research Study

Dear Ms xxx ,
You are scheduled to attend the Colposcopy smear clinic within the next number of weeks. We are writing to invite you to take part in a research study to be carried out at the Coombe Women and Infants University Hospital (CWIUH) led by Professors John O'Leary and Cara Martin of Trinity College Dublin, Dr. Helen Keegan of CWIUH and their study team.

The study will look at new ways to improve how we screen and test for cervical cancer, using using molecular biomarkers or tests. An information leaflet about the study is included here. We would like you to read this in advance of your visit to the clinic so you can take time to decide if you would like to participate or not. On the day of your appointment a member of the research team will be available to answer any questions you may have and to discuss the study in more detail. If you agree to participate she/he will get your consent on the day.

You do not have to participate it is entirely voluntary and your decision alone. If you decide not to take part it won't affect your future medical care in any way, you will undergo the scheduled examination as planned. Similarly, if you decide you wish to take part, you will still undergo your scheduled examination as planned.
We look forward to meeting you at your visit and answering any questions you might have. If you have any questions about the study at this stage or would like further information please do not hesitate to contact Dr Cara martin (cara.martin@tcd.ie or 01 4085430).

Thank you for taking the time to read this letter.

Yours sincerely,
The Colposcopy Clinic
The Coombe Women and Infants University Hospital

Appendix II

PATIENT CONSENT FORM

Study title: Molecular Biomarkers for Improved Cervical Screening [HistoCerv]

General		
I have read and understood the **Information Leaflet** about this research study. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study. I understand that not taking part won't affect my future medical care. I understand that participating in this research is my own decision.	**Yes** ☐	**No** ☐
I understand that I can ask that information about me be deleted from the study within 4 weeks of enrolment. I understand that I don't have to give a reason for opting out (Please note that after four weeks, data may form part of aggregated results and therefore it will not be possible to remove it after this point in time.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
I am aware of the potential risks and benefits of this research study.	**Yes** ☐	**No** ☐
Consent		
I consent to allow researchers to access my leftover tissue specimen for this research study once all routine investigations have been completed.	**Yes** ☐	**No** ☐
I consent to information from my medical records, and results of analysis of the specimen (personal data) to be used for this research study by the research team. I have been assured that information about me will be kept private and confidential by the research team.	**Yes** ☐	**No** ☐
I give permission to be contacted by a CERVIVA researcher in relation to future studies approved by a Research Ethics Committee. Please note that no contact information will be stored by the CERVIVA study team. We will ask the colposcopy clinic to provide this information if you are happy for us to contact you.	**Yes** ☐	**No** ☐

STORAGE AND FUTURE USE OF INFORMATIONAND SAMPLES		
RETENTION OF RESEARCH MATERIAL IN THE FUTURE [please choose one or more as you see fit]		
I consent to coded samples and information collected for this study being stored for 10 years after the study end date for use by the research team for future research on cervical screening and cervical cancer. I understand any future research will need ethical approval.	Yes ☐	No ☐
I consent to coded information collected for this study being stored for 10 years after the study end date to be shared worldwide with industry or other institutions for research related to cervical screening and cervical cancer, only if research is approved by a Research Ethics Committee in Ireland.	Yes ☐	No ☐
I consent to coded information and samples collected for this study being used for teaching and or training related to cervical cancer and cervical screening by CWIUH and Trinity College Dublin	Yes ☐	No ☐

Patient Name (Block Capitals)	Patient Signature	Date
Translator Name (Block Capitals)	Translator Signature	Date

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above participant the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

Name (Block Capitals)	Qualifications	Signature	Date

3 copies to be made: 1 for participant, 1 for PI and 1 for hospital records.

Patient Information Leaflet

Study Title: Molecular biomarkers for improved cervical screening [HistoCerv Project]

Principal investigator: Prof. John O'Leary, Director of Pathology, CWIUH, Tel: 01 4085200
Consultant co-investigator: Dr Tom D'Arcy, Professor Obstetrics and Gynaecology and Lead Clinician Colposcopy Clinic, CWIUH, Tel: 01 4085200
Joint Data Controllers: Coombe Women & Infants University Hospital and Trinity College Dublin
Data Protection Officers: Siobhan Lyons (CWIUH) dataprotection@coombe.ie and John Eustace (TCD), dataprotection@tcd.ie

You are being invited to take part in a research study to be carried out at The Coombe Women and Infants University Hospital & Trinity College led by Prof. John O'Leary and his study team.

Before you decide whether you wish to take part or not, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision. You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.

You don't have to take part in this study. If you decide not to take part, it won't affect your future medical care. You have the option to opt out within 4 weeks of enrolling. After that your data will be anonymized and combined with the rest of the study data and it will not be possible to remove it. You don't have to give us a reason to opt out and if you do please rest assured it won't affect the quality of treatment you get in the future.

What is the Research/Study about?

The new method of examining cervical screening samples is called HPV cervical screening. HPV cervical screening was introduced in Ireland in 2020, and involves your cervical screening sample being checked for Human Papillomavirus (HPV). If HPV is found the same test sample is checked under the microscope to see if you have an abnormal (pre-cancerous) cell changes in your cervix. HPV is a common virus that is linked with changes in the cells of the cervix (neck of the womb) and cervical cancer. The presence of HPV and or abnormal cells does not always lead to the development of cervical cancer but often women who are positive for HPV and have abnormal cells detected will be referred to colposcopy for further investigation.

Our research programme, CERVIVA has identified a number of biomarkers related to HPV which have potential to distinguish those women with HPV positive and abnormal cell changes, who are more likely to develop cervical cancer/pre-cancer. Our research is looking at new

markers or tests (called biomarkers) that might be helpful in the future in determining women who have a higher risk of developing cervical pre-cancer or cancer.

Who is organising and funding this study?

This research is being managed by CERVIVA, led by Trinity College Dublin. This study forms part of a research project which will be submitted to Trinity College Dublin for the award of PhD. It is funded by the Coombe Women and Infants University Hospital and Trinity College Dublin. As the study progresses we may get further funding from various sources including industry to continue this work.

Why am I being asked to take part?

You are being invited to take part in this because you are scheduled to have a colposcopy examination for the further investigation of HPV and/or cervical abnormalities detected on your cervical screening test. We hope to recruit approximately 500 women into the study.

How will the study be carried out?

Recruitment to the study will take place in your colposcopy clinic. You will have received notification of the potential to participate in this study in advance of your clinic visit. You will be asked to review this leaflet and the study will be explained in greater detail by a member of the research team when you arrive at the clinic. You will have the opportunity to ask questions. Following this, study staff will ask if you would like to participate in the study. If you agree then you will be asked to sign a consent form.

Participation in the study will not require any additional procedures or visits, we are just asking for your permission to use some of your tissue sample that remains after the routine diagnosis for our research which will be carried out in the Trinity College Dublin, Molecular Pathology Laboratory based at the Coombe Women and Infants University Hospital. We may also collect some information relating to your cervical screening history prior to the start of the study and we are also interested to know what happens to you with respect to your cervical diagnosis after you agree to participate. This is to help us determine how good the new markers we are developing are at picking up disease.

Your identity will remain confidential, you will be assigned a study ID that will be used by the researchers.

What will happen to me if I agree to take part?

If you agree to take part, you do not have to do anything extra other than attend your colposcopy visit as normal at Coombe Women and Infants University Hospital where you will be asked to sign copies of a consent form at the colposcopy clinic. You may ask as many questions as you like about the study. You will not be asked to give extra samples outside of those taken by your treating clinician or colposcopy nurse as per routine practice.

What are the benefits?

There is no direct benefit to you from taking part, but the results of this study may benefit you and other women in the future by improving the tests we use for cervical screening and diagnosis.

Appendix 4

Table 1 Clinical Performance of HPV Genotypes Individually or in pooled groups for detecting CIN2+ disease.								
HPV Type	**CIN2 + Pos**	**CIN2 + Neg**	**Sensitivity % 95% C.I.**	**Absolute Risk% 95% C.I.**	**PPV% 95% C.I.**	**<CIN2 Pos**	**<CIN2 Neg**	**Specificity % 95% C.I.**
HPV16 N=404	132	161	45.0 (39.3-51.0)	32.7 (28.1-37.5)	49.2 (43.1-55.4)	136	440	76.4 (72.7-80.0)
HPV18 N=128	41	252	14.0 (10.2-18.5)	32.0 (24.1-40.8)	47.7 (37.0-58.7)	45	531	92.2 (90.0-94.2)
HPV45 N=122	26	265	9.0 (6.0-12.8)	21.3 (14.4-30.0)	33.0 (22.7-44.4)	53	523	90.8 (88.1-93.0)
HPVP1 N=203	52	241	17.7 (13.5-22.6)	17.7 (12.7-23.7)	40.0 (31.5-50.0)	78	498	86.5 (83.4-89.1)
HPV31 N=240	57	236	19.4 (15.1-24.5)	23.7 (18.5-29.6)	39.0 (31.1-47.4)	89	487	84.5 (81.3-87.4)
HPVP2 N=398	58	235	19.8 (15.4-24.8)	14.6 (11.3-18.4)	25.5 (20.1-31.7)	169	407	70.7 (66.8-74.3)
HPV51 N=160	33	260	11.5 (8.0-15.4)	20.6 (14.6-28.0)	34.7 (25.5-45.2)	62	514	89.2 (86.4-91.6)
HPV52 N=189	49	244	16.7 (12.6-21.5)	26.0 (20.0-33.0)	40.2 (31.4-49.4)	73	503	87.3 (84.3-90.0)
HPVP3 N=290	56	237	19.1 (14.8-24.1)	19.3 (15.0-24.3)	35.0 (27.7-42.7)	105	471	81.8 (78.4-84.8)

* All infections observed were counted regardless of whether they were present as single infections or multiple infections.

Table 2 Clinical Performance of HPV Genotypes Individually or in pooled groups for detecting CIN3+ disease.								
HPV Type*	**CIN3 + Pos**	**CIN3+ Neg**	**Sensitivity % 95% C.I.**	**Absolute Risk% 95% C.I.**	**PPV% 95% C.I.**	**<CIN3 Pos**	**<CIN 3 Neg**	**Specificity % 95% C.I.**
HPV16 N=404	101	93	51.6 (44.3-59.0)	25.0 (20.8-29.5)	41.0 (34.6-47.4)	165	510	75.5 (72.1-78.7)
HPV18 N=128	30	164	15.6 (10.8-21.5)	23.4 (16.4-31.7)	38.0 (27.3-49.6)	56	619	91.7 (89.3-93.6)
HPV45 N=122	13	181	6.8 (3.6-11.3)	10.7 (5.8-17.5)	19.4 (10.7-31.0)	66	609	90.2 (87.7-92.3)
HPVP1 N=203	39	155	20.3 (15.0-26.7)	19.2 (14.0-25.3	33.0 (24.7-42.3)	90	585	86.6 (83.8-89.0)
HPV31 N=240	42	152	22.0 (16.2-28.4)	17.5 (13.0-23.0)	34.1 (25.8-43.2)	102	573	85.0 (82.0-87.5)
HPVP2	32	162	16.1	10.4	17.0	191	484	71.7

N=298			(11.2-22.1)	(7.2-14.4)	(12.0-23.3)			(68.1-75.0)
HPV51 N=160	20	174	10.4 (6.5-15.6)	12.5 (7.8-18.6)	25.0 (16.0-36.0)	74	601	89.0 (86.4-91.3)
HPV52 N=189	35		18.2 (13.0-24.4)	18.5 (13.2-24.8)	32.4 (23.7-42.1)	87	588	87.1 (84.3-89.5)
HPVP3 N=290	31		16.1 (11.2-22.1)	10.7 (7.4-14.8)	21.4 (15.0-29.0)	130	548	81.2 (78.0-84.0)

* All infections observed were counted regardless of whether they were present as single infections or multiple infections.

Table 3 Individual HPV genotypes and Individual group genotypes in 293 CIN2+ and 576 <CIN2

HPV Type	CIN2+ Pos	Sensitivity% 95% C.I.	Abs Risk	PPV% 95% C.I.	CIN2 + Neg	<CIN2 Pos	<CIN2 Neg	Specificity% 95% C.I.
HPV16 N=212	58	19.8% (15.4-25.0)	27.4% (21.5-34.0)	49.1 (39.7-58.6)	235	74	502	86.7% (83.2-89.7)
HPV18 N=51	11	3.7% (2.0-6.6)	21.6% (11.3-35.3)	42.3 (23.3-63.1)	282	18	558	96.6% (94.5-98.1)
HPV45 N=59	7	2.4% (1.0-5.0)	12.0% (5.0-23.0)	26.0 (11.1-46.3)	286	27	549	95.5% (93.1-97.2)
HPVP1 N=97	14	4.8% (2.6-8.0)	14.4% (8.1-23.0)	32.5 (19.1-48.5)	282	39	537	93.5% (90.8-95.6)
HPV31 N=120	21	7.2% (4.5-10.7)	17.5% (11.2-25.5)	40.4 (27.0-55.0)	272	46	530	93.0% (90.3-95,2)
HPVP2 N=210	18	6.1% (3.7-9.5)	8.6% (5.2-13.2)	23.4 (14.5-34.4)	2	84	492	86.7% (83.2-89.7)
HPV51 N=67	2	0.7% (0.08-2.4)	3.0% (0.4-10.4)	9.5 (1.2-30.4)	289	31	545	95.7% (93.4-97.4)
HPV52 N=86	12	4.1% (2.1-7.0)	14.0% (7.4-23.1)	30.8 (17.0-47.6)	279	39	537	94% (91.3-96.0)
HPVP3 N=137	16	5.5% (3.1-8.7)	11.7% (6.8-18.3)	28.1 (17.0-41.5)	275	46	530	90.8% (87.7-93.3)

* Only single infections were counted, regardless of whether they were present as single infections or multiple infections

Table 4 Individual HPV genotypes and Individual group genotypes in 194 CIN3+ and 675 <CIN3								
HPV Type	**CIN3+ Pos**	**Sensitivity % 95% C.I.**	**Absolute Risk% 95% C.I.**	**PPV% 95% C.I.**	**CIN3 + Neg**	**<CIN3 Pos**	**<CIN3 Neg**	**Specificity**
HPV16 N=212	43	22.0% (16.2-28.4)	19.8% (14.7-25,8)	36.2 (27.5-45.6)	151	87	588	87.1% (84.3-89.5)
HPV18 N=51	7	3.6% (1.5-7.4)	13.7% (5.7-26.3)	27.0 (11.6-47.8)	187	30	645	95.5% (93.7-97.0)
HPV45 N=59	0	0	0	0	194	31	644	95.4% (93.5-96.8)
HPVP1 N=97	10	5.2% (2.5-9.4)	10.3% (5.1-18.1)	23.2 (11.7-38.6)	184	42	633	93.8% (91.7-95.5)
HPV31 N=120	16	8.8% (5.2-13.8)	14.2% (8.5-21.7)	32.1 (20.0-46.3)	178	50	625	92.6% (90.3-94.4)
HPVP2 N=210	7	3.6% (1.5-7.4)	3.3% (1.3-6.7)	9.1 (3.7-17.8)	187	92	583	86.4% (83.5-89.0)
HPV51 N=67	0	0	0	0	194	32	643	95.2% (93.3-96.7)
HPV52 N=86	7	3.6% (1.5-7.4)	8.1% (3.3-16.0)	18.0 (7.5-33.5)	187	43	632	93.6% (91.5-95.3)
HPVP3 N=137	8	4.2% (1.8-8.0)	5.8% (2.5-11.2)	14.0 (6.2-25.8)	186	54	621	92.0% (89.7-94.0)

* Only single infections were counted regardless of whether they were present as single infections or multiple infections

Table 5 Clinical performance of HPV16 combined with other genotypes and cytology and CINTec PLUS				
HPV genotypes with ASCUS	**Sensitivity**	**Specificity**	**PPV**	**NPV**
16/18	45.0 (39.3-51.0)	88.0 (85.1-90.6)	65.7 (58.7-72.2)	76.0 (72.5-79.1)
16/45	44.0 (38.3-50.0)	87.1 (84.1-89.8)	63.5 (56.5-70.2)	75.4 (72.0-78.6)
16/P1	48.8 (43.0-54.7)	85.1 (82.0-88.0)	62.4 (55.8-68.7)	76.7 (73.1-79.8)
16/31	46.8 (41.0-52.6)	85.1 (82.0-88.0)	61.4 (54.7-68.0)	75.8 (72.4-79.1)
16/P2	49.1 (43.3-55.0)	79.7 (76.2-83.0)	55.2 (49.0-61.3)	75.5 (72.0-79.0)
16/51	42.0 (36.3-48.0)	87.0 (84.0-89.6)	62.1 (55.0-69.0)	74.7 (71.2-78.0)
16/52	45.4 (39.6-51.3)	86.6 (83.6-89.3)	63.3 (56.4-70.0)	75.7 (72.3-79.0)
16/P3	49.1 (43.3-55.0)	83.0 (79.6-86.0)	59.5 (53.0-65.7)	76.2 (72.7-79.5)
16/18/45	51.2 (45.3-57.0)	84.9 (81.7-87.7)	63.3 (56.4-86.9)	86.4 (83.5-89.0)
16/18/P1	53.6 (48.7-60.4)	82.5 (79.1-85.5)	61.3 (55.1-67.2)	87.6 (85.0-90.2)
16/18/31	53.2 (47.3-59.0)	82.5 (79.1-85.5)	60.7 (54.4-66.7)	87.0 (84.0-89.5)
16/18/52	52.0 (46.0-57.7)	84.2 (81.0-87.1)	62.5 (56.1-68.7)	86.7 (83.8-89.3)
P1/P2/P3	40.0 (34.3-45.8)	75.2 (71.4-78.6)	45.0 (38.8-51.3)	79.6 (76.2-82.8)
P1/31/52	39.0 (33.3-44.7)	84.4 (81.1-87.2)	56.0 (48.8-62.8)	82.7 (79.6-85.5)
HPV genotypes with CINTec PLUS	**Sensitivity**	**Specificity**	**PPV**	**NPV**
16/18	28.7 (23.6-34.2)	88.0 (85.1-90.6)	55.0 (46.7-63.0)	70.8 (67.3-74.1)
16/45	29.7 (24.5-35.3)	87.7 (84.7-90.2)	55.1 (47.0-63.0)	71.0 (67.5-74.3)
16/P1	33.1 (27.7-38.8)	85.4 (82.3-88.2)	53.6 (46.0-61.0)	71.5 (68.0-75.0)
16/31	31.1 (25.8-36.7)	86.8 (83.8-89.5)	54.5 (46.6-62.2)	71.2 (67.7-74.5)
16/P2	29.7 (24.5-35.3)	83.8 (80.6-86.8)	48.3 (40.8-56.0)	70.8 (67.2-74.2)
16/51	27.3 (22.3-32.8)	88.5 (85.6-91.0)	54.8 (46.3-63.0)	70.5 (67.1-73.8)
16/52	28.7 (23.6-34.2)	86.3 (83.2-89.0)	51.5 (43.6-59.4)	70.4 (67.0-73.7)

16/P3	32.1 (26.8-37.8)	85.1 (82.0-88.0)	52.2 (44.7-59.7)	71.1 (67.6-74.5)
16/18/45	33.1 (27.7-38.8)	90.4 (87.7.-92.7)	63.8 (55.6-71.4)	72.7 (69.2-76.0)
16/18/P1	35.5 (30.0-41.3)	87.8 (85.0-90.4)	59.8 (52.1-67.1)	72.8 (69.3-76.1)
16/18/31	34.1 (28.7-40.0)	88.5 (85.6-91.0)	60.2 (52.4-67.7)	72.5 (69.1-75.8)
16/18/52	32.1 (26.7-37.7)	88.7 (85.8-91.2)	59.1 (51.0-66.8)	72.0 (68.5-75.2)
P1/P2/P3	22.2 (17.6-27.4)	88.2 (85.3-90.7)	49.0 (40.1-57.7)	69.0 (65.5-72.3)
P1/31/52	25.3 (20.4-30.6)	89.2 (86.4-91.6)	54.4 (45.7-63.0)	70.1 (66.6-73.4)

Table 6 Comparison of triage strategies

Triage strategy				Referral rate	PPV	Specificity
Cytology				38%	50.7 (46.0-55.3)	59.7 (55.6-63.7)
CINtes PLUS				34%	53.8 (47.7-59.7)	70.0 (63.1-72.5)
HPV16				27%	49.2 (43.1-55.4)	76.4 (72.7-80.0)
HPV18				8.6%	47.7 (37.0-58.7)	92.2 (90.0-94.2)
	Referral rate with cytology	**PPV**	**Specificity**	**Referral rate with CINtec PLUS**	**PPV**	**Specificity**
HPV16/18	**15.1 (13.3-17.0)**	**65.7 (58.7-72.2)**	**88.0 (85.1-90.6)**	17.1 (15.0-19.5)	55.0 (46.7-63.0)	88.0 (85.1-90.6)
HPV16/45	**14.8 (13.1-16.7)**	**63.5 (56.5-70.2)**	**87.1 (84.1-89.8)**	17.2 (15.0-19.6)	55.1 (47.0-63.0)	87.7 (84.7-90.2)
HPV16/P1	**17.2 (15.3-19.2)**	**62.4 (55.8-68.7)**	**85.1 (82.0-88.0)**	17.9 (15.7-20.3)	53.6 (46.0-61.0)	85.4 (82.3-88.2)
HPV16/31	**17.1 (15.2-19.1)**	**61.4 (54.7-68.0)**	**85.1 (82.0-88.0)**	14.0 (12.0-16.1)	54.5 (46.6-62.2)	86.8 (83.8-89.5)
HPV16/51	**15.1 (13.3-17.0)**	**62.1 (55.0-69.0)**	**87.0 (84.0-89.6)**	17.2 (15.0-19.6)	54.8 (46.3-63.0)	88.5 (85.6-91.0)
HPV16/52	**17.2 (15.3-19.2)**	**63.3 (56.4-70.0)**	**86.6 83.6-89.3)**	16.0 (13.8-18.3)	51.5 (43.6-59.4)	86.3 (83.2-89.0)
HPV16/18/45	17.6 (15.7-19.7)	63.3 (56.4-86.9)	84.9 (81.7-87.7)	12.0 (10.3-13.6)	63.8 (55.6-71.4)	90.4 (87.7.-92.7)
HPV16/18/P1	20.0 (18.0-22.0)	61.3 (55.1-67.2)	82.5 (79.1-85.5)	13.8 (12.0-15.6)	59.8 (52.1-67.1)	87.8 (85.0-90.4)
HPV16/18/31	20.0 (18.0-22.0)	60.7 (54.4-66.7)	82.5 (79.1-85.5)	13.8 (12.1-15.7)	60.2 (52.4-67.7)	88.5 (85.6-91.0)
HPV16/18/52	18.6 (16.6-20.6)	62.5 (56.1-68.7)	84.2 (81.0-87.1)	13.0 (11.4-14.8)	59.1 (51.0-66.8)	88.7 (85.8-91.2)
HPV16/18/45	17.6 (15.7-19.7)	63.3 (56.4-86.9)	84.9 (81.7-87.7)	12.0 (10.3-13.6)	**63.8 (55.6-71.4)**	**90.4 (87.7.-92.7)**
HPV16/18/P1	20.0 (18.0-22.0)	61.3 (55.1-67.2)	82.5 (79.1-85.5)	13.8 (12.0-15.6)	59.8 (52.1-67.1)	87.8 (85.0-90.4)
HPV16/18/31	20.0 (18.0-22.0)	60.7 (54.4-66.7)	82.5 (79.1-85.5)	13.8 (12.1-15.7)	60.2 (52.4-67.7)	88.5 (85.6-91.0)
HPV16/18/52	18.6 (16.6-20.6)	62.5 (56.1-68.7)	84.2 (81.0-87.1)	13.0 (11.4-14.8)	59.1 (51.0-66.8)	88.7 (85.8-91.2)