Correlating $p16^{INK4a}$/Ki-67 co-expression and gene methylation with HIV infection and high-risk HPV in abnormal cervical squamous intraepithelial cells

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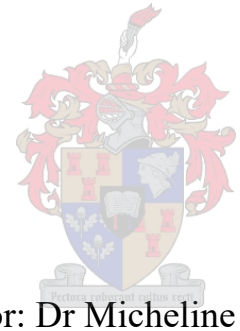

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March 2023

Declaration

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Abstract

Globally, cervical cancer is the fourth most common cancer amongst women. Persistent infection with high-risk human papillomavirus (HPV) is shown as the causal factor in cervical cancer development. Women living with HIV is six times more prone to cervical cancer development. The aim of this study was to identify the HR-HPV types, investigate the simultaneous expression of $p16^{INK4a}$ and Ki-67 and evaluate the methylation status of *CADM1, MAL* and *miR124-2* genes in cytology samples from HIV-positive and HIV-negative women with LSIL, HSIL, ASC-US, and ASC-H Pap smear results. Study participants were women between the ages of 21 years to 60 years referred to the Colposcopy clinic at Tygerberg Academic Hospital.

Exfoliated cervical intraepithelial cells were collected in Surepath medium and HR-HPV types were determined using the Hybrispot HPV direct flow chip kit, whereas the co- expression of $p16^{INK4a}$/Ki-67 proteins was evaluated with the CINtec® Plus cytology immunocytochemistry kit. For methylation assays, DNA was isolated from the left-over exfoliated cells followed by the assessment of quantity and purity of isolated DNA using fluorometry and spectrophotometry, respectively. Isolated DNA was bisulfite converted and the methylation assays for the *CADM 1, MAL* and *miR-124-2* genes were done using the respective EpiMelt assays.

HPV DNA detection results were associated with cytological diagnosis as well as HIV status. In our study group 74 % (51/69; 95% CI: 62,1%-83%) of woman tested positive for HPV of which 70.6% (36/51) were of WLWH and 29.4% (15/51) of HIV negative women. Our results showed that $p16^{INK4a}$ /Ki-67 dual-staining was detected in 43.8% (25/57) of the samples with the HSIL cytology showing the highest $p16^{INK4a}$ /Ki-67 co-expression rate of 64% (16/25) compared to the other cytology groups. The proportion of the LSIL group with $p16^{INK4a}$ /Ki-67 dual staining was 33,3% (4/12), whereas that of the ASC-US and ASC-H groups were 25% (2/8) and 23% (3/13) ASC-H, respectively. *CADM1* methylation was detected in 12.3% (7/57) of samples, while *MAL* and the *miR-124-2* genes showed methylation in 14% (8/57) and 12.3% (7/57)of the samples, respectively. HPV infection was detected in 28.1% (16/57) of the samples with methylated *CADM1, MAL* and *miR-124-2* genes. A significant relationship was found between HR-HPV and *miR-124-2* methylation.

The logistic regression model analysis employing predictors such HR-HPV, $p16^{INK4a}$ and Ki-67 co-expression, as well as the methylation status of the *CADM1, MAL*, and *miR-124-2* genes, for LSIL cytology showed low sensitivity and high specificity, contrasting to that of the HSIL model with high sensitivity and low specificity. Therefore, we conclude that a larger study is warranted, with removing or adding predictors for model improvement.

Opsomming

Wêreldwyd is servikale kanker die vierde mees algemene kanker onder vroue. Aanhoudende infeksie met hoë-risiko menslike papillomavirus (HR-MPV) word getoon as die oorsaaklike faktor in servikale kanker ontwikkeling. Vroue wat met MIV leef is ses keer meer geneig tot servikale kanker ontwikkeling. Die doel van hierdie studie was om HR-MPV tipes te identifiseer in sitologiemonsters van MIV-positiewe en MIV-negatiewe vroue wat, met behulp van ‘n Papsmeer, met LSIL, HSIL, ASC-US, en ASC-H gediagnoseer is. Die gelyktydige uitdrukking van $p16^{INK4a}$ en Ki-67 in hierdie monsters was ook geëvalueer tesame met die metileringstatus van *CADM1*, *MAL* en *miR124-2* geen. Die studiedeelnemers was vroue tussen die ouderdom van 21 jaar tot 60 jaar wat na Kolposkopie-kliniek by Tygerberg Akademiese Hospitaal verwys is.

Servikale intraepiteelselle is in Surepath-medium versamel. Die Hybrispot MPV direktevloeiskyfiestel is gebruik om HR-MPV tipes in hierdie monsters te op te spoor, terwyl die mede-uitdrukking van $p16^{INK4a}$/Ki-67 proteïene met die CINtec® Plus sitologie-immunositochemie-stel geëvalueer is. DNS is uit die oorblywende servikale selle geïsoleer en die kwaliteit en kwantitieit van die DNS was met behulp van fluorometrie en spektrofotometrie bepaal. Geïsoleerde DNS is bisulfiet omgeskakel en die metileringstoetse vir die *CADM1*, *MAL* en *miR-124* geen is gedoen met behulp van die onderskeie EpiMelt-toetse.

Die resultate van MPV DNA analise was geassosieer met sitologiese diagnose sowel as MIV status. Die proporsie van vroue wat positief getoets het vir MPV was 74 % (51/69; 95% CI: 62,1%-83%), waarvan 70.6% (36/51) van vroue met MIV en 29.4% (15/51) MIV-negatiewe vroue was. Dubbele kleuring van $p16^{INK4a}$/Ki-67 kon in 45.6% (25/51) van monsters opgetel word. Monsters met HSIL-sitologie het ‘n $p16^{INK4a}$/Ki-67 mede-kleuring patroon van 66,6% (16/24) getoon, wat die hoogste was in vergelyking met die ander sitologiegroepe. Die proporsie van die LSIL-groep waaring positiewe $p16^{INK4a}$/Ki-67 dubbele kleuring aangetref was, was 33,3% (4/12), terwyl dié van die ACS-US- en ASC-H-groepe 25% (2/8) en 23% (3/13) was. *CADM1* metilering is in 12.3% (7/57) van monsters opgespoor, terwyl *MAL* en die *miR-124-2* gene metilering in onderskeidelik 14% (8/57) en 12.3% (7/57) van die monsters getoon het. HPV-infeksie is opgespoor in 28.1% (16/57) van die monsters met gemetileerde *CADM1*, *MAL* en *miR-124-2* gene. Beduidende verwantskap is gevind tussen HR-MPV en *miR-124-2* metilering.

Die logistiese regressiemodel-analise wat voorspellers soos HR-MPV, $p16^{INK4a}$ en Ki-67 mede-uitdrukking gebruik, sowel as die metileringstatus van die *CADM1, MAL* en *miR-124-2*-gene, vir LSIL-sitologie het lae sensitiwiteit en hoë spesifisiteit getoon , in teenstelling met dié van die HSIL-model met hoë sensitiwiteit en lae spesifisiteit. Daarom kom ons tot die gevolgtrekking dat 'n groter studie geregverdig is, met die verwydering of byvoeging van voorspellers vir modelverbetering.

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List of abbreviations

%: Percentage

µl: microlitre

°C: Degree Celsius

A260nm: Absorbance at 260 nanometers

A230nm: Absorbance at 230 nanometers

ART: Antiretroviral Therapy

ASC-US: Atypical Squamous Cells of Undetermined Significance

ASC-H: Atypical Squamous cell does not exclude High-grade lesion

CADM1: Cell adhesion Molecule 1

CDK: Cyclin dependent kinase

CIN: Cervical Intraepithelial Neoplasia

DNA: Deoxyribonucleic Acid

DNMTs: DNA Methyltransferases

EGFR: Epidermal Growth Factor Receptor

et al: and others

FDA: Food and Drug Administration

g: G force

HIV: Human Immunodeficiency Virus

HPV: Human Papilloma Virus

HR-HPV: High Risk HPV

HSIL: High-grade Intraepithelial Lesion

ISCC: Invasive Squamous Cell Carcinoma

LBC: Liquid- based Cytology

LCR: Long Control Region

LLETZ: Large Loop Excision of the Transformation Zone

LR-HPV: Low Risk HPV

LSIL: Low-grade Squamous Intraepithelial Lesion

MAL: T- cell differentiation protein

ml: millilitre

miRs: Micro RNAs

MS-HRM: Methylation- Sensitive High-Resolution Melting

NILM: Negative Intraepithelial Lesion or Malignancy

NHLS: National Health Laboratory Services

ng: nanograms

ORF: Open Reading Frame

Ori: origin of replication

Pap: Papanicolaou

PCR: Polymerase Chain Reaction

pRB: Retinoblastoma protein

RNA: Ribonucleic acid

rpm: revolutions per minute

SCC: Squamous Cell Carcinoma

UNAIDS: United Nations Programme on HIV/AIDS

WLWH: Woman Living With HIV

WHO: World Health Organisation

v/v: volume/ volume

Chapter 1

Introduction

1.1 Background

Cervical cancer is the fourth most common cancer amongst women worldwide, with an estimated 604 127 new cases and 341 831 deaths being reported for the year 2020 (WHO 2022; Bruni *et al*., 2021; Kovacevic *et al.,* 2021; Rajaram and Gupta, 2021). Human papillomavirus (HPV) is known as the main etiological factor in the development of cervical cancer (WHO 2022; Balasubramaniam *et al*., 2019; Kose and Naki, 2014; Arney and Bennet, 2010). According to the Human papillomavirus and related disease report for 2019, there is an estimated amount of 569 847 new cases of HPV infections and 311 365 HPV-related cancer deaths worldwide (Bruni *et al.,* 2021).

Women living with HIV (WLWH) are 6 times more likely to develop cervical cancer, which is the most common malignancy in HIV-infected women (WHO 2022; Clark *et al.,* 2021; Stelzle *et al.,* 2020). South Africa ranks fourth highest in terms of the percentage of HIV-positive women who develop cervical cancer (Stelzle *et al*., 2020). Effective HPV infection prevention strategies and early identification of cervical cancer and its precursor lesions are critical in WLWH due to their compromised immune systems. However, predicting which HPV infections will progress to high-grade cervical lesions or cancer and which infections are temporary and will regress, remains difficult. The use of ancillary molecular tests as diagnostic tools has been shown to be valuable.

An HPV positive diagnosis at Tygerberg Academic Hospital is now mostly based on the histological observation of HPV-induced changes such koilocytosis and, less frequently, Ki-67 immunostaining. It is essential to identify additional molecular assays that may improve the early cytological evaluation of Low-grade Squamous Cell Intraepithelial Lesions (LSIL), Atypical Squamous Cells of Undetermined Significance (ASC-US), High-Grade Squamous Intraepithelial Lesion (HSIL) and Atypical Squamous Cells, cannot exclude a High-grade Squamous Intraepithelial Lesion (ASC-H) at Tygerberg Hospital. Ancillary molecular tests

using biomarkers, are often routinely used for early detection of cervical dysplasia, intraepithelial neoplasia, and carcinoma (Meraková *et al*., 2018; Sahasrabuddhe *et al*., 2001).

$p16^{INK4a}$, a surrogate marker for HPV infection, and Ki-67, a marker for proliferation index, are utilised as ancillary tests to differentiate between neoplasia and non-neoplastic lesions (Hebbar and Murthy, 2017). Overexpression of $p16^{INK4a}$ resulting from infection with high-risk HPV infection and Ki-67 occurs in pre-cancerous and cancerous lesions (Ding *et al.*, 2020; Hebbar and Murthy, 2017). Ki-67 could also be used to better discern low grade cervical intraepithelial lesions that are difficult to morphologically resolve and to avert misdiagnosis (Yu *et al.*, 2022)

Numerous potential genes for methylation biomarker diagnostics have been identified (Sandra Meršaková *et al.,* 2018; Sahasrabuddhe *et al*., 2001). Promising candidate genes for methylation biomarkers to discern between neoplastic and non-neoplastic lesions are *CADM1* (Cell adhesion molecule 1), *MAL* (gene encoding T-lymphocyte maturation-associated protein), and *miR-124-2* (Wilting *et al*., 2010). Micro RNAs (miR) can also be down-regulated as a result of methylation of CpG-rich regulatory regions, and intriguingly, down-regulation of certain miR, such *miR-124*, has been associated to increased promoter methylation of the *CADM1* and *MAL* genes (Del Pino *et al*., 2019; DeVuyst *et al.*, 2015; Wilting *et al*., 2010). *CADM1* and *MAL* tumour suppressor genes and *miR-124-2,* a post-transcriptional regulator of these genes, have been suggested to contribute to the development of cervical cancer through promoter hypermethylation-mediated silence (Steenbergen *et al*., 2014). Recent research has indicated that the length of HR-HPV infection and the severity of the cervical intraepithelial neoplasia (CIN) lesion appear to be correlated with the combination of methylation-mediated silencing of the *CADM1* and *MAL* promoter genes in cervical swabs (Bierkens *et al.*, 2012). The methylation of *CADM1, MAL* and *miR-124-2* is well recognized for the tendency to develop cancer and may thus be used as techniques for predicting progressive lesions (Del Pino *et al*., 2019; Steenbergen *et al*., 2014).

The findings of this study could help researchers better understand the utility of $p16^{INK4a}$ /Ki-67 dual staining and the methylation status of *CADM1, MAL,* and *miR-124-2* genes as biomarkers for the early detection or risk assessment of the oncogenic transformation of cervical epithelial cells into precancerous lesions or carcinoma. Data may be useful to design

larger studies for the development of algorithms, whether single or combined, that can help with the diagnosis of pre-cancerous cervical intraepithelial neoplasia and aberrant squamous cells of the cervix uteri. The implementation of such algorithms may result in fewer referrals for colposcopy-guided biopsies in the clinic. Reduced colposcopy referrals will not only lower the number of women for whom colposcopy is irrelevant, averting wasteful yet minimally invasive surgical procedures, but it will also relieve the burden on health care systems and economies by diverting such services from those really in need of them. Furthermore, the HPV typing data may contribute to the South African HPV Information Centre database to help elucidate the common HPV types in the country.

1.2 Study rationale

Cervical cytology, the gold standard for the detection of cellular abnormalities of the cervix uteri, such as CIN, has been shown to be more sensitive to detect higher grade lesions (i.e., CIN II/III). The traditional Papanicolaou (Pap) smear or more contemporary liquid-based cytology (LBC) techniques are used in cytology screening to examine the morphology of exfoliated cervical epithelial cells (Phaliwong *et al*., 2018). However, even though each technique has its own benefits, several restrictions may affect how successful cytology-based testing are, particularly in nations with insufficient resources to guarantee accurate test findings and enough coverage of the population at risk. The conventional Pap smear test has a higher specificity (86 to 100%) in detecting high-grade lesions (Kamineni *et al*., 2019; Nanda *et al.,* 2000) than LBC but a low sensitivity rate of 60 to 66% (Hebbar and Murthy, 2016; Chrysostomou *et al*., 2018). The Pap smear method is labour-intensive and is not easily adjusted to high throughput automated screening and in cases where cytology slides are of inadequate quality, patients often must undergo repeat smears, which can add burden to healthcare systems as well as be detrimental to patient wellbeing (Kamineni *et al.,* 2019).

Liquid-based cytology (LBC) is utilised in middle to high income countries where it is affordable. This method is recommended due to its ability to reduce patient follow-ups, by means of multi-testing with a single sample, hereby making it convenient for the patient (Chrysostomou *et al.,* 2018; Karimi-Zarchi *et al.,* 2013). Furthermore, poor cytology results

due to operator errors such as sampling and interpretation errors may contribute to low sensitivity as well as low reproducibility of both the Pap smear and LBC tests (Kamineni *et al*., 2019; Chrysostomou *et al*., 2018). For example, sampling errors may create large numbers of false negatives or false positives, which may lead to the over diagnosis/misdiagnosis and over treatment/ under treatment of patients and may ultimately affect both the patients and the health care system. Unnecessary treatment of patients (in a case of over diagnosis) exposes the patients and put them at increased risk hereby affecting the patient's physical health due to side effects of medication as well as their psychological or mental wellbeing (Singh *et al*., 2018).

To offset the limitations of the Pap smear and LBC and to improve their sensitivity and specificity for colposcopy referral, novel approaches such as using triage algorithms that combine cytology-based methods with molecular testing are explored (Hebbar and Murthy, 2017). Thus, in a bid to improve the sensitivity and specificity of the existing methods for cytology screening as well as the diagnostic accuracy, ancillary molecular tests using biomarkers for cervical dysplasia, intraepithelial neoplasia, and carcinoma such as $p16^{INK4a}$ and Ki-67 are frequently routinely used for early detection (Voidăzan *et al*., 2022; Diouf *et al*., 2020; Meršaková *et al.,* 2018). Novel molecular tests such as those determining the extent of gene methylation has also recently emerged (Meršaková *et al.,* 2018; Lai *et al*., 2008).

Moreover, the simultaneous detection of $p16^{INK4a}$ and Ki-67 expression in cells has drawn much interest of late. In fact, in the year 2020 the FDA has approved the Cintec® Plus Cytolology kit for $p16^{INK4a}$/Ki-67 dual staining from Roche for cytology screening. The test can be used to determine the risk of a patients infected with a high-risk HPV type to develop pre-cancerous lesions or cancer. On the other hand, molecular-based test relying on epigenetic changes occurring during the progression to pre-cancer and cancer, such as DNA methylation that result in the activation or exclusion of certain genes and/or the hypermethylation of cytosine residues leading to the switching off genes are also being explored (Meršaková *et al.,* 2018). For instance, the hypermethylation of the *CADM1, MAL* and *miR-124-2* genes have been implicated in the progression of cervical intraepithelial neoplasia (Zhang *et al.,* 2022; De Strooper *et al.,* 2014).

DNA methylation analysis might be a useful supplementary test for triaging cervical neoplasia for colposcopy to detect clinically relevant cervical lesions missed by cytology. Combined triage consisting of both DNA methylation analysis and cytology testing reached higher CIN II to CIN III sensitivities compared with sole cytology at a slight drop in specificity (De Strooper *et al.,* 2014). Therefore, it is an attractive candidate triage tool for HR-HPV positive women. It is suggested by Steenbergen *et al.* (2014) that an increase in methylation level of *CADM1* and *MAL* correspond to an increase in severity and duration of cervical disease, it is therefore expected that additional methylation analysis measures will provide a safety precaution in terms of missing women with advanced CIN disease or cervical cancer.

Utilisation of combined algorithms may improve the diagnostic accuracy to provide more reliable detection of HPV infections, especially in cases with uncertain diagnoses. Additionally, these methods may help in the early identification of the risk of cellular transformations that may result in the development of cancer, identifying individuals who need to be closely monitored and maybe informing the care approach for those patients (De Strooper *et al*., 2014).

1.3 Study Aim and Specific Objectives:

The aim of this study was to identify the HR-HPV types as well as investigate the simultaneous expression of $p16^{INK4a}$ and Ki-67 and the degree of methylation of *CADM1*, *MAL* and *miR124-2* genes in cytology specimens collected from HIV positive and HIV negative women diagnosed with LSIL, HSIL, ASC-US, and ASC-H.

1.4 The specific objectives are to:

1) Identify the HR-HPV types in cytology samples from HIV positive and HIV negative woman diagnosed with LSIL, HSIL, ASC-US and ASC-H using the Hybrispot HPV direct flow chip kit (Master Diagnostica, Granada, Spain).

2) To evaluate the simultaneous expression of $p16^{INK4a}$/Ki-67, in collected HSIL, LSIL, ASC-US, and ASC-H cytology samples, using the CINtec PLUS kit (Roche Diagnostics, Pleasanton, California, USA).

3) To examine the degree of methylation of the *CADM 1, MAL* and *miR-124-2* genes in exfoliated cervical cells using the respective EpiMelt assays (MethylDetect, Aalborg, Denmark).

1.5 Thesis Outline

This works has 8 Chapters. Chapter 1 is the introduction to this work. Chapter 2 comprise the literature review which covers the following aspects: the epidemiology of cervical cancer and HPV is discussed, followed by a detailed explanation of the HPV mechanism of infection. A brief background of the utilization of HPV vaccines is given followed by the diagnostic tools used for screening and detection of abnormal cervical intraepithelial lesions in South Africa. Lastly, insight into novel biomarkers for early detection of pre-malignant cervical neoplasia is given. Chapter 3 covers the Materials and Methods used in this study, whereas Chapter 4 comprises the Results. The Discussion of this work is given in Chapter 5. Chapter 6 comprises the conclusion to this study. The references used are indicated in Chapter 7 and the supporting material for this work is compiled in the Addendum in Chapter 8.

Chapter 2

Literature Review

Cervical cancer and HPV infection in South Africa and the role of biomarkers to improve early detection of cervical squamous intraepithelial neoplasia

2.1 Epidemiology of cervical cancer and HPV in South Africa

In South Africa, cervical cancer ranks as the second most leading female cancer with an estimate of 10 702 new cervical cancer cases diagnosed annually and 5 870 deaths (Bruni *et al.,* 2021). The burden of HPV-associated cervical cancer is exacerbated by HIV infection. The Southern and Eastern African regions have the highest amount of HIV infections worldwide (Parker *et al.*, 2021; UNAIDS 2019). In 2018, 800 000 new HIV infections have been reported in these regions, representing 54% of individuals living with HIV in the world (UNAIDS, 2019). South Africa appears to be the country with the highest HIV incidence rate within Africa, contributing to more than a quarter of new infections within Southern and Eastern Africa regions in the year 2019 (UNAIDS 2019). The burden of HIV-associated cervical carcinoma in Southern Africa is escalating, representing 63.8 % of the newly diagnosed HIV-positive women with cervical carcinoma in 2018 (Stelzle *et al*., 2020). South Africa is considered the country with the fourth highest rank for HIV-positive woman diagnosed with cervical carcinoma (Stelzle *et al*., 2020). The highest regional occurrence and mortality rates for cervical carcinoma were observed in Sub- Saharan Africa, which could be attributable to the high HIV prevalence, which is still increasing, despite the wide usage of antiretroviral therapy (ART) (UNAIDS 2019).

The most prevalent viral infection of the reproductive system is the HPV, which is frequently transmitted through sexual contact. (WHO 2022). Early sexual initiation may raise the risk of HPV infection (Njue *et al*., 2021; Stelzle *et al*., 2020; Mbulawa *et al*., 2018; Ribeiro *et al*., 2015). Long-term use of hormonal contraceptives and those with impaired immune systems are two additional risk factors for HPV infection. (Njue *et al*., 2021; Stelzle *et al*., 2020). As

previously stated, HIV infection, a known risk factor for HPV infection is linked with increased HPV acquisition rates, decreased HPV and precancerous lesion clearance, and higher cervical cancer risk (Liu *et al.*, 2018).

To date, more than 200 HPV genotypes have been identified (Kovacevic *et al.*, 2021; Jung *et al* ., 2004; Burd, 2003). Forty of these genotypes infect the mucous membrane epithelium of the genital tract in both females and males (Zhu *et al.*, 2019; Findink *et al*., 2019; Kocjan *et al*., 2015; Shikova *et al.*, 2009). The HPV types that mostly infect the genital area are categorized into high and low-risk HPV types (Balasubramaniam *et al*., 2019). Infections with low-risk HPV types (HPV 6, HPV 11, HPV 40, HPV 42, HPV 43, HPV 44, HPV 54, HPV 61, HPV 70, HPV 72, HPV 81, CP6108) affect the cutaneous epithelia and are not associated with cancer but can cause genital warts or low-grade cervical cellular changes. The high-risk group consisting of approximately 18 HPV types (HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 68, HPV 73, and HPV 82) affect the mucosal regions and are classified as potential oncogenic HPV types (Balasubramaniam *et al*., 2019; Findink *et al*., 2019; Kines *et al*., 2009; von Knebel Doeberitz *et al*., 2012). A probably high-risk HPV group also exist which consist of 8 types: HPV26, HPV53, HPV66, HPV67, HPV68, HPV70, HPV73 and HPV82 (Halec *et al*., 2014).

Cervical cancer can develop from ongoing high-risk HPV infection (Tafadzwa *et al*., 2021) and can take up to 5 years to manifest in immunocompromised women while taking 15 to 20 years in immune-competent women (WHO 2022). The most common HPV types found in women in South Africa with normal cervical cytology, precancerous cervical lesions, and invasive cervical cancer are HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV51, HPV 52, HPV 56, HPV58 and HPV 59 (Bruni *et al.,* 2021). Conversely, Mbulawa *et al.* (2018) found that the most common HR-HPV types detected in participants from Cape Town, South Africa were HPV 58, HPV 16, HPV 51 and HPV 66, HPV 53, HPV 68 and HPV 81, HPV 61 and HPV 83. Although most of these HR-HPV types are shared between the shown studies, the order of prevalence differed. However, HPV16 and HPV51 seemed to be the only HR-HPV types common between these two studies. It is known that prevalent HPV types differ in different geographical regions (Reade *et al*., 2014). These differences in prevalence of HR-HPV types between Bruni *et al.* (2021) and Mbulawa *et al*. (2018) could reflect this as Bruni *et al.* (2021) reports on the HPV prevalence in South Africa as a whole.

2.2 The HPV mechanism of infection

The human papilloma virus is a small, non-enveloped circular double-stranded DNA virus that spreads by micro-wounds or micro-abrasions to the basal epithelial cells of the skin or the inner lining of tissues (Burd, 2003). The HPV genome is divided into three functional regions: I the long control region (LCR), (ii) the early region (E), which contains the open reading frames (ORFs) for the E1, E2, E4, E5, E6, and E7 proteins, and (iii) the late region (L), which is known for encoding major (L1) and minor (L2) capsid proteins or structural proteins (Gheit, 2019; Burd, 2003). Between the L1 and E6 ORFs, the LCR is home to several cis-elements that regulate the viral DNA's transcription and replication, particularly the origin of replication (ori) (Gheit, 2019; Vonsky *et al*., 2019) (Figure 2.1). According to Narisawa-Saito and Kiyono (2007), the early genes E1 and E2 control the transcription and replication of the viral genome within the host cell, while the two structural proteins L1 and L2 facilitate entrance into the host cell (Narisawa-Saito and Kiyono, 2007). The expression of epidermal growth factor receptor (EGFR), by the host cells, which promotes their progression to the G1 cell cycle phase, is increased by the HPV E5 early gene (Saxena *et al.,* 2018). Two tumour suppressor genes, p53 and retinoblastoma (RB), can be damaged by the viral oncogenes E6 and E7, altering cellular pathways and promoting uncontrolled cell growth (Narisawa-Saito and Kiyono, 2007). While the E7 protein binds to pRB to circumvent cell cycle restriction points, the HPV E6 oncogene binds to p53, a protein known to promote cell differentiation and cell survival (Saxena *et al*., 2018).

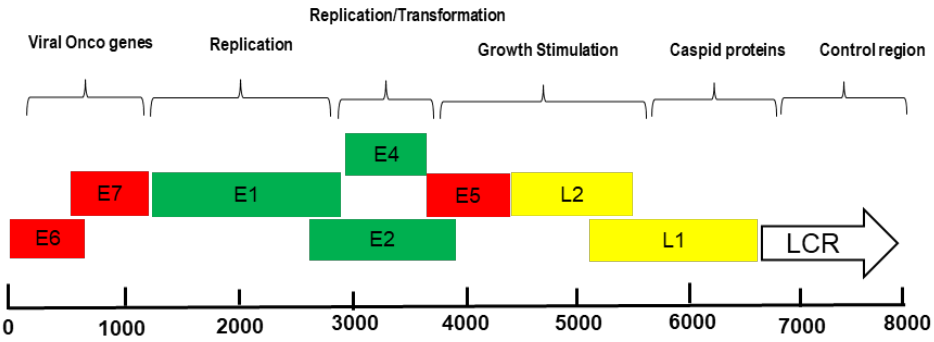

Figure 2.1: Illustration of the genome organization of human papillomaviruses. The image is adapted from The Health Professional's Handbook: Human papilloma virus and Cervical Cancer by Walter Prendiville, 2006.

The E1 and E2 viral proteins facilitate viral genome replication in basal cells (Vonsky *et al*., 2019). High volumes of viral DNA are produced and packaged in virions when the infected basal cells develop and migrate towards the epithelium's surface (Senapati *et al*., 2016; Doorbar *et al*., 2015). At the viral origin of replication, E1 and E2 interact to start viral replication. Once HPV has attached to the cell surface receptors, it can either integrate itself into the DNA of the basal cell or not. (Yao *et al.;* 2017; Abreu *et al.,* 2012; Schiller *et al*., 2010). Viruses known as episomal HPV take advantage of the DNA damage response pathway as the cell attempts to heal itself by employing this route (Senapati *et al.,* 2016).

The pRB tumour suppressor protein interacts with cyclin-dependent kinase (CDK) 4 and CDK 6, which typically forms protein complexes with cyclin D to phosphorylate pRB under normal circumstances (Yu *et al*., 2019) (Figure 2.2). When pRB is phosphorylated, it separates from the transcription factor E2F1 (E2F1), allowing E2F1 to go into the nucleus and activate the transcription of target genes that help cells move from the G1 phase to the S phase (von Knebel Doeberitz *et al*.,2012; Duncan *et al*., 2008). By blocking CDK from interacting with cyclin D and inactivating pRb, $p16^{INK4a}$ functions as a CDK inhibitor, inhibiting the advancement of the cell cycle (Yu *et al*., 2019). (Figure 2.2). When the E7 protein competes with pRB for binding, E2F1 is released from the pRb protein, which causes the cell cycle to advance in HPV-infected cells (Yu *et al*., 2019; Hebbar and Murthy, 2017). (Figure 2.2). Through a negative feedback loop, E7's disruption of the pRb-E2F1 pathway causes the overexpression and accumulation of $p16^{INK4a}$ in the cells (Hebbar and Murthy, 2017).

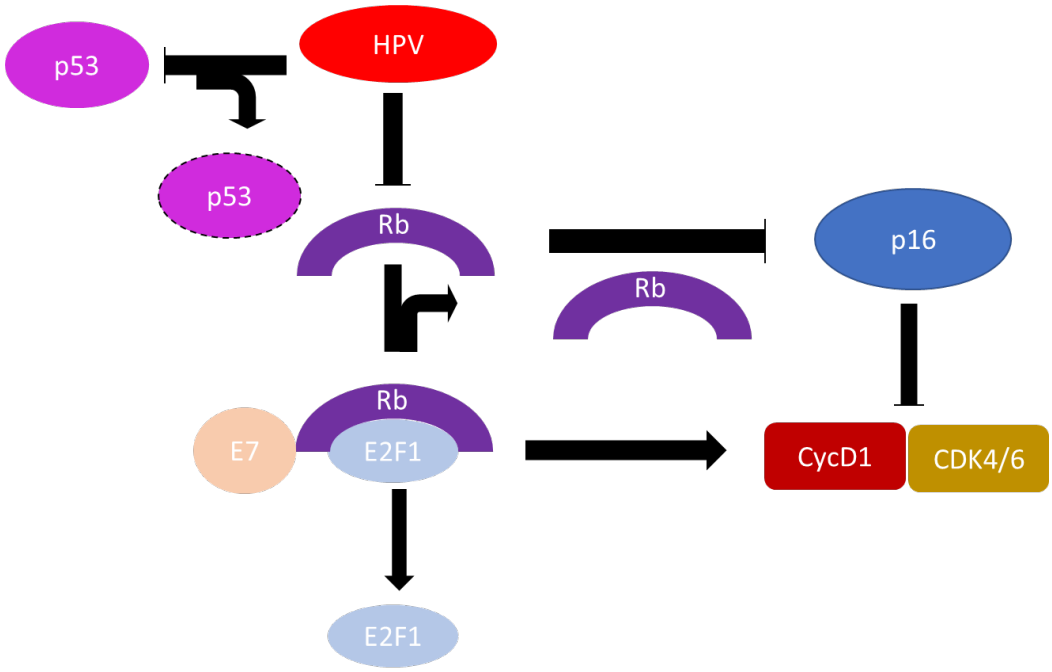

Figure 2.2: The following image is adapted from Litjens *et al.*, 2013 and Suh *et al.*, 2014. The diagram illustrates how E7 oncogene inactivates retinoblastoma (RB) which increases the amount of free E2F1 in the cell which will lead to an increase in $p16^{INK4a}$ expression and increase in cell proliferation as discussed in the previous section.

Most cervical precancers and malignancies have been shown to have $p16^{INK4a}$ overexpression (Ventana, 2016; Pauck *et al.*, 2014). HR-HPV infection is primarily linked to $p16^{INK4a}$ strong and widespread cytoplasmic and nuclear expression in cervical squamous cell carcinomas. $p16^{INK4a}$ is therefore viewed as a stand-in marker for ongoing HR-HPV infection. (Ventana, 2016; Pauck *et al.,*2014). Cervical cancer may arise as a result of overexpression of $p16^{INK4a}$, production of the HPV E6 and E7 oncogenes, and subsequent interruption of the function of the p53 and pRB tumour suppressor genes (Zhu *et al.,* 2019; Knopp *et al.,* 2009).

Cervical cancer remains a major public health problem in women worldwide, contributing to increased cancer incidence and mortality (Arbyn *et al.,* 2019). With South Africa ranked as the fourth highest country with HIV positive woman diagnosed with cervical cancer, effective prevention strategies for HPV infections are required. However, due to South Africa's low to middle income status, basic healthcare services are often underdeveloped, and cytological screening adoption is poor (Botha and Richter, 2015). In developed countries the necessary

healthcare facilities are in place for woman with invasive or cervical intraepithelial neoplasia. The appropriate resources are available for patients to utilise, hereby decreasing potential development of invasive carcinoma because of untreated cervical intraepithelial neoplasia and burden on their healthcare systems.

Cervical cytology screening programs were started in developing nations to prevent the growth of cervical carcinoma by screening to find precancerous lesions and treat them before they develop into aggressive malignancy (Burger *et al*., 2011; Sankaranarayanan *et al.,* 2001). Sexually active women were checked annually as part of such programs in industrialized nations like the United States, and this drastically reduced the incidence and fatality rate of cervical cancer (Sankaranarayanan *et al.,* 2001). In contrast, due to the lack of screening programmes in high-risk developing countries such as Southeast Asia, South Asia, and Sub-Saharan Africa, the risks of advanced cervical disease and death from such lesions have remained largely uncontrolled. In developing countries cytology-based tests have not been as effective, leading to an investigation of cervical screening approaches more suited to low-resource settings such as HPV DNA testing (Siebers *et al*.,2009).

2.3 The use of vaccines to manage HPV infections and reduce cervical cancer development

Infection with HPV is so common that it affects most sexually active women at some point in their life (WHO 2022). According to Schiffmann and Castle (2005), HPV prevalence peaks throughout adolescence, shortly after sexual debut, and then declines with age. As a result, women who are exposed to early sexual debut are at a higher risk of contracting HPV. Furthermore, HIV-positive women are more likely to be infected with HPV (Delany-Moretlwe *et al.,* 2018). The global burden of HPV-related cancers varies, with roughly 6% of cancers in the developed countries compared to 20–24% in Sub-Saharan Africa (Williamson *et al*., 2002). This is owing to less developed countries' lack of access to cervical screening programs and treatment for cervical neoplasia. Cervarix, Gardasil, and Gardasil-9 are the three preventive HPV vaccines now available worldwide (Mbulawa *et al*., 2018). Cervarix is a bivalent vaccination that protects against two high-risk HPV types, HPV 16 and HPV 18, and Gardasil is a quadrivalent vaccine that protects against two HR-HPV and two LR-HPV types, HPV 6, HPV 11, HPV 16, and HPV 18. Gardasil-9 (a nine-valent vaccine), on the other hand, protects

against 9 HPV types, including those covered by Gardasil vaccine as well as 5 additional HPV types (HPV 31, HPV 33, HPV 45, HPV 52, and HPV 58) HR kinds (Mbulawa *et al.,* 2018). In 2014, the bi-valent vaccine (Cervarix) was released in South Africa as part of the country's national school HPV vaccination program (Mbulawa *et al*., 2018). Cervarix and Gardasil are the two vaccines currently available in the South African public health system. The efficacy of these HPV vaccinations, as well as the impact of HPV school vaccination programs in South Africa, have yet to be determined. It will thus be important to understand the incidence and prevalence of specific HPV types in South Africa, as different HPV types are prevalent in different regions of the world, especially in low-to middle income countries (Kombe Kombe *et al.*, 2021; Bruni *et al*., 2021).

2.4 Diagnostic tools for screening and detection of abnormal cervical intraepithelial lesions

The Papanicolaou (Pap) stain is the gold standard cytological screening test for HPV-induced changes (Dankai *et al*., 2019, Burd, 2003). Cells from the cervix's transformation zone are exfoliated with a cytology brush, smeared on glass slides, and stained with the Papanicolaou stain, hence the Pap smear, for microscopic analysis (von Knebel Doeberitz *et al*., 2012; Arney and Bennet, 2010). Light microscopy is used to assess for abnormalities in the cell morphology of the nuclei of squamous epithelial cells for any alterations. The standard Pap smear test has a low sensitivity rate of 60% to 66% for identifying high grade lesions (Hebbar and Murthy, 2016). False positive and false negative findings from the routine Pap test are often the result of poor sample quality and processing (Bedell *et al*., 2020; Siebers *et al.,* 2009). Liquid-based cytology (LBC) is a technique created as an alternative to standard cytology in which cervical epithelial cells are collected using a regular sample instrument and put into a vial containing a preservation solution (Siebers *et al.,* 2009). The traditional Pap smear and LBC are extensively used worldwide and have been attributed with significantly lowering cervical cancer incidence and death in underdeveloped countries. Although well-trained personnel and cytopathologists perform the cell collection, spreading, fixation, staining, and reporting procedures with high standards, the sensitivity of the method is still only modest (Kremer *et al.,* 2019; Sankaranaravanan *et al*., 2001). At the moment, it is impossible to morphologically identify which patients are at danger of developing advanced cervical illness (Schiffman and Adrianza, 2000). As a result, directed biopsy and colposcopy are used to treat both low and high-grade lesions (Schiffman and Adrianza, 2000). The pressure on clinic workers and healthcare systems

increases as a result. In order to lessen the burden of cervical cancer and its precursor lesions on colposcopy clinics and eventually healthcare systems, more effective screening, management, and preventive techniques are required (Denny *et al.,* 2009). The South African Department of Health guidelines for referral for Colposcopy for patient treatment and management are: (i) two consecutive LSIL or ASC-US lesions, (ii) one HSIL lesion and (iii) one ASC-H lesion. However due to the limitation of early identifying which abnormal cervical intraepithelial lesions will progress to a high-grade lesion, alternative tests in combination with the Pap smears could better identify woman that truly needs colposcopy referral. The improved and specific referral of women for colposcopy examination may decrease the unnecessary visits to colposcopy for women whose lesions will regress.

2.4.1 Classification of cervical intraepithelial neoplasia

The growth of abnormal cells grow on the surface of the cervix is known as cervical intraepithelial neoplasia (CIN). These pre-malignant lesions may progress slowly, with some even regressing spontaneously (Bedell *et al*., 2020; Sankaranaravanan *et al.,* 2001). There are currently two systems used to categorize cervical precursor lesions, the CIN system, and the Bethesda system. In cytology, the CIN system is being phased out and substituted with the Bethesda system, which is being updated regularly. For histological diagnosis of cervical intraepithelial lesions, however, both the CIN and the Bethesda systems are still currently in use at Tygerberg Hospital. The progression of cervical cancer starts from moderate cervical intraepithelial neoplasia (CIN I) to more severe degrees of neoplasia (CIN II and CIN III), which may eventually result in the development of invasive squamous cell carcinoma (Burd, 2003). Precursor lesions are categorized into three phases by the CIN classification, which describes the percentage of the cervix that is afflicted by abnormal cells in each stage (Nayar and Wilbur, 2015). CIN I, also known as low-grade neoplasia, is a dysplasia that affects around one-third of the epithelium's thickness. An aberrant alteration that affects one-third to two-thirds of the epithelial layer is referred to as CIN II. A condition known as CIN III (the most severe kind) affects more than two thirds of the epithelium (Nayar and Wilbur, 2015).

The Bethesda system categorizes squamous cell abnormalities that are detectable by cervical cytology into four groups: ASC, LSIL, HSIL and squamous cell carcinoma (SCC) (Ndifon and Al-Eyd, 2021; Burd, 2003). The moderate dysplasia CIN I cases are categorized in this

classification scheme as LSIL whereas the CIN II and CIN III cases are categorized as HSIL (Burd, 2003). Atypical squamous cells are divided into ASC-US and ASC-H by the updated 2014 Bethesda method of cytological categorization. The updated version of Bethesda includes the endometrial cells category that is present in woman older than 45 years and is used to manage squamous intraepithelial lesion (SIL) cases accordingly. Atypical squamous cells of uncertain significance are aberrant cytologic alterations that are indicative of a SIL but are qualitatively and quantitatively different from those of a diagnosis of a definite squamous intraepithelial lesion (SIL) (Ndifon and Al-Eyd, 2021). HPV infection frequently results in an ASC-US result. ASC-US is a frequent diagnostic category that is viewed as a challenging and indeterminate diagnosis that is between a negative and a confirmed LSIL (Liu *et al*., 2019; Nayar and Wilbur, 2015).

2.4.2 Biomarker expression for early detection of pre-malignant cervical intraepithelial neoplasia

It has been demonstrated that the use of biomarkers can alleviate issues with false-positive and false-negative results in cervical cytology and histology, increasing the positive predictive value of cervical screening results (Brown *et al.,* 2012). The capacity to prioritize moderately abnormal and uncertain cytology cases was demonstrated in published studies on the use of biomarkers in Pap cytology samples (Brown *et al.,* 2012). As an illustration, instances with higher levels of $p16^{INK4a}$/Ki-67 expression were more likely to be cases of actual high-grade cervical illness (Brown *et al.,* 2012). The biomarkers can also be used to identify cells that may be aberrant (Brown *et al.,* 2002). The clinical utilisation of ancillary molecular testing such as the $p16^{INK4a}$ /Ki-67 dual staining and gene methylation, for example methylation of the *CADM1, MAL* and *miR124-2* genes, either in various combinations with extensive HPV genotyping could positively impact the management of patients with transformative HPV-induced disease and may be a promising approach for cervical screening and triaging, especially for colposcopy referral. To improve early diagnosis and treatment, cervical cancer screening is steadily migrating from Papanicolaou cytologic screening to HPV testing or co-testing with HPV (Onyango *et al*., 2020). However, there is still a need for an effective triage method to determine which HPV positive women should have additional diagnostic examination to avoid unnecessary colposcopy referrals (Onyango *et al.,* 2020). The

identification of ancillary molecular tests to improve the performance for cytological evaluation abnormal cervical intraepithelial cells is warranted.

2.4.2.1 The utilization of p16^{INK4a} and Ki-67 dual-staining as alternative biomarkers for identifying oncogenic transformation in abnormal cervical squamous intraepithelial cells

Biomarkers p16^{INK4a} and Ki-67 are used as prognostic and proliferation markers for cervical neoplasia and cervical carcinoma, respectively. Normally, p16^{INK4a} and Ki-67 are rarely overexpressed together in one cell (Ventana, 2016). Therefore, concurrent increased p16^{INK4a} and Ki-67 expression may identify cells are undergoing HR-HPV-induced carcinogenesis and may be indicative of women having high-grade cervical disease (Ventana, 2016). The CINtec® PLUS cytology kit is an immunocytochemistry assay for the simultaneous qualitative detection of the p16^{INK4a} and Ki-67 proteins in cervical cytology preparations (Ventana Medical Systems, 2019). It may therefore aid in identifying women that can benefit from immediate colposcopy. Studies have demonstrated the increased sensitivity of the CINtec® PLUS dual detection compared to LBC test (86.7% vs. 68.5%) for HSIL (Tjalma, 2017; Ikenberg *et al.,* 2013). Since women living with HIV present with advanced cervical disease at a much younger age, this test may aid in the effective management of such cases.

According to the Ventana CINtec® PLUS cytology kit interpretation guide, p16^{INK4a} staining may occur in cytoplasm only or both the cytoplasm and the nucleus of a cell in a brown colour whereas Ki-67 stains the nucleus and/or nucleoli red (Sussex, 2013). Furthermore, it was mentioned that p16^{INK4a} /Ki-67-negative cells cytoplasm and nucleus would be counterstained by haematoxylin and look blue. To compare the stain intensity of other cells with particular p16^{INK4a} and Ki-67 staining cells, these cells are utilized as "reference cells" (Ratnam *et al*., 2020). One additional measure to improve the sensitivity for finding cervical pre-cancerous lesions is genomic DNA methylation (Dankai *et al*., 2019; Gustafson *et al*., 2004). In a transforming HPV infection, changes in host cell methylation patterns accumulate over time and can be used to detect HR-HPV-mediated oncogenic transformed cervical intraepithelial lesions (Steenbergen *et al.,* 2014).

2.4.2.2 Utilizing the methylation status of genes as an additional molecular test for cervical neoplasia

DNA methylation is a direct chemical modification where a methyl group is added to DNA (Mathiasen *et al*., 2022; Villicaña and Bell, 2021; Moore, Le and Fan, 2013). The family of DNA methyltransferases (DNMTs) catalyses the transfer of a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to produce 5-methylcytosine or 5mC (Dhar *et al.*, 2021; Moore *et al*., 2013). There are two forms of DNMTs: DNMT1 and DNMT3 (DNMT3a and DNMT3b) (Moore *et al.*, 2013). DNMT1 is responsible for copying the DNA methylation pattern from the parent DNA strand onto the freshly synthesized daughter strand (Moore *et al.*, 2013). While DNMT3a and DNMT3b oversee de novo DNA methylation, which is the process of adding new methylation patterns to unmodified DNA during DNA replication (Cui *et al*., 2018; Moore *et al*., 2013). The enzymes known as writers are responsible for adding methyl groups to cytosine residues. Erasers change the methyl group and eliminate it. Readers identify methyl groups and bind to them in order to ultimately affect gene expression (Moore *et al*., 2013). Methylation plays an important role in altering gene expression without causing any change to the gene sequence.

DNA methylation in cancer causes the deregulation of various genes (Phillips *et al.,* 2022, Mikeska *et al*., 2010). The epigenetic expression that causes changes in genes primarily takes place at CpG dinucleotides. These CpG dinucleotides are naturally methylated, and their distribution throughout the genome is uneven (Han and Zhao, 2009). CpG islands are clusters of CpG dinucleotides in GC-rich regions. (Mikeska *et al*., 2010; Han and Zhao, 2009). These CpG islands are typically unmethylated and frequently found around the promoter regions of genes (Saxonov, 2006). It is believed that CpG methylation controls and stabilizes chromatin structure, possibly by influencing the transcription machinery's accessibility to DNA regions. As a result, unmethylated CpG islands next to a gene allow for the expression of that gene whereas methylation of such CpGs limit transcription and hence may lead to reduced gene expression and/or silencing (Saxonov, 2006). Human cancers naturally exhibit changes in DNA methylation, with usually hypomethylated areas (such CpG island promoters) being methylated (Baylin and Jones, 2002). Multiple systems can be used to evaluate DNA methylation levels. The four most used techniques are pyrosequencing, methylation-specific

high-resolution DNA melting (MS-HRM), quantitative methylation-specific polymerase chain reaction, and methylation-specific restriction endonucleases (Šestáková *et al*., 2019). Of these the most convenient methods identified by Šestáková *et al*. (2019) were pyrosequencing and MS-HRM. The sequencing method may be used to examine each CpG site in a specific area however this method may be expensive (Šestáková *et al*., 2019). Methylation specific high-resolution DNA melting, on the other hand, is an easy PCR-based technique and is cost effective. MS-HRM assays can be done qualitatively and quantitatively based on real-time PCR methods using a housekeeping reference gene to assess methylation levels.

Several DNA methylation markers involved in carcinogenesis are being developed as tools to predict the progression of lesions into advanced disease. Gustafson *et al*. (2004) states that the methylation profiles of tumour suppressor genes can be identified that distinguish HSIL from LSIL samples. Low grade squamous intraepithelial lesions that have the potential to progress to HSIL can thus be identified early. Several candidate genes for diagnostic methylation biomarkers have been identified (Lorincz, 2014), of which the *CADM1* (gene encoding cell adhesion molecule 1), *MAL* (gene encoding T-lymphocyte maturation-associated protein) and *miR-124-2* genes are promising (Phillips *et al.,* 2022; Del Pino *et al.,* 2019; DeVuyst *et al*., 2015 and Wilting *et al*., 2010).

The immunoglobulin superfamily's transmembrane protein, which is involved in epithelial cell adhesion, is encoded by the *CADM1* gene. In a cervical cancer cell line of advanced stage cervical intraepithelial neoplasia as well as in HR-HPV positive women with cervical squamous cell carcinoma, the functional involvement of the *CADM1* in cervical carcinogenesis has been described (Steenbergen *et al.,* 2004). Suppression of *MAL* gene expression by promoter methylation is associated with cervical carcinogenesis (Steenbergen *et al.,* 2004). In HR-HPV positive samples with high-grade cervical lesions, including CIN III, Squamous cell carcinoma, and adenocarcinoma, the hypermethylation of the *MAL* promoter was commonly seen. Additionally, a bi-marker panel consisting of combined *CADM1* and *MAL* promoter methylation has been suggested for cervical screening in HR-HPV positive women (De Strooper *et al.,* 2014). The *CADM1* and *MAL* methylation genes have been found to be involved in the development of cervical cancer through promoter hypermethylation-mediated silence. (Del Pino *et al.,* 2019; Steenbergen *et al*., 2004). Studies showed that the methylation-induced

silencing of *CADM1* and *MAL* in cervical swabs is likely due to the duration of HR-HPV infection and the severity of the cervical intraepithelial lesion (Bierkens *et al.,* 2012; Bierkens *et al.,* 2013). Furthermore, the expression of micro-RNAs (miRs), such as *miR-124* (encoding the *miR-124-2*), is downregulated through the methylation of CpG-rich regulatory sequences in genes. According to literature it is found that an increase in methylation level of the promotor region of *miR-124-2* will lead to a more severe lesion (Cook *et al.,* 2019; Overmeer *et al.,* 2008). miR-124 has been linked to the increased promoter methylation of the *CADM1* and *MAL* genes (Wilting *et al.,* 2010; Del Pino *et al*., 2019; DeVuyst *et al.,* 2015). Therefore, the methylation of tumour suppressor genes such as *CADM1,* and *MAL* and their post-transcriptional regulators such as *miR-124-2*, are involved cervical carcinogenesis and may thus be developed as tools for predicting progressive lesions (Gustafson *et al*., 2004). The methylation status of such biomarkers may be valuable in cervical cancer screening.

In this study, the degree of methylation was analysed with the Methyl detect assay which employs a high-throughput technology for highly sensitive analyses of locus specific methylation known as Methylation-Sensitive High-Resolution Melting (MS-HRM) (Wojdacz *et al.,* 2008; Clark *et al.,* 1994). After bisulfite conversion, the method makes use of the variance in melting characteristics of the PCR product produced from methylation and unmethylated DNA strands. Additionally, the use of methylation markers in conjunction with HR-HPV testing might provide a comprehensive molecular screening plan for treating women who have concurrent HR-HPV and HIV infections (Del Pino *et al.,* 2019; DeVuyst *et al.,* 2015).

Chapter 3

Materials and Methods

3.1 Materials

All materials used in this research project are detailed in the methods section accompanied by the manufacturer and catalogues numbers.

3.2 Methods

3.2.1 Ethics Approval

Ethics approval was obtained from the Health Research Ethics Committee at the Faculty of Medicine and Health Sciences at Stellenbosch University (S21/03/050) and was annually renewed for the duration of this study (Addendum A; Addendum B). Approval was also received from Tygerberg Academic Hospital for the collection of cytology samples at the Colposcopy and Family Planning Clinics in the Department of Gynaecology and Obstetrics. Approval for the use of patient data was obtained from the Central Data Warehouse of the National Health Laboratory Service (NHLS).

3.2.2 Study design

3.2.2.1 Inclusion criteria:

This prospective, observational pilot study included woman between ages 18 to 60 with known HIV status who were referred to the Colposcopy Clinic at Tygerberg Academic Hospital with the following cytology results: (i) two consecutive LSIL or ASC-US lesions, (ii) one HSIL lesion and (iii) one ASC-H lesion. Women with known HIV and confirmed normal cytology were also included. Participants were recruited at the Colposcopy (abnormal cytology) and Family Planning (normal cytology) clinics at Tygerberg Academic Hospital.

3.2.2.2 Exclusion criteria:

Women that were pregnant at the time of their colposcopy visit were excluded as excisional procedures such as Large Loop Excision of the Transformation Zone (LLETZ) are not part of the routine workup for such patients at Tygerberg Academic Hospital. Colposcopy examinations during pregnancy are safe for expectant mothers, but excisional operations of HSIL are deemed risky since they may cause foetal loss (Ciavattini *et al*., 2018). Woman with unknown HIV status were also excluded from the study.

3.2.2.3 Sample collection

The following group sizes per diagnosis were determined: LSIL (12 HIV+; 12 HIV-), HSIL (12 HIV+; 12 HIV-), ASC-US (12 HIV+; 12 HIV-), ASC-H (12 HIV+; 12 HIV-) and NILM (6 HIV+; 6 HIV-) (negative controls). Training was received for obtaining participant informed consent. A checklist for the informed consent process to keep track of all important points covered in the informed consent form was used. Consenting of patients was done at the respective clinics in a private area. Eligible participants were enrolled from October 2021 to June 2022. A HIV test result of 3 months or less was considered as a known HIV status. Participants with no testing or testing outside of the 3-month window period were invited to have HIV testing done. Counselling for HIV testing was done by a professional research nurse before bloods were drawn for testing. Should any positive results have been obtained from these HIV tests, the routine counselling at Tygerberg Hospital would have been followed. All samples and data were de-identified and given unique study numbers, thus no patient data (name, hospital folder number, etc.) was shared publicly, either verbally or in any publication or presentation.

3.2.3 Collection of exfoliated epithelial cells from the cervix uteri

A trained clinician performed the cervical swabbing of consented participants by using a Rovers Cervex-Combi cytobrush (Catalogue #491462, Rovers Medical Devices B.V, Netherlands). The cervical brush containing the exfoliated cervical cells was then placed into a sterile 50 ml skirted centrifuge tube containing 15 ml BD SurePath™ preservation fluid

(Catalogue #491337, BD TriPath, Burlington, NC). Samples were stored at 4ºC until required for further analysis.

3.2.4 Estimating the number of exfoliated cervical cells using an automated cell counter.

The Bio- Rad TC20™ automated cell counter (BioRad, Singapore) was used to estimate the number of exfoliated cervical cells collected in 15 ml SurePath™ preservative medium (Catalogue #491337, BD TriPath, Burlington, NC). The Bio-Rad TC20™ automated cell provides a estimation of total cell counts with or without the use of the trypan blue stain (TC20™ automated cell counter instruction manual, 2011). Due to the interest in the total amount of cells (dead and live) all cell counts were done without the use of the Trypan blue viable stain (BioRad, Singapore). The collection tube containing the exfoliated cells were mixed for 2 minutes using a vortex to ensure maximum cell removal from cytology brushes. The cytology brush was aseptically removed from the collection vial using sterile flat-tip tweezers. Prior to the initial cell count, cells were mixed again with a vortex to ensure a homogenous cell suspension. One hundred micro litres of the cell suspension were then aliquoted into a microcentrifuge tube, of which 10 µl was pipetted into a disposable cell counting chamber (BioRad, Singapore) and counted using TC20 Cell counter (BioRad, Singapore).

3.2.5 Detection of HPV DNA in exfoliated cervical cells using the Hybrispot HPV Direct Flow Chip Kit

The Hybrispot HPV Direct Flow Chip Kit (Master Diagnostica, Spain) was used to determine the HPV types in exfoliated cervical cells collected in SurePath™ (Catalogue #491337, BD TriPath, Burlington) following the manufacturer's instructions. The assay could detect 18 HR-HPV (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82,) and 18 LR-HPV (HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81 and 84) types by Polymerase Chain Reaction (PCR), followed by reverse hybridization on a membrane containing specific probes.

The kit contained biotinylated primers for the amplification of the human genomic DNA fragment (beta-globin gene) that was used as an endogenous amplification control as well as hybridization controls which served as a quality control. Additionally, the kit included universal HPV primers targeting a broad range of mucosal HPV types including genotypes excluded from the assay. Each chip contained the range of target-specific probes plus probes for the amplification and hybridization controls to which the biotinylated PCR products hybridized. Once the binding between the specific amplicons and their corresponding probes has occurred, the signal was visualized by an immunoenzymatic colorimetric reaction with Streptavidin−Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization.

Since the kit does not require prior DNA extraction from cervical swabs in a cytology collection medium, samples were directly used for PCR amplification. Briefly, 600 µl of the homogenous cervical cell suspension was placed into a 2 ml microcentrifuge tube and spun at room temperature for 1 min at 2000 rpm in a benchtop centrifuge. The rest of the cell suspension was stored at 4 ˚C for DNA extraction and biomarker analysis. The supernatant fraction was discarded, and the cell pellet was washed with 400 µl DNase/RNase-free water (provided with Hybrispot HPV Direct Flow Chip Kit) and spun at room temperature for 1 min at 2000 rpm in a benchtop centrifuge. The supernatant was removed, and the cell pellet was resuspended in 300 µl DNase/RNase-free water. These resuspended cells were then vortex to homogenise the cell suspension. A volume of 30µl of the homogenized cell suspension was used as DNA template for PCR reactions. Once the sample was added to the lyophilized PCR mix it was placed in a Thermal cycler (Veriti 96, Thermofisher Scientific). The PCR cycling conditions were 25 ˚C for 10 minutes, 94 ˚C for 3 minutes, 15 cycles at 94 ˚C for 30 seconds, 47 ˚C for 30 seconds, 72 ˚C for 30 seconds, 35 cycles at 94 ˚C for 30 seconds, 65 ˚C for 30 seconds, 72 ˚C for 30 seconds, 72 ˚C for 5 minutes and hold at 8 ˚C. Upon completion of the PCR amplification, the PCR product was denatured by heating at 95 °C for 10 minutes in a thermocycler (Veriti 96, Thermofisher Scientific) followed by immediate cooling on ice for at least 2 minutes.

The chip hybridization was performed using the HybriSpot 12 (HS12) semi-automated reverse hybridisation system (Master Diagnostica, Spain) following the instructions of the manufacturer. Briefly, an HPV chip was placed in the HS12 Platform. Three hundred

microliters of preheated (41 ºC) Hybridization solution were pipetted onto the HPV chip and incubated for 2 minutes and then removed. All solutions were removed by suction using the on-board vacuum pump. A mixture containing 30µl of PCR product and 270 µl Hybridization solution was dispensed onto each chip and incubated at 41 ºC for 8 minutes. Subsequently, fluids were removed and the chip was washed three times with 300 µl of Hybridization solution. After removal of the Hybridization solution, 300 µl of Blocking solution was then dispensed onto each chip and incubated for 5 minutes. Streptavidin-Alkaline Phosphatase (300 µl) was then added to each chip and incubated for 5 mins at 29 ºC. After removal of the fluid by vacuum pump, the chip was washed 4 times with Washing Buffer I. Three hundred microliters of Reagent E were aliquoted onto each chip, followed by an incubation of 10 minutes at 36 ºC. Finally, 300µl of Washing Buffer II was dispensed onto each chip and removed using the on-board vacuum pump. The HPV chips was then removed from the HS2 Platform for analysis. The HybriSoft camera and software (Master Diagnostica, Spain) were used to capture and analyse the HPV chips.

HPV DNA testing was done on 69 collected samples in batches of 10 to 12 reactions. Each batch of assays included an “in-house” external positive control comprising 20 ng of HPV 16 (catalogue #45113D, ATCC, Manassas, United States of America), 20 ng of HPV 18 plasmid DNA (catalogue #45152D, ATCC, Manassas, United States of America) and 20 ng human genomic DNA (catalogue# PRG3041) (Promega, Madison, WI, USA). A non-template control (NTC) for detecting external DNA contamination was also included in each batch. The “in-house” external positive control and non-template control were analyzed using the HybriSpot software. Results were obtained as automatically generated reports that indicated the HPV types detected as well as reporting on the adequacy of the sample, where applicable. Examples of such reports for the “in-house” external positive control and the NTC were included as Addendum C and Addendum D, respectively.

Data were interpreted either as single HR-HPV or LR-HPV, multiple HR-HPV or LR-HPV and mixed HR/LR HPV infections. A single HPV infection indicated cases where only one high risk or low risk HPV were detected. A multiple HPV infection designated cases where two or more high risk or low risk HPV type were detected. Mixed HR/LR-HPV infected described cases where a mixture of HR-HPV and LR-HPV types were detected.

3.2.6 Immunocytochemistry staining of $p16^{INK4a}$/Ki67 dual staining using the CINtec® PLUS Cytology Kit]

The CINtec® PLUS Cytology kit (Roche Diagnostics, Pleasanton, California, USA) was used to simultaneously detect $p16^{INK4a}$ and Ki-67 in exfoliated cervical cells collected in SurePath™ (Catalogue #491337, BD TriPath, Burlington). The CINtec® PLUS Cytology kit (Roche Diagnostics, Pleasanton, California, USA) is a ready-to-use kit for the Ventana automated immunostaining platform. A manual method was tested as specialised equipment, which we did not have access to, was required for the automated CINtec® PLUS Cytology immunocytochemistry test. The method was first optimised before samples were tested.

3.2.6.1 Optimization of CINtec plus kit: Manual staining method.

The optimization of the manual method entailed:

i) Determination of the ideal number of cells per slide required for optimal staining and result interpretation.
ii) Selection of the most suitable slide brand and best possible drying conditions in terms of optimal cell adhesion.
iii) Effective minimum volume of antibody used per sample for staining
iv) Optimal haematoxylin staining times.

3.2.6.1.1 Determination of the ideal number of cells and slide type required for optimal staining and result interpretation.

The ideal number of cells per slide required for optimum staining and meaningful result interpretation was determined. As 5000 epithelial cervical cells are deemed satisfactory for liquid-based cytology by Nayar and Wilbur (2015), we tested 10 000 and 15 0000 cells, respectively in triplicate. Based on the evaluation for adequate cellularity on a slide after manual staining, the Cytotechnologist checked for the presence of at least 10 endocervical cells as this would be an indication that the transformation zone was sampled. A cellularity score was given based on an average of at least 12 squamous cells per 40x magnification field equates

to 5000 and more cells in total (Nayar and Wilbur, 2015). The starting number of 15 000 cells were determined to be adequate for manual staining using the CINtec® Plus cytology kit.

3.2.6.1.2 Selection of the most suitable slide brand and air-drying method in terms of optimal cell adhesion

The use of inadequate slides and sub-optimal drying of cell suspensions could result in loss of cells during the staining procedure and introduce cellular artefacts, respectively, which could interfere with staining interpretation. The Histobond Plus, SurePath, and Shandon Thermofisher Scientific brands of positively charged slides were compared for best cellularity after drying and staining. An amount of 15 000 (in 100 µl) cells were placed on slidesThe 100 µl volume of cell suspension resulted in the best cellularity (Airdrying cells in a BSCII hood to limit dust carryover was compared to drying in an oven at 65 ºC for 4 minutes. Slides were then subjected to the staining procedure. Only a slight difference in cellularity between the two drying methods was observed by the Cytotechnologist. We therefore decided on the air drying of the specimen overnight in a BSC II hood which ensured that all samples are dried sufficiently and fixed to the slide ensuring higher cellularity. Different volumes of the cell suspensions were also tested (10 µl, 20 µl, 50 µl, and 100 µl) and airdried against the cytospin (Cytospin 4, Thermofisher, California, USA) method on Thermo Fisher Shandon, Histobond and SurePath slides to determine which resulted in the best cellularity. The Thermo Fisher Shandon slide brand using 100 µl of cell suspension and airdried, showed the highest cellularity with manual staining procedure and was thus the volume and slide brand of choice after validation.

3.2.6.1.3 Effective minimum volume of antibody used per sample for staining

As we were optimizing a manual method for the CINtec® PLUS Cytology kit and to absorb costs as these kits are rather expensive, we wanted to determine whether reducing the manufacturer's prescribed of 200 µl would still result in successful staining and result interpretation. Antibody volumes of 50 µl and 100 µl against 200 µl were tested. We reasoned that since 200 µl of antibody covers the entire slide in the automated instruments and that we demarcated the cell area with a hydrophobic pen to contain the antibody solution, volumes of 50 µl and 100 µl should sufficiently cover the cell area. Using light microscopy, sufficient

p16^{INK4a}/Ki-67 staining was observed slides stained with 100 µl of antibody solution. The use of 50 µl antibody, however, resulted in sub-optimal staining with high background staining, thus that hampering interpretation. It was difficult to distinguish between the brown p16^{INK4a} from the and the red Ki-67 stain when using 50 µl of antibody solution.

3.2.6.1.4 Most favourable haematoxylin staining times.

The optimal duration for the Haematoxylin counterstaining was determined for manual staining of cervical intraepithelial cells with the CINtec Plus kit. Haematoxylin staining times of 2, 5 and 10 minutes were tested for the most suitable method of staining reference cells. The optimum duration of the Haematoxylin counterstaining was determines to be 10 minutes.

3.2.6.2 Optimised p16^{INK4a}/Ki67 dual staining CINtec® PLUS Cytology method

The BD Density Reagent was used to clarify the cervical cell suspensions from obscuring agents such as mucus and blood that may interrupt the interpretation and analysis of a specimen. The BD Density Reagent was added to the 3 ml samples previously aliquoted in a separate 15 ml conical tube and mixed by pipetting up and down. The sample containing BD Density Reagent and cervical epithelial cells was placed into a benchtop centrifuge and spun for 2 minutes at 200 x g. Taking care not to disturb the pellet, the supernatant was aspirated off and discarded into a waste container using a sterile pasteur pipette. The pellet was resuspended in 1.5 ml BD Density Reagent and centrifuged at 800x g for 10 minutes. The supernatant was then discarded, and the cell pellet was resuspended in 250 µl of BD SurePath^{TM} preservation fluid. The number of cells in the cell suspension was again determined as previously mentioned. A volume of 100 µl containing up to 15 000 cells was added onto a Menzel Shandon superfrost plus slide (Cat #J1800AMNZ) and left overnight in a BSCII Hood to airdry. The following day, slides were manually stained with the CINtec Plus kit following the manufacturer's instructions.

Briefly, cells were fixed in 99 % (v/v) ethanol for 10 minutes and left to air dry for one hour. The slides were rehydrated by placing the air-dried slides into a fresh 100 % (v/v) xylene bath for 2 minutes followed by sequential incubation in 99 % (v/v) ethanol for 2 minutes, 70 % (v/v)

ethanol for 2 minutes and distilled water for 2 minutes. Slides were then placed in pre-warmed Epitope retrieval solution for 15 minutes at 95 ºC. The staining jars containing the epitope retrieval solutions was then placed onto a bench and left to cool to at least 50 ºC before manual staining with the CINtec plus kit continued. Once cooled, the cell area on the slide was demarcated by encircling with a hydrophobic pen as a measure of containment to ensure sufficient coverage of kit reagents. Wash buffer was then pipetted onto the cells and left to incubate for 5 minutes. The slide was then tilted to allow wash buffer to run off the slide. Peroxidase Blocking Reagent was pipetted onto the cell area and left to incubate for 5 minutes followed by washing with Wash buffer. The $p16^{INK4a}$/Ki-67 Primary antibody solution was pipetted onto cells and left to incubate for 30 minutes. To remove the $p16^{INK4a}$/Ki-67 primary antibody, the slide was tilted so that the solution can run off. The slide was then washed by incubating with wash buffer for 5 minutes before the Horseradish Peroxidase (HRP) Visualization reagent was applied. The samples were then rinsed twice with wash buffer before applying the Visualization reagent AP. After incubation with visualization reagent samples were counterstained with haematoxylin and mounted with liquid mounted provided with the CINtec plus kit before cover slipping slides.

For each batch of staining, a histologically confirmed HSIL was used as a positive control and a cytologically confirmed NILM as a negative control. The HSIL positive controls used were MLMPath-001, MLMPath-005, MLMPath-007, MLMPath-024 and MLMPath-035. The NILM negative controls used were MLMPath-058, MLMPath-059, MLMPath-061, MLMPath-063 and MLMPath-065. $p16^{INK4a}$/Ki-67 dual staining of specimens was interpreted blindly by a Cytotechnologist by using a light microscope (BX41 Olympus, Olympus Optical Co, Germany). A positive control showed $p16^{INK4a}$/Ki-67 dual staining within the same cell whereas a negative control showed only the haematoxylin counterstaining.

3.2.7 Isolation of DNA from exfoliated cervical intraepithelial squamous cells using the Qiagen QIAmp DNA FFPE Tissue Kit

Total DNA was extracted from exfoliated cervical cells using the QIAamp DNA FFPE Tissue Kit (Catalogue #56404) (Qiagen, Hilden, Germany) according to manufacturer's recommendations. A total cell count containing at least 3 million cells were pelleted by spinning the sample at 500 x g for 10 minutes in a benchtop centrifuge. Pelleted cervical intraepithelial squamous cells were resuspend the in 180 µl ATL Buffer and lysed using 20 µl proteinase K. Specimens were then incubated at 56 °C for 1 h (or until the sample has been completely lysed) and immediately thereafter placed at 90 °C for 15 minutes. To digest residual RNA, 2 µl RNase A (100 mg/ml) was added to each specimen and incubated for 2 minutes at room temperature. Each specimen was mixed with Buffer AL and molecular grade Ethanol (Catalogue #E7023) (Sigma-Aldrich, St. Louis, Missouri, United States), before the lysates were added to a QIAamp minelute column. The columns were washed with buffer AW1 and buffer AW2, respectively and spun down at 11 000 x g in a benchtop centrifuge. DNA was then double eluted using a total volume of 65 µl ATE buffer and stored at -20 °C until further analysis.

3.2.8 Nucleic acid purity and quantity assessment

Nucleic acid purity was assessed by measuring the A260nm/A280nm and A260nm/A230nm absorbance ratios using the Biodrop µLite spectrophotometer (Biodrop LTD, Cambridge, UK) while the nucleic acid concentrations was assessed by using the DNA High sensitivity Qubit kit with the Qubit 3.0 fluorometer (Life Technologies, Thermo Fisher Scientific, California, USA) according to manufacturer's recommendations. It is generally accepted that the A280nm/A260nm for pure DNA is 1.8 and an A260nm/A230nm ratio between 2.0 and 2.2 is indicative of pure nucleic acids. In our study a A280nm/A260nm ratio of 1.7-1.8 was acceptable with a A260nm/A230nm ratio of 2.2-2.4.

3.2.9 Bisulfite conversion of genomic DNA for methylation assays

Deoxynucleic acids isolated from exfoliated cervical intraepithelial squamous cells were bisulfite treated using the Epitect Bisulfite conversion kit (Catalogue #59104, Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The procedure consists of two steps, (i) the bisulfite conversion step followed by (ii) the DNA clean-up step. The bisulfite reactions were prepared in 200 µl PCR tubes. An amount of 1µg of isolated DNA in a volume of 20 µl was subjected to bisulfite conversion. Briefly, a PCR reaction for the bisulfite treatment of genomic DNA was prepared by adding the bisulfite mix and DNA protect buffer to the 1 µg DNA (20 µl) in a volume not exceeding 20 µl. The total reaction volume was thus 40 µl.

The bisulfite conversion of DNA was performed in a thermal cycler with cycling conditions of: (i) denaturation for 5 minutes at 95 °C, incubation at 25 minutes at 60 °C, (ii) denaturation for 5 minutes at 95°C, incubation for 85 minutes at 60 °C, (iii) denaturation for 5 minutes at 95°C followed by 175 minutes at 60°C and then (iv) hold at 20 °C. The bisulfite converted specimens were then stored at room temperature until clean-up of bisulfite converted DNA. After briefly spinning the reaction tubes, the bisulfite converted DNA was transferred to a 1.5 ml microcentrifuge tube containing freshly prepared Buffer BL and 10 µg/ml carrier RNA. This mixture was then transferred to the Epitect spin column that was spun at maximum speed for 1 minute. Five hundred microlitre of Buffer BW was added to the spin column and centrifuged at full speed for 1 minute. This was followed by the addition of 500 µl Buffer BD to each Epitect spin column and incubation at room temperature for 15 minutes. The spin columns were spun down at full speed for 1 minute in a benchtop centrifuge. Buffer BW was then added to Epitect column and centrifuged at full speed for 1 minute. The Epitect spin columns were incubated at 56°C for 5 minutes and DNA was eluted in 40µl EB Buffer. Purified bisulfite converted DNA was stored at -20°C until methylation assays were done.

3.2.10 Assays to determine methylation status of *CADM1*, *MAL* and *miR-124-2* genes in exfoliated cervical intraepithelial squamous cells.

The degree of methylation of the *CADM1, MAL* and *miR-124-2* genes was investigated using the respective EpiMelt test assays (Methyl Detect, Aalborg, Denmark) and methylation-sensitive high-resolution melting (MS-HRM) analysis with the Quant Studio 5 real time PCR machine (Thermo Fisher Scientific, California, USA). Bisulfite converted DNA equivalent to 50 ng were used for DNA methylation assays. A 14 µl PCR master mix containing the relevant primers and probes was used for each reaction. A methylation positive, methylation negative and assay calibration controls were provided with the kit and was included on each prepared plate per assay. Bisulfite converted DNA from 4 NILM samples from HIV negative participants without HPV infection and $p16^{INK4a}$ /Ki67 positivity were pooled and used as the external negative control in the respective assays. The MS-HRM data was analysed using the Quantstudio Design and Analysis software (Thermo Fisher, Massachusetts, USA). All samples were done in technical triplicates for the methylation assays. Ready to use positive methylation control, negative methylation control and methylation assay controls were provided with each kit and included in each run. Respective external negative controls consisted of 4 pooled HPV-negative NILM specimens from HIV positive (MLMPath-058, MLMPath-059, MLMPath-061, MLMPath-063) and 4 pooled HPV-negative NILM samples from HIV negative participants (MLMPath-065, MLMPath-066, MLMPath-067, MLMPath-068). Therefore, 2 separate negative controls (one HIV positive and one HIV negative) with no HPV infection and $p16^{INK4a}$/Ki-67 dual staining were used as external negative controls. A non-template control as quality control measure for the detection of DNA cross contamination was also included for each assay.

MS-HRM analysis employing the resultant melt curves and specific melting temperatures of methylation positive control, methylation negative control and methylation assay controls were used for interpretation. A qualitative method was used to assess the DNA methylations status of the *CADM1*, *MAL* and *miR-124-2* genes. The concordance to the melting temperatures of the respective assay positive, negative and assay controls was used to determine the methylation status of our unknown samples. Therefore, the methylation status of an unknown samples could either be scored positive, heterogenous, or negative based on the correspondence to the methylation positive control, methylation assay control and methylation negative control, respectively.

According to the MethylDetect manufacturer's guide, the melting temperatures for the provided methylation controls as determined when using a Roche LightCycler® 480 platform (Roche Applied Science, Laval, PQ, Canada) for *CADM1, MAL* and *miR-124-2* genes were as follows: *CADM1* methylation positive control: Tm = 79.5 ºC, *CADM1* methylation negative control: Tm=76.5 ºC and the *CADM1* methylation assay control: Tm=78 ºC, *MAL* methylation positive control: Tm = 77 ºC, *MAL* methylation negative control: Tm=80 ºC, *MAL* methylation assay control: Tm=79 ºC, *miR-124-2* methylation positive control: Tm = 79 ºC, *miR-124-2* methylation negative control: Tm=76.5 ºC and the *miR-124-2* methylation assay control: Tm=76.5 ºC.

3.2.11 Statistical analysis

No sample size calculation was done, but samples size was determined using the "sample size of 12 per group rule of thumb for a pilot study" of Julious (2005). We assumed that 24 cases per diagnosis group (N = 96) with 12 samples each for samples collected from HIV positive and HIV negative women, will be sufficient to determine feasibility of the methylation assays as well as the $p16^{INK4a}$/Ki67 dual staining in cervical exfoliated specimens (Lewis *et al.,* 2021 and Bell *et al.,* 2018). Data was analyzed using Stata version 13.1. To analyze categorical variables, such as HR- HPV positivity and type, $p16^{INK4a}$/Ki67 positivity vs. negativity, and methylated DNA vs. unmethylated DNA, frequencies and proportions was used to summarize the data. Associations between HPV strains and categorical variables such as HIV status, cytological diagnosis, and $p16^{INK4a}$/Ki-67 biomarker expression was assessed using Fisher's Exact test or the Chi-Square test. The respective performance of the tests for HR-HPV typing, the methylation markers (*CADM1, MAL* and *miR-124-2*), and $p16^{INK4a}$/Ki-67 co-expression to was evaluated by performing Receiver Operator Characteristic (ROC) analysis. A ROC analysis was done separately for the LSIL (LSIL/ASC-US) and HSIL (HSIL/ASC-H) cytology, using the LSIL and HSIL histology diagnostic results as the respective gold standards. A logistic regression analysis was used to determine the separate associations of LSIL and HSIL cytology with HR-HPV testing, $p16^{INK4a}$/Ki-67 co-expression, gene methylation (*CADM1, MAL* and *miR-124-2*). All statistical tests were performed at an alpha level of 5 % level for significance where $p < 0.05$ was considered significantly different and 95 % confidence intervals were be reported.

Chapter 4

Results

4.1 Specimen collection

Specimens for this prospective observational laboratory-based pilot study were collected from October 2021 to August 2022. A subset of specimens negative for intraepithelial lesions (NILM) were collected as negative controls. As the NILM samples were prospectively collected at the Family Planning Clinic at Tygerberg Hospital, samples were only included after the routine cytology results were finalised to ensure that only confirmed NILM cases were used. The proportion of samples collected were 71.9 % (69/96) (Table 4.1). Study groups were subdivided according to their cytology diagnosis and HIV status.

Table 4.1: The proportion of cases collected for each cytology diagnosis group for HIV positive and HIV negative participants.

LSIL		HSIL		ASC-US		ASC-H		NILM	
HIV +	**HIV -**	**HIV +**	**HIV-**	**HIV +**	**HIV -**	**HIV +**	**HIV -**	**HIV +**	**HIV -**
12/12 (100%)	0/12 (0%)	12/12 (100%)	12/12 (100%)	4/12 (33.3%)	4/12 (33.3%)	11/12 (91.6%)	2/12 (16.6%)	6/6 (100%)	6/6 (100%)

A significant relationship between Pap cytology diagnosis and HIV status was determined using a Pearson's Chi-Squared tested (alpha level = 0.5, p =0.011).

4.2 Assessment of the relationship between Age and Cytology diagnosis in woman.

The age range for the study participants was between 21 years and 60 years, with a median age of 37 years. Our results show that most (85.5%; 59/69) of our study participants were younger than 45 years old (Figure 4.1). Age at diagnosis was generally positively skewed for all cytology diagnosis as the medians were closer to the 25th percentile (Q1) than the 75th percentile

(Q3) for all groups (Figure 4.1). The medians for the LSIL, HSIL, ASC-H and ASC-US groups were 36.5 years, 35 years, 39 years, and 36 years, respectively. The range of ages for LSIL was between 31 years to 46 years with the age distribution of the HSIL group from 27 years to 60 years depicted by the whiskers in Figure 4.1.

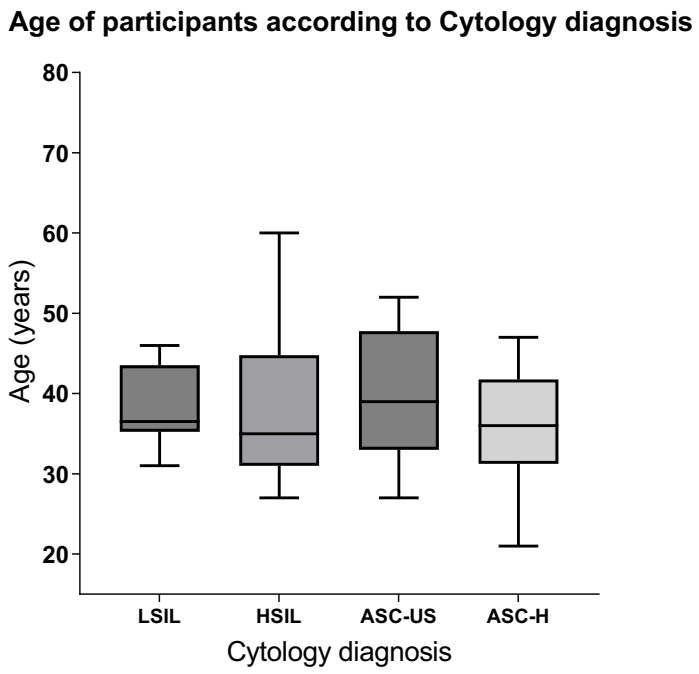

Figure 4.1: A box and whisker plot shows the variation of age variation of participants for each cytology diagnosis. The median is indicated by the thin line inside the boxes. The 25th and 75th percentiles (boundaries of the boxes), and 5th and 95th percentiles (whiskers above and below box plots) are depicted.

The age range of ASC-H was between 21 years and 47 years, whilst that of ASC-US was between 27 years to 52 years. The HSIL and ASC-US groups showed more variability in age at diagnosis as the interquartile ranges of these groups were larger compared to that of the LSIL and ASC-H groups. For the HSIL group the Q1 was 31 years and the Q3 was 44.7 years, whereas for the ASC-US group the Q1 was 33 years and the Q3 was 47.5 years. The Q1 for the LSIL groups was 35.2 years and the Q3 was 43.5 years, whilst for the ASC-H group the Q1 was 31.2 years and the Q3 was 41.7 years. Moreover, most participants with a cytology diagnosis of HSIL were between 32 years and 44 years, whereas for the LSIL group, the majority were aged between 35 years and 43 years.

4.3 Assessment of the relationship between Age and HIV status in woman with abnormal cervical cytology.

The proportion of WLWH in our study was 65.2% (45/69) while 34.7% (24/69) had no HIV infection. Our results show that the age range of the HIV positive group was between 21 years and 52 years with a median age of 37 years (Figure 4.2). The ages of the HIV negative group spanned from 26 years to 60 years with a median age of 35.5 years (Figure 4.2). The age distribution of WLWH and of women with no HIV infection was positively skewed as the median for both groups were closer to the 25th percentile (Q1) than the 75th percentile (Q3) (Figure 4.2).

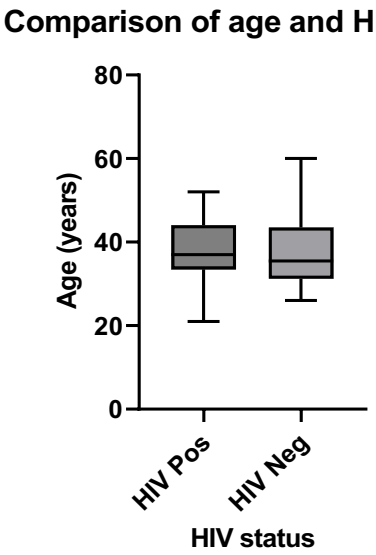

Figure 4.2: The distribution of ages among HIV positive and HIV negative research participants is displayed using a box and whisker plot. The line inside the boxes represents the median. The box and whisker plot shows the 25th and 75th percentiles (box boundaries), as well as the 5th and 95th percentiles (whiskers above and below box plots).

The variability in age at presentation seemed similar as only a marginal difference was observed in the interquartile ranges between the two groups. For the HIV negative group, the Q1 was 31.2 years and the Q3 was 43.5 years, whereas for the HIV positive group the Q1 was

33.5 years and the Q3 was 44 years. Therefore, overall, the participants of this study were relatively young, irrespective of HIV status.

4.4 HPV genotype determination in exfoliated cervical epithelial cells

4.4.1 Detection of HPV DNA in cervical swabs from HIV positive and HIV negative woman.

Immunocompromised individuals, for example WLWH, are at increased risk in contracting HPV and subsequently developing precancerous lesions and cervical cancer (WHO, 2022; Saidu *et al*., 2016; Mbulawa *et al*., 2012). We tested for HPV DNA in the exfoliated cervical cells collected from HIV positive and HIV negative women using the Hybrispot HPV direct flow chip kit (Figure 4.3). As expected, the HybriSpot assay endogenous hybridization and amplification controls were positive for all the samples tested. Similarly, the external HPV16/HPV18 positive control was detected. No PCR products were detected on the NTC chip, indicating that no external DNA contamination.

Overall, 74 % (51/69; 95% CI: 62,1%-83%) of our study samples tested positive for HPV. Of these, 80.3% (41/51; 95% CI: 47.3%-70.4%) were positive for HR-HPV types (Figure 4.3). In turn, 23.5% (12/51; 95% CI: 10% - 28,4%) represented single HR-HPV infections while 19.6% (10/51; 95% CI:7,8% - 25,1%) were multiple HR-infections (Figure 4.3). Low-risk HPV types were detected in 56.8% (29/51; 95% CI: 30,8% - 54,1%) of cases, of which 7.8% (4/51; 95% CI: 2,1% - 14,6%) were single LR-HPV infections and 11.7% (6/51; 95% CI: 3,9% - 18,2%) multiple LR-HPV infections (Figure 4.3). Mixed infections consisting of LR- and HR-HPV types were also detected in 37.2% of cases (19/51) (95% CI: 18,1% - 39,4%) (Figure 4.3).

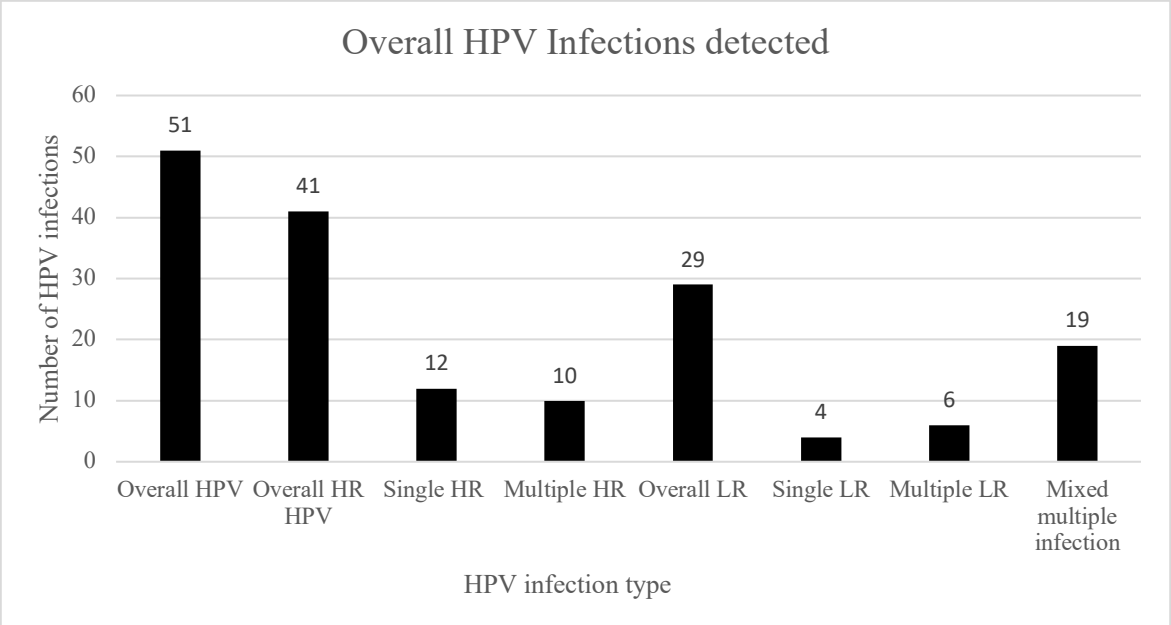

Figure 4.3: The frequencies of the overall HPV infections detected in the cervical swabs collected from participants. The frequencies of HR-HPV as well as LR-HPV types in terms of single and multiple infections as well as mixed HPV types are represented. Single HPV denotes cases where only one high risk or low risk HPV was detected. Multiple HPV detection signifies cases where two or more high risk or low risk HPV type was detected. Mixed LR-or HR-HPV type detection indicates cases where a mixture of the two types were detected.

Our results showed that HPV infection was detected in 70,6% (36/51) of WLWH and 29,4% (15/51) of HIV negative women. The incidence of LR-HPV types observed in our study group for WLWH, and HIV negative women was 82.8% (24/29) and 17,2% (5/29), respectively (Figure 4.4). High-risk HPV was detected in 68.3% (28/41) of HIV positive and 31.7% (13/41) of HIV negative participants (Figure 4.4). No significant association between HIV status and HPV infection ($p = 0.115$) was determined when using the Pearson Chi-square test.

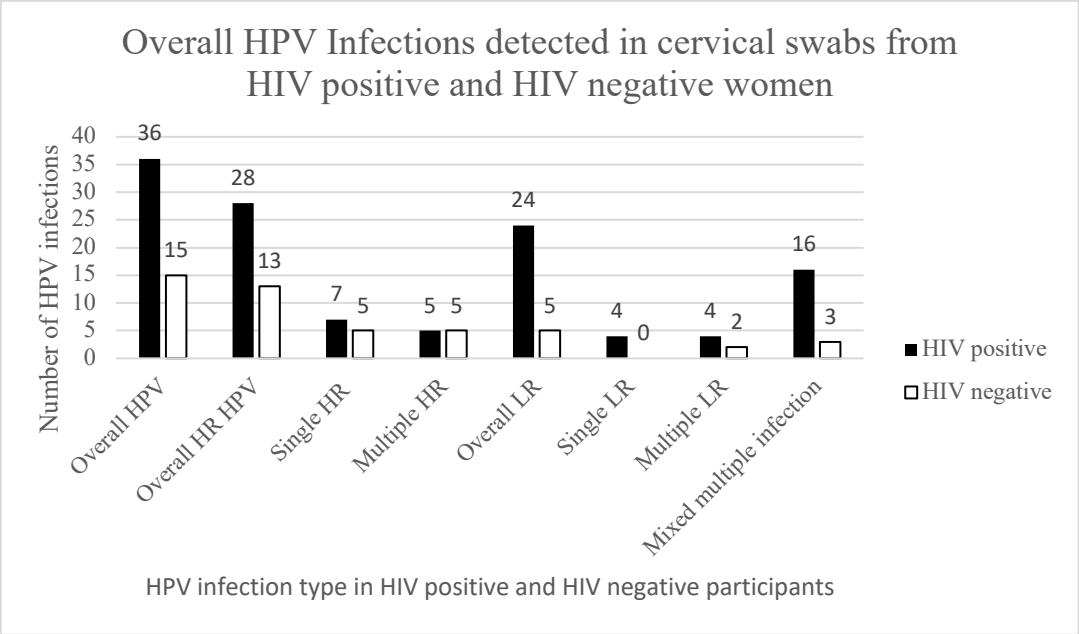

Figure 4.4: The overall HPV detected in the cervical swabs collected from in HIV positive and HIV negative woman. The frequencies of HR- HPV as well as LR-HPV types in terms of single and multiple infections are represented.

It was observed that HR-HPV infections were more common than LR-HPV infections in our study group (Figure 4.4). Moreover, the proportion of study participants who were co-infected with more than one HR-HPV types 19.6% (10/51) was higher than those with multiple LR-HPV types 11.8% (6/51). As stated earlier, the proportion of participants with multiple LR and HR-HPV infection was 27,5%. Our findings also showed WLWH harboured the most HPV infections, albeit HR-HPV types (68.3%; 28/41), LR-HPV (85.7%;24/28) or mixed LR/HR-HPV (89.5%; 17/19). A Pearson Chi-square test revealed no significant difference between HIV status and HPV detection, whether single, mixed, or multiple HPV infections (p= 0.115).

When comparing the HPV detection rate with the cytological diagnosis, we found that the HSIL group harboured the most HPV infections (21/51; 41.1%), followed in turn by the LSIL (12/51; 23.5%), ASC-H (9/51; 17.6%) and ASC-US (6/51; 11.7%). The HPV infection status of the NILM negative control samples were also evaluated. Our results showed that 3 (3/51; 5.8%) of these samples hosted HPV infections. MLMPath-060 tested positive for HPV 35 and HPV 40, MLMPath-062 tested positive for HPV 61while MLMPath-064 tested positive for HPV61 and HPV 71. A significant association between the Pap cytology diagnosis and HPV was

determined using a Pearson's Chi-Squared test. At an alpha level set at 0.5, p= 0.001 was obtained.

Comparison of HPV detection between cytology diagnosis and HPV in WLWH and women HIV uninfected, it was seen that all LSIL (12/12; 100%) samples from WLWH tested positive for HPV, whereas for the HSIL group, 11 (91.6%) of the samples from WLWH and 10 (83.3%) from HIV uninfected women with were positive for HPV. For the ASC-H group, 8 (72.7%) samples from WLWH and 1 (50%) HIV from infected woman tested positive for HPV (Table 4.2).

Table 4.2: Distribution of the frequency of HPV genotypes detected in HIV-positive and HIV-negative women according to cytological findings.

	LSIL		HSIL		ASC-US		ASC-H	
	HIV +	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-
HPV positive	12	0	11	10	3	3	8	1
HPV negative	0	0	1	2	1	1	3	1
Total	12	0	12	12	4	4	11	2

Our findings show that irrespective of the diagnosis, most samples from WLWH tested positive for HPV, except for the ASC-US. Therefore, the known association of HIV infection with HPV infection is seen (Figure 4.5 and Table 4.2). Our results also showed that HPV incidence increased with increasing severity of cervical lesions (HSIL, ASC-H) in WLWH (Figure 4.5 and Table 4.2).

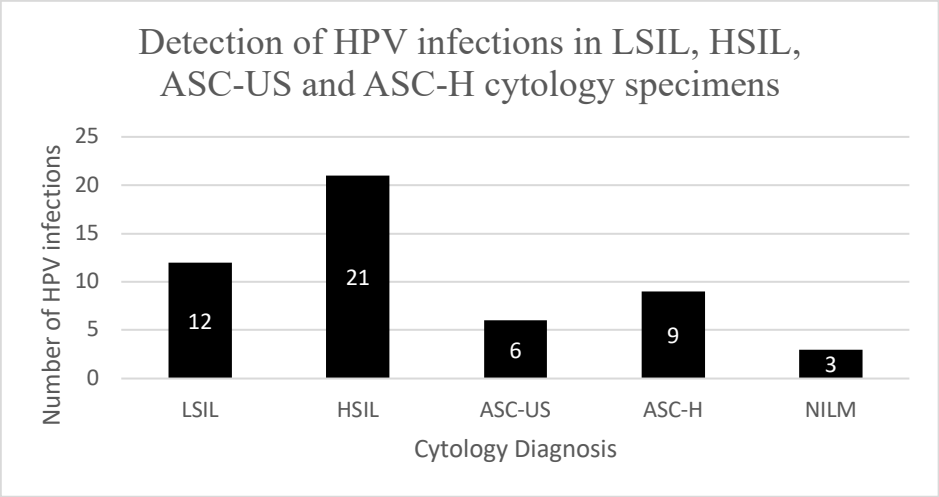

Figure 4.5: The number of HPV infections detected per cervical cytology diagnosis. The plot displays the frequency of HPV infections detected per cytology diagnosis groups.

4.4.2 HPV genotypes detected in HIV positive and HIV negative women with abnormal cytology diagnosis.

Several HR-HPV and LR-HPV types were detected in the exfoliated cervical intraepithelial cell collected from WLWH and HIV uninfected women. As shown in Figure 4.6, the 5 most common HR-HPV types detected were HPV 16 (27.4%, 14/51), HPV 35 (17.6%, 9/51), HPV 33 (17.6%, 9/51), HPV 82 (15.6%, 8/51), HPV 58 (15.6%, 8/51), HPV 18 and HPV 52 had similar frequencies of (13.7%, 7/51). (Figure 4.6). The most detected LR-HPV types in our study group, in descending order, were HPV 81 (13.7%, 7/51), HPV 62 (13.7%, 7/51), HPV 67 (11.7%,6/51) HPV70 (9.8%, 5/51), HPV 54 (9.8%, 5/51) and HPV 6 (9.8%, 5/51) (Figure 4.7).

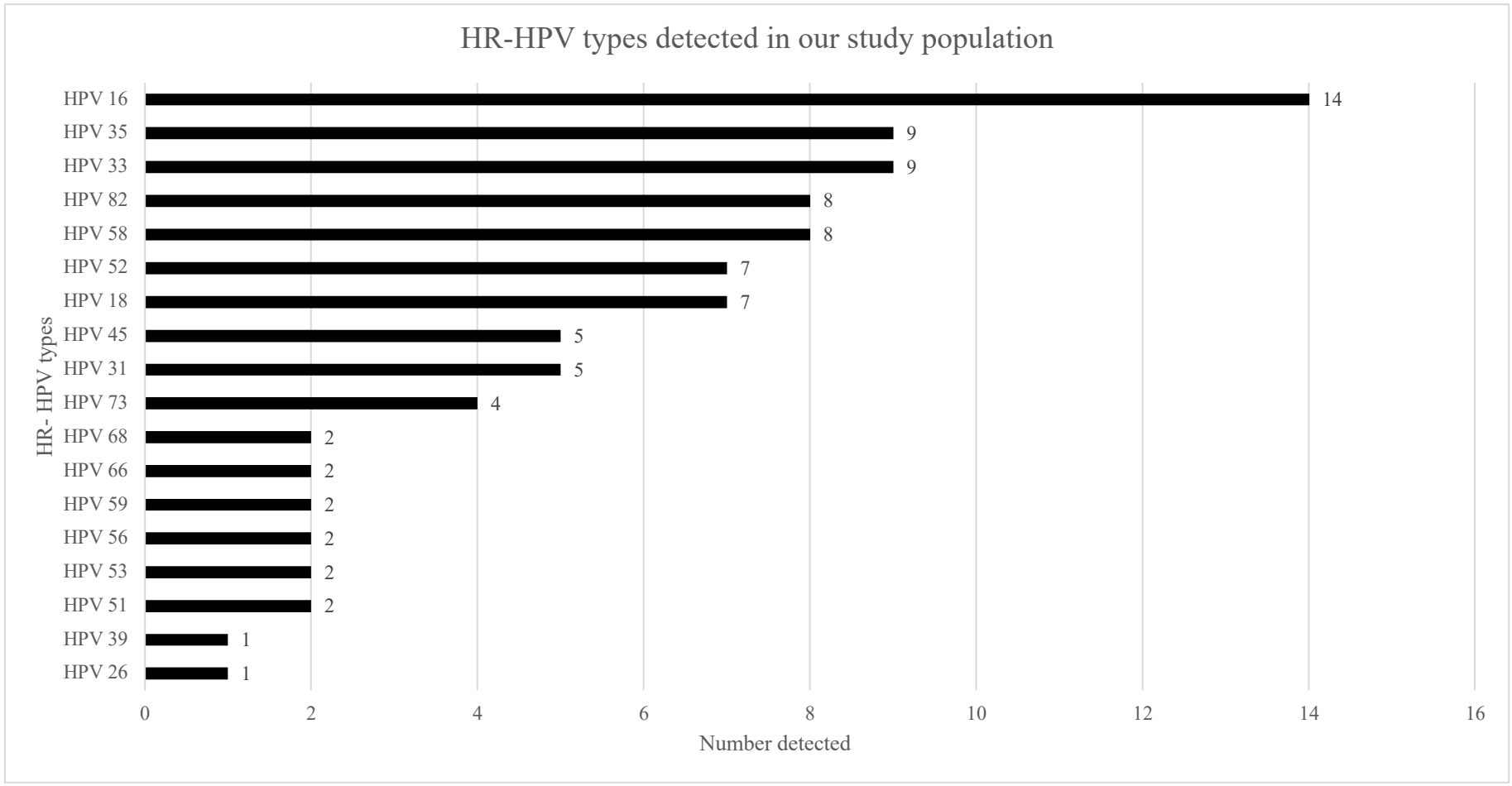

Figure:4.6: Illustration of the high-risk HPV genotypes detected in our study group.

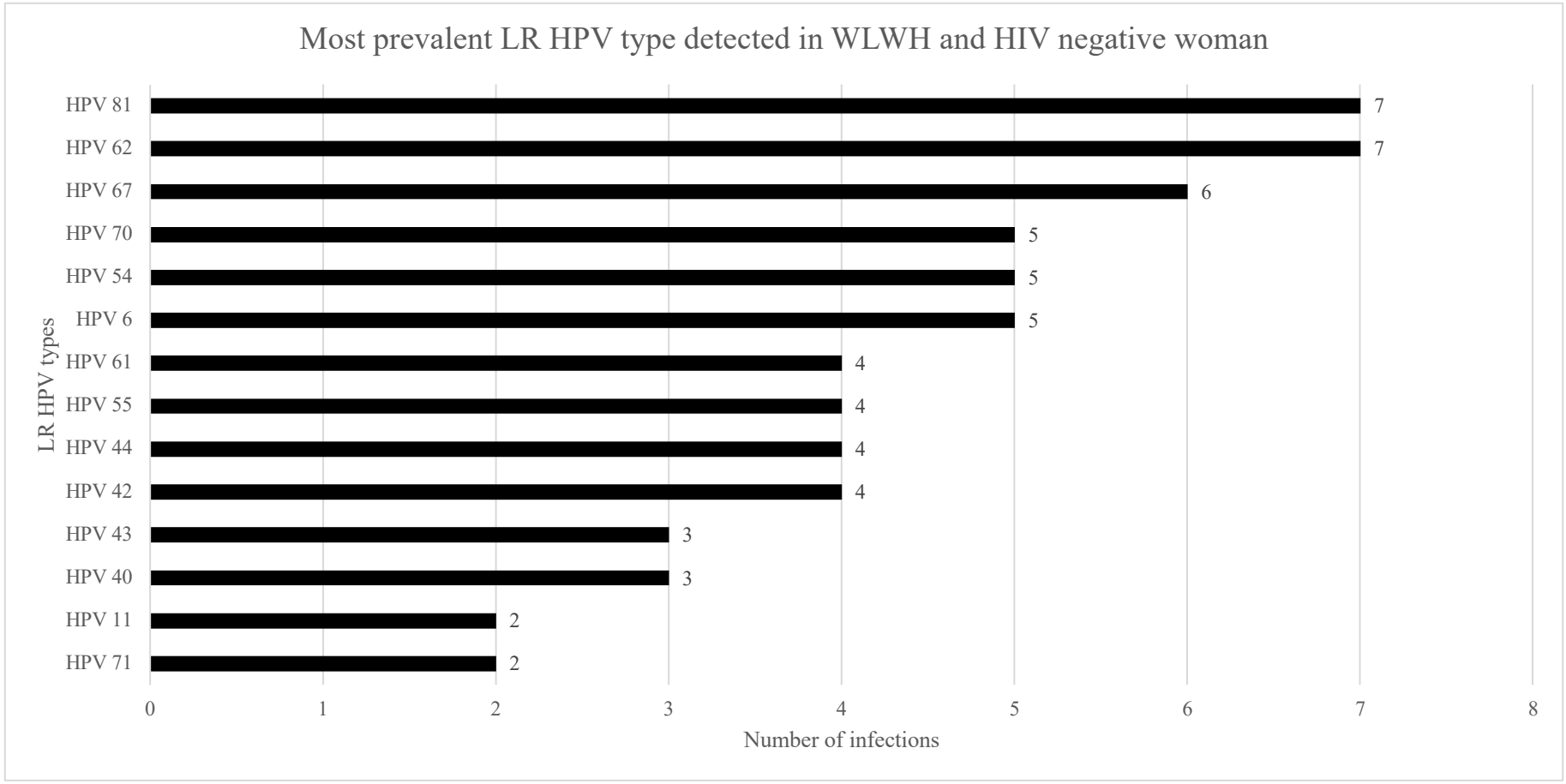

Figure:4.7: Illustration of the low-risk HPV genotypes detected in our study group.

Our findings illustrated that HR-HPV types were predominantly detected in samples from WLWH (Table 4.3). However, no statistically significant association was found between HR-HPV types detected and HIV status when doing a Fisher's Exact test ($p = 0.609$). Specifically, of the 14 woman that tested positive for HPV 16, 9 were HIV positive while 5 were HIV negative woman. A total of 9 participants tested positive for HPV 35 of these 6 were HIV positive and 3 HIV negative participants. Similarly for HPV 33, 7 were HIV positive participants and 2 were HIV negative participants. While HPV 82 was detected in only WLWH. Of the 8 woman that tested positive for HPV58, 6 were HIV positive and 2 HIV negative (Table 4.3).

Table 4.3: High-risk HPV type showed in HIV positive and HIV negative woman.

HPV type	HIV Pos	HIV Neg	Total
HPV 16	9	5	14
HPV 35	6	3	9
HPV 33	7	2	9
HPV 82	8	0	8
HPV 58	6	2	8
HPV 52	3	4	7
HPV 18	3	4	7
HPV 45	4	1	5
HPV 31	4	1	5
HPV 73	2	2	4
HPV 68	2	0	2
HPV 66	2	0	2
HPV 59	2	0	2
HPV 56	1	1	2
HPV 53	2	0	2
HPV 51	2	0	2
HPV 39	0	1	1
HPV 26	0	1	1
Total	63	27	90

4.5 Evaluation of the simultaneous expression of $p16^{INK4a}$ /Ki-67 proteins in exfoliated cervical epithelial cells collected from HIV positive and HIV negative woman

As mentioned previously the simultaneous expression of $p16^{INK4a}$ and Ki-67 in the same cell may be used as biomarker to identify cells undergoing HR-HPV-associated carcinogenesis.

4.5.1 Comparison of $p16^{INK4a}$/Ki-67 dual staining with HIV status relative to cytology diagnosis

All abnormal cytology specimens and 12 NILM specimens (6 HIV positive and 6 HIV negative) were subjected to $p16^{INK4a}$ /Ki-67 manual dual staining with the CINtec plus kit, which was performed in 5 batches of 14 samples each. As mentioned previously samples were scored positive if cytoplasmic $p16^{INK4a}$ (brown) and nuclear Ki-67 (red) dual-staining was detected within the same cell (Figure 4.8 A). Therefore, if a specimen only had one cell with dual $p16^{INK4a}$/ Ki-67 staining, it was scored positive (Figure 3.8 B). Haematoxylin was used to counterstain (blue) reference cells (normal cells with no HPV-associated changes) in the specimens (Figure 4.8 D).

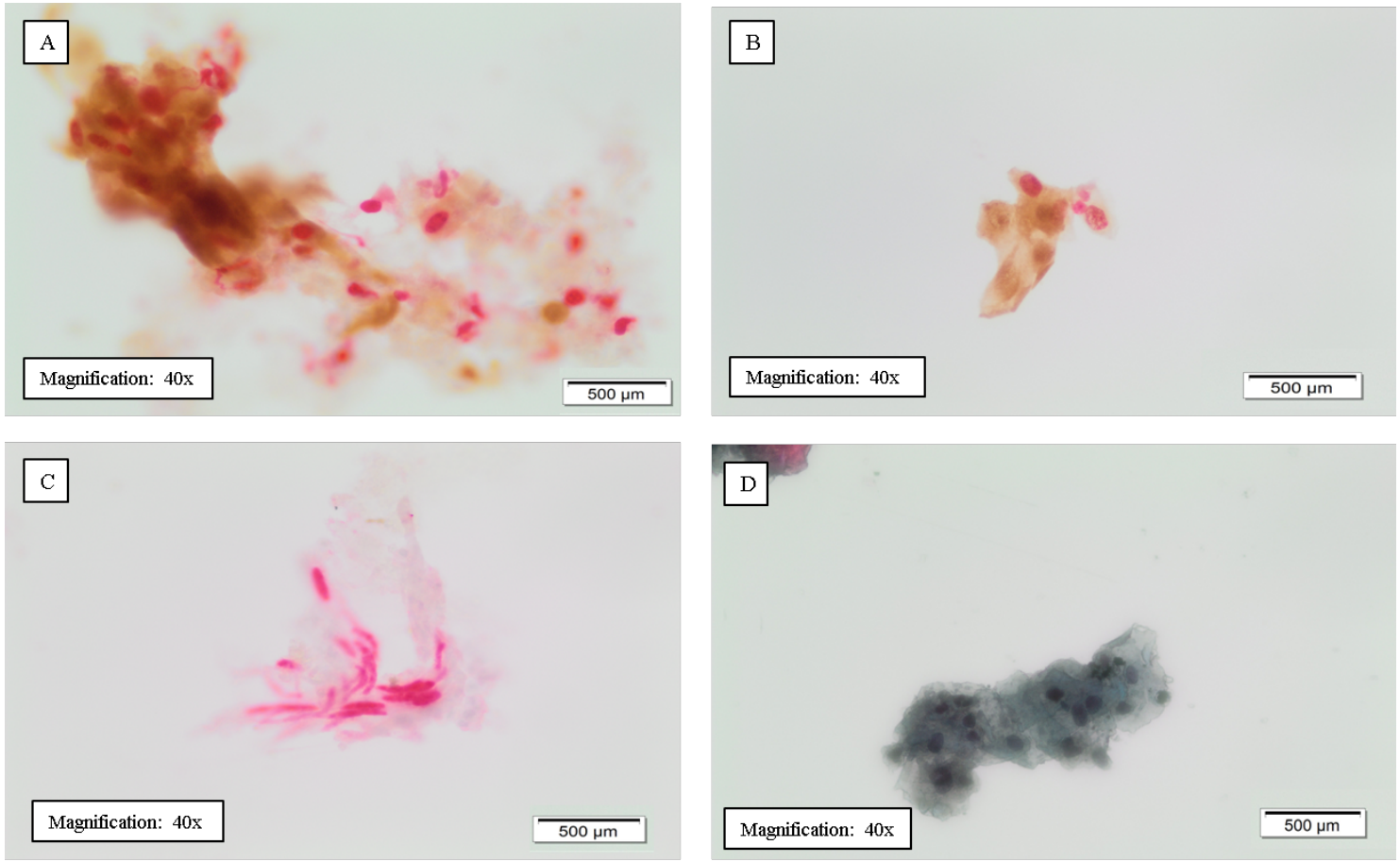

Figure 4.8: Examples of p16^{INK4a}/Ki-67 dual staining in cervical intraepithelial cells with the CINtec plus kit. (A) Picture Shows cluster of HSIL cells with p16 ^{INK4a} /Ki-67 dual-staining. (B) LSIL cells considered positive if one or more cervical intraepithelial cells are stained with a red nuclear stain for Ki-67 and a brown cytoplasmatic stain for p16 ^{INK4a}. (C) represents specimen's that stained negative for p16 ^{INK4a} /Ki-67 dual staining with only one type of stain present, no dual staining. While image (D) is an example of as reference cell counterstained with haematoxylin.

Figure 4.9 shows a representative image of a HSIL cervical intraepithelial lesion identified by the presence of cell clusters and the high cross-sectional area displaying the large nuclear-to-cytoplasmic ration (N:C) of cells in the cluster. However, the dual staining of the cell nucleus (Ki-67 red stain) and cytoplasm ($p16^{INK4a}$ brown stain) cannot be clearly seen due to reduced intensity of the stain. The following result was interpreted as a false negative. For these reasons, only the results of the first round $p16^{INK4a}$/Ki-67 dual staining was analysed.

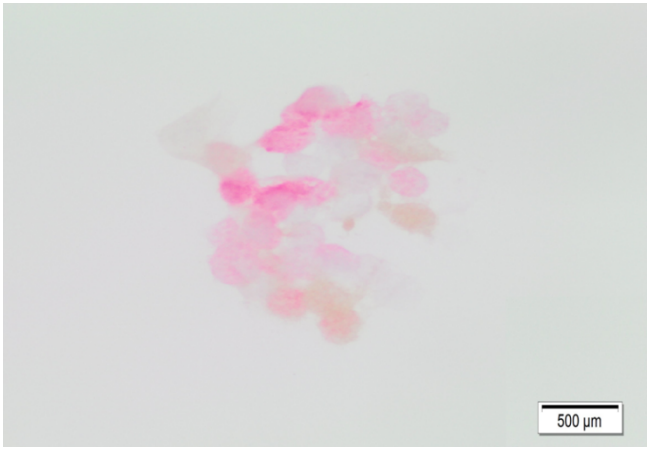

Figure 4.9: Representative image of a false negative HSIL samples. HSIL cell cluster stained with CINtec plus kit shows poor $p16^{INK4a}$ (brown) and Ki-67 (red) staining and Hematoxylin counterstaining.

The cellularity of slides was scored using a scale of 1 to 5 and samples with a score between 3 and 5 were deemed to be adequate. As previously mentioned, the presence of at least 10 endocervical cells was used as indication that the transformation zone was sampled. Again, scoring for cellularity was based on the presence of least 12 squamous cells per 40x magnification field that would equate to 5000 and more cells in total (Bethesda system, 2014).

Our results showed that the HSIL cytology group had the highest $p16^{INK4a}$ /Ki-67 positivity rate of 66,6% (16/24) when compared to the other cytology groups. The majority of HSIL samples (66.6%; 16/24) stained positive for $p16^{INK4a}$ /Ki-67 (Table 4.4). Results showed that $p16^{INK4a}$ /Ki-67 was co-expressed in 33,3% (4/12) of LSIL samples, whereas the proportion for ASC-US 25% (2/8) and ASC-H 23% (3/13) samples were lower (Table 4.4). Using a Pearson

Chi-Squared test, a significant relationship between $p16^{INK4a}$ /Ki-67 dual-staining and Pap cytology diagnosis was demonstrated in this study (p= 0.003).

Table 4.4: $p16^{INK4a}$ and Ki-67 co-expression results in abnormal cytology groups

	LSIL	HSIL	ASC-US	ASC-H	Total
CINtec Pos	4 (33,3%)	16 (66,6 %)	2 (25%)	3 (23%)	25 (42,1%)
CINtec Neg	8 (66,6%)	8 (37,5%)	6 (75%)	10 (76,9%)	32 (56,2%)
Total	12 (100%)	24 (100%)	8 (100%)	13 (100%)	57 (100%)

A correlation between $p16^{INK4a}$ /Ki-67 dual staining and HPV detection was done in this pilot study. $p16^{INK4a}$ /Ki-67 co-expression was observed in 43.8% (25/57) abnormal cervical cytology cases (Table 4.5). Of these 92% (23/25) were positive for HR-HPV types. Using a Pearson Chi-Squared test, a significant relationship between $p16^{INK4a}$ /Ki-67 dual-staining and HPV status was demonstrated in this study (p= 0.020).

Table 4.5: $p16^{INK4a}$ and Ki-67 co-expression results with HPV positivity

	HPV Pos	HPV Neg
CINtec Pos	23	2
CINtec Neg	25	7
Total	57	9

4.5.2 Comparison of p16^{INK4a}/Ki-67 immunocytochemistry dual staining with p16^{INK4a} immunohistochemistry on corresponding histology samples

The histology diagnoses for all LSIL, HSIL, ASC-US and ASC-H were reviewed by a pathologist for confirmation. It was found that 91.2% (52/57) of the diagnoses were concordant. However, the pathologist requested that p16^{INK4a} IHC be done on 5 cases that were challenging to diagnose due to denuded tissue present (Table 4.6).

Table 4.6: Comparison of p16 ^{INK4a} IHC requested by Pathologist and the p16^{INK4a} and Ki-67 dual staining results of the 5 cases with denuded tissue.

Study ID	Routine Histology Diagnosis	Confirmatory Diagnosis	p16 ^{INK4a} IHC	p16 ^{INK4a} / Ki-67
MLMPath006	Cervicitis	LSIL	Pos	Pos
MLMPath037	LSIL	LSIL	Neg	Neg
MLMPath047	Cervicitis	HSIL	Pos	Neg
MLMPath048	HSIL	HSIL	Pos	Pos
MLMPath054	HSIL	HSIL	Pos	Pos

The review of the p16^{INK4a} immunohistochemistry (IHC) of the routine diagnostic workup was done blinded by a pathologist. After completion of the histology review, the pathologist requested that additional p16^{INK4a} IHC be done on 5 cases (Table 4.6). Of these cases, 4 were p16^{INK4a} positive (Table 4.5). The p16^{INK4a} IHC results aided in confirming the LSIL and HSIL diagnosis for 2 respective cervicitis cases (routine histology). The LSIL and HSIL results for the remaining 3 reviewed cases were also confirmed. However, the total number of p16^{INK4a} cases in this study was 6 (MLMPath002 had concordant diagnosis and a positive result for p16^{INK4a} as well as p16$^{INK4a/}$Ki-67 dual-staining). When comparing the p16^{INK4a} IHC and the p16$^{INK4a/}$Ki-67 immunocytochemistry (ICC) results, only 3 (3/6; 50%) cases were found be positive for both IHC and ICC.

4.6 Evaluation of the methylation status of the *CADM1, MAL* and *miR-124-2* genes in abnormal cytology specimens

The methylation status of the *CADM1, MAL* and *miR-124-2* genes were evaluated in exfoliated cervical intraepithelial cells using the Epimelt methylation assays (MethylDetect, Aalborg, Denmark).

4.6.1 Assessment of DNA quality extracted from exfoliated cervical cells

The average concentration of DNA extracted from exfoliated cervical cells were 56 ng/µl per 3 X 10^6 cells. Generally, the DNA yields were acceptable, but 20 samples had to be concentrated using an Eppendorf centrifugal vacuum concentrator 5301(Merck, Germany). The purity of isolated DNA was measured with a Biodrop spectrophotometer and samples had acceptable $A260_{nm}/A280_{nm}$; $A230_{nm}/A260_{nm}$ ratio's, ranging between 1.6-1.8 and 1.8-2.4, respectively. The average A260/280 for isolated DNA was 1.90 while an average of 2.3 was seen for $A230_{nm}/A260_{nm}$ ratios. Thus, all samples yielded DNA of adequate quality and quantities and 1 µg or more of DNA were sufficient for the bisulphite conversion and subsequent cleaning of the bisulfite converted DNA.

4.6.2 Assessment of the *CADM1* gene methylation

Out of a total amount of 69 specimens collected, 82.6% (57/69) abnormal cervical cytology specimens and two pooled NILM was subjected to MS-HRM to examine the DNA methylation status of the *CADM1* gene. No product was detected in NTC for *CADM1* gene methylation qPCR plates, therefore indicating that no DNA contamination or non-specific amplification was present in samples. Heterogenous *CADM1* methylation was observed in 12.3% (7/57) specimens, while 87.7% (50/57) were unmethylated. No samples with homogenous *CADM1* methylation were obtained. The samples for which a heterogenous methylation pattern were detected were MLMPath-014, MLMPath-015, MLMPath-024, MLMPath-026, MLMPath-027, MLMPath-035 and MLMPath-055. Out of the 7 specimens that showed heterogenous methylation of *CADM1*, 6 were positive for HPV detection while 1 was negative for HPV (MLMpath-015). No significant relationship was seen between the methylation status of *CADM1* gene and HPV infection when doing a Pearson Chi- square test (p=0.065). (Table 4.7).

Table 4.7: Relationship between methylation status of *CADM1* promotor gene and HPV detection

Gene	**HPV positive**	**HPV negative**	**Total**
CADM1 heterogenous methylated	6	1	7
CADM1 unmethylated	42	8	50
Total	48	9	57

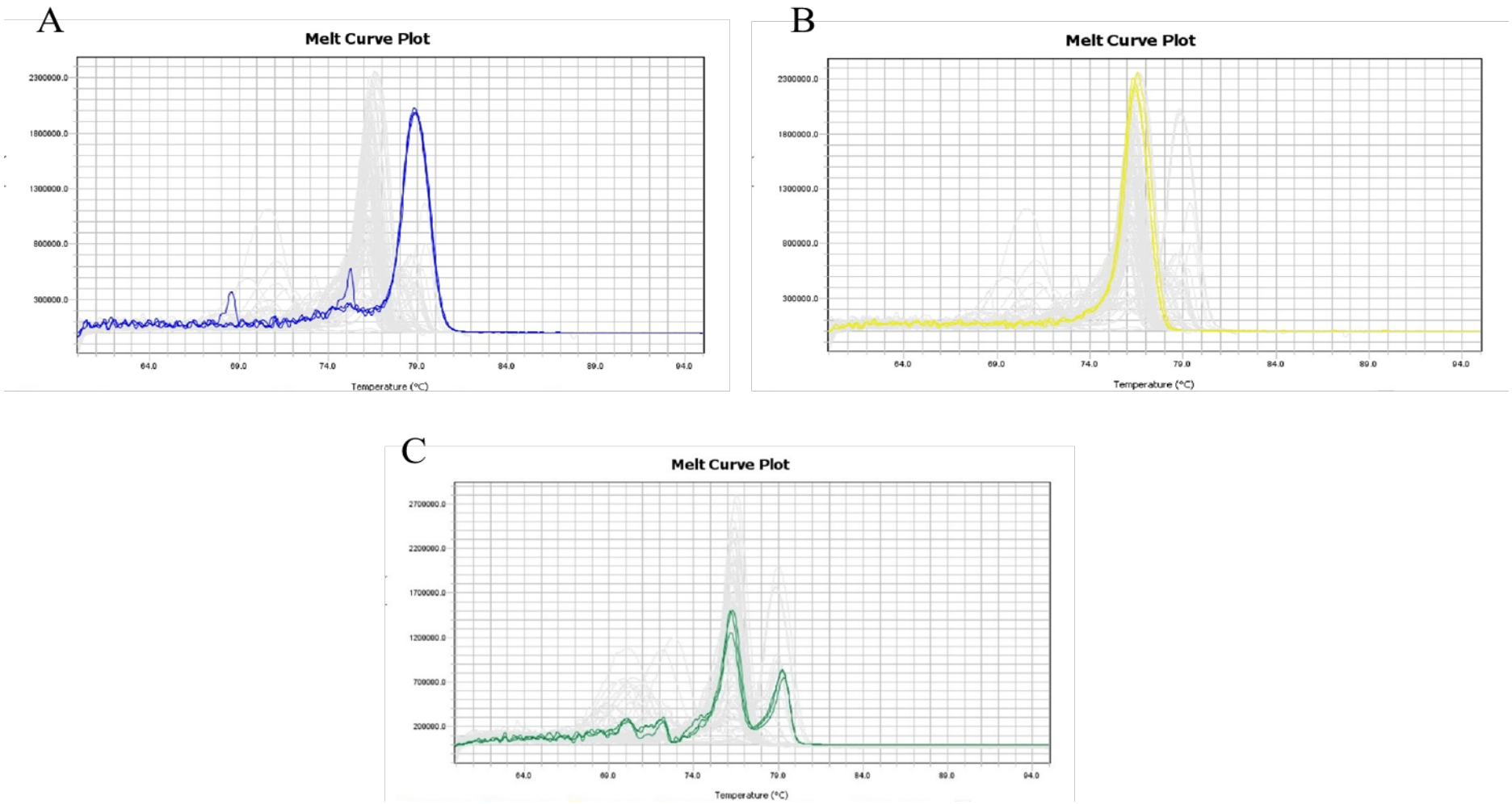

Figure 4.10: Graph showing the methylation positive control (A), methylation negative control (B) and the methylation assay control (C) for the *CADM1* gene. The melting temperature for the *CADM1* methylation positive control was Tm=78.9 ºC, the *CADM1* negative was Tm=76.4 ºC and the *CADM1* assay control was Tm=76.3 ºC using the QuantStudio 5 real-time PCR machine.

4.6.3 Assessment of the *MAL* gene methylation

Similarly, to the *CADM1* MS-HRM assays, a marginal difference in melting temperatures between the manufacturer's *MAL* assay controls and ours were observed but remain constant between runs. No product was detected in NTC for the *MAL* methylation assays, therefore indicating there were no DNA contamination or non-specific amplification.

No samples showed homogenous methylation for the *MAL* gene however 14% (8/57) showed heterogenous DNA methylation for *MAL*. A proportion of 85.9% (49/57) of samples were unmethylated for *MAL*. The samples that were heterogeneous for *MAL* was MLMPath-007, MLMPath-008, MLMPath-019, MLMPath-020, MLMPath-024, MLMPath-032, MLMPath-041 and MLMPath-055. Out of the 8 specimens that where heterogenous for methylation status of *MAL*, 100% (8/8) were positive for HPV detection (Figure 3.13 showing methylation curve for MLM-024). When doing a Pearson chi square test, no significant relationship was seen between the methylation status of *MAL* gene and HPV infection (p= 0.002). (Table 4.8).

Table 4.8: Relationship between methylation status of *MAL* promotor gene and HPV detection

Gene	**HPV positive**	**HPV negative**	**Total**
MAL Positive	8	0	8
MAL Negative	40	9	49
Total	48	9	57

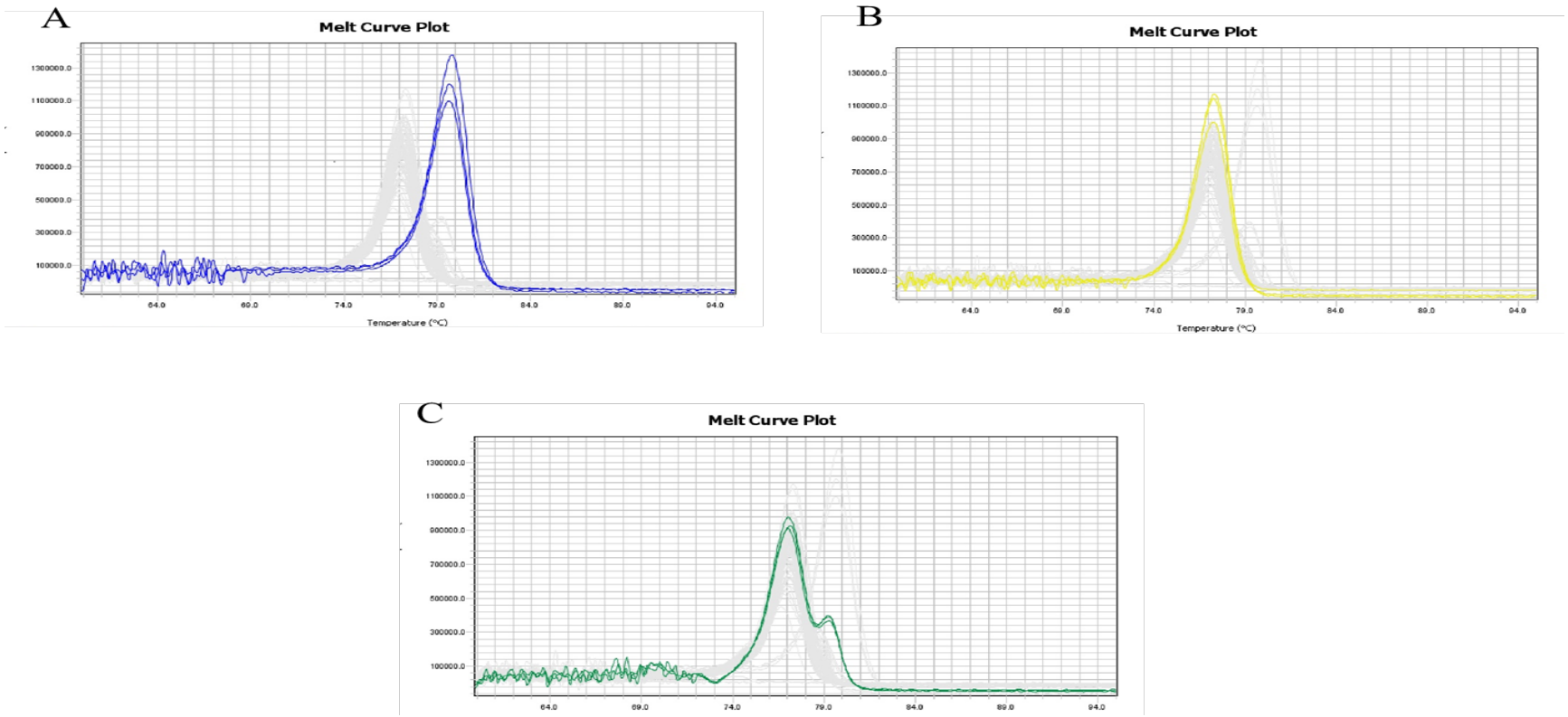

Figure 4.11: Graph showing the methylation positive control (A), methylation negative control (B) and the methylation assay control (C) for the *MAL* gene. The melting temperatures for the methylation positive, negative and assay controls in our runs when using a QuantStudio 5 real-time PCR machine were Tm=78.9 ºC., Tm=80.2 ºC. and Tm=78 ºC, respectively

4.6.4 Interpretation of the *miR-124-2* gene methylation

All controls for the *miR-124-2* methylation assay were successful. No product was detected in our NTC for *miR-124-2* assays, therefore indicating that no DNA contamination or non-specific amplification was present. No samples showed homogenous methylation for the *miR-124-2* gene however specimens showed heterogenous DNA methylation for *miR-124-2* while 87.7% (50/57) had negative methylation results. The proportion of specimens that were heterogenous for *miR-124-2* methylation was 12.2% (7/57) samples. Theses samples were MLMPath-005, MLMPath-021, MLMPath-022, MLMPath-026, MLMPath-027, MLMPath- 042 and MLPath-055 (Table 4.9).

Table 4.9: Relationship between methylation status of *miR-124-2* promotor gene and HPV detection

Gene	HPV positive	HPV negative	Total
miR-124-2 Positive	7	0	7
miR-124-2 Negative	41	9	50
Total	48	9	57

Our results suggest that *CADM1* and *MAL* are likely indicated in HSIL and higher severity lesions, which are in concordance with that of Meršaková *et al*., (2018) and Del Pino *et al.* (2019). (Table 4.9). Conversely, it seems that *miR-124-2* heterogenous methylation might be indicated in HSIL and cervical intraepithelial lesions with lower grade severity. MLMPath-055, a sample with cytology diagnosis of ASC-US with multiple HR-HPV, but was confirm HSIL with histology, showed methylation of all three *CADM1, MAL* and *miR-124-2* genes. The cytology diagnosis for MLMPath-026 changed from HSIL to Invasive Squamous Cell Carcinoma (ISCC) upon confirmation of the histological diagnosis and showed a heterogenous methylation pattern for *CADM1* (Table 4.10). One sample (MLMPath-055) showed heterogenous methylation of all three genes *CADM1, MAL* and *miR-124-2*. The Cytology of this sample was an ASC-US while the histology for this specific sample was an HSIL.

Table 4.10: **Comparison of cytology and histology diagnosis with results from HR-HPV typing and gene methylation (*CADM1*, *MAL* and *miR-124-2*).**

Study ID	Cytology Diagnosis	Histology Diagnosis	HR-HPV (Single, multiple, mixed)	*CADM1* methylation	*MAL* methylation	*miR-124-2* methylation
Mpath-005	LSIL	HSIL	Mixed	-	-	*miR-124-2*
Mpath-007	LSIL	LSIL	LR-HPV	-	*MAL*	-
Mpath-008	LSIL	LSIL	LR-HPV	-	*MAL*	-
Mpath-014	HSIL	HSIL	Single	*CADM1*	-	-
Mpath-015	HSIL	HSIL	Neg	*CADM1*	-	-
Mpath-019	HSIL	HSIL	Single	-	*MAL*	-
Mpath-020	HSIL	LSIL	Multiple	-	*MAL*	-
Mpath-021	HSIL	LSIL	Single	-	-	*miR-124-2*
Mpath-022	HSIL	HSIL	Single	-	-	*miR-124-2*
Mpath-024	HSIL	HSIL	Single	*CADM1*	*MAL*	-
Mpath-026	HSIL	ISCC	Single	*CADM1*	-	*miR-124-2*
Mpath-027	HSIL	HSIL	Single	*CADM1*	-	*miR-124-2*
Mpath-035	HSIL	HSIL	Multiple	*CADM1*	-	-
Mpath-041	ASC-H	HSIL	Multiple	-	*MAL*	-
Mpath-042	ASC-H	HSIL	Single	-	-	*miR-124-2*
Mpath-044	ASC-H	LSIL	LR-HPV	-	*MAL*	-
Mpath-055	ASC-US	HSIL	Multiple	*CADM1*	*MAL*	*miR-124-2*

All specimens for which heterogenous methylation patterns for *miR-124-2* were detected, were positive for HR-HPV types (Table 4.8 and Table 4.9). A significant relationship was found between HR-HPV and *miR-124-2* methylation (p= 0.005) after a Pearson Chi-square analysis. No significant relationship was seen between HPV infection and the methylation status of *CADM1* and *MAL* genes when doing a Pearson Chi-square test (p=0.052).

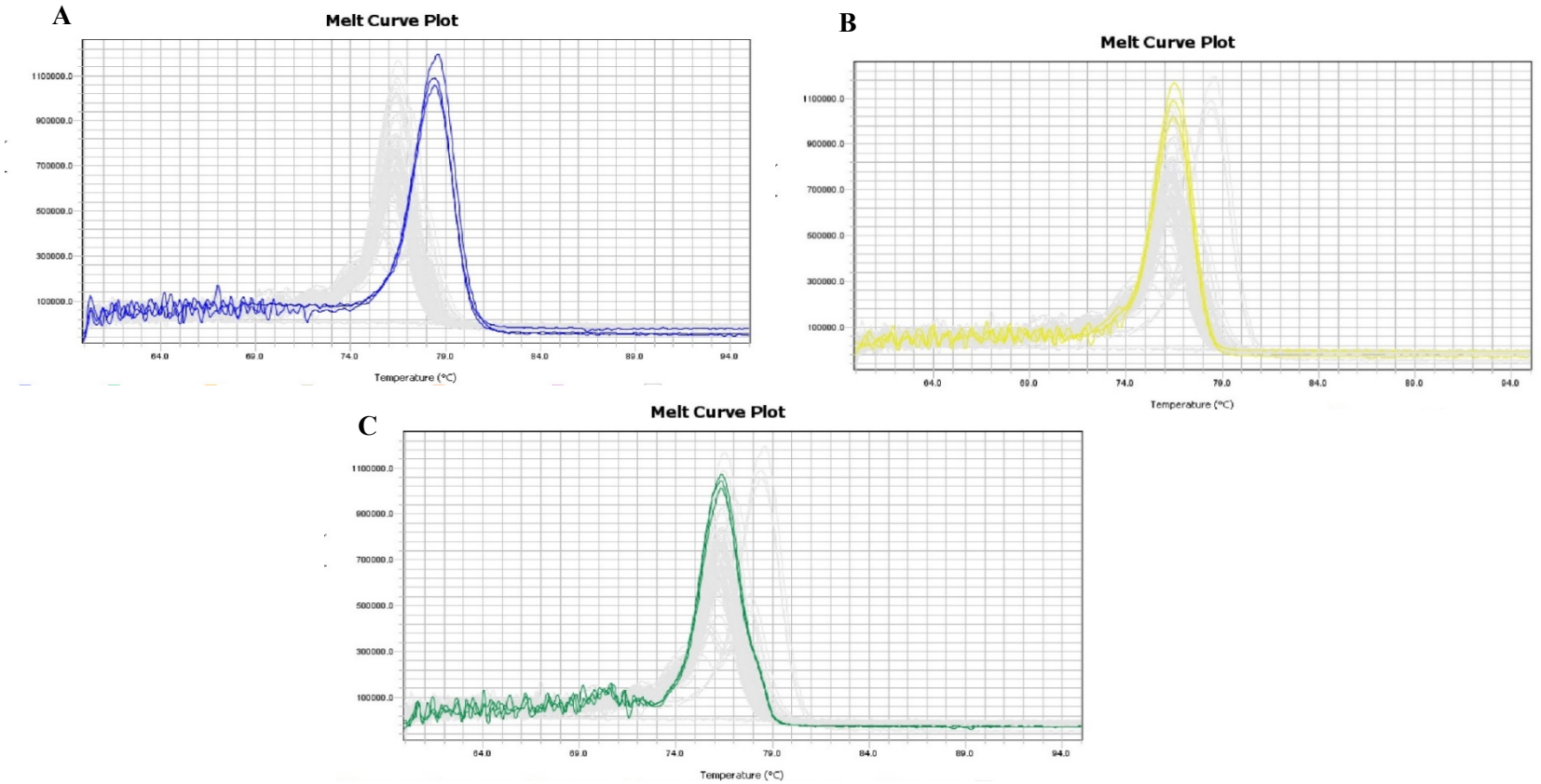

Figure 4.12: Graph showing the methylation positive control (A), methylation negative control (B) and the methylation assay control (C) for the *miR-124-2* gene. The melting temperatures for controls in our runs were: *miR-124-2* positive control Tm=79 ºC, negative control Tm= 76 ºC and assay control Tm=76.5 ºC, respectively.

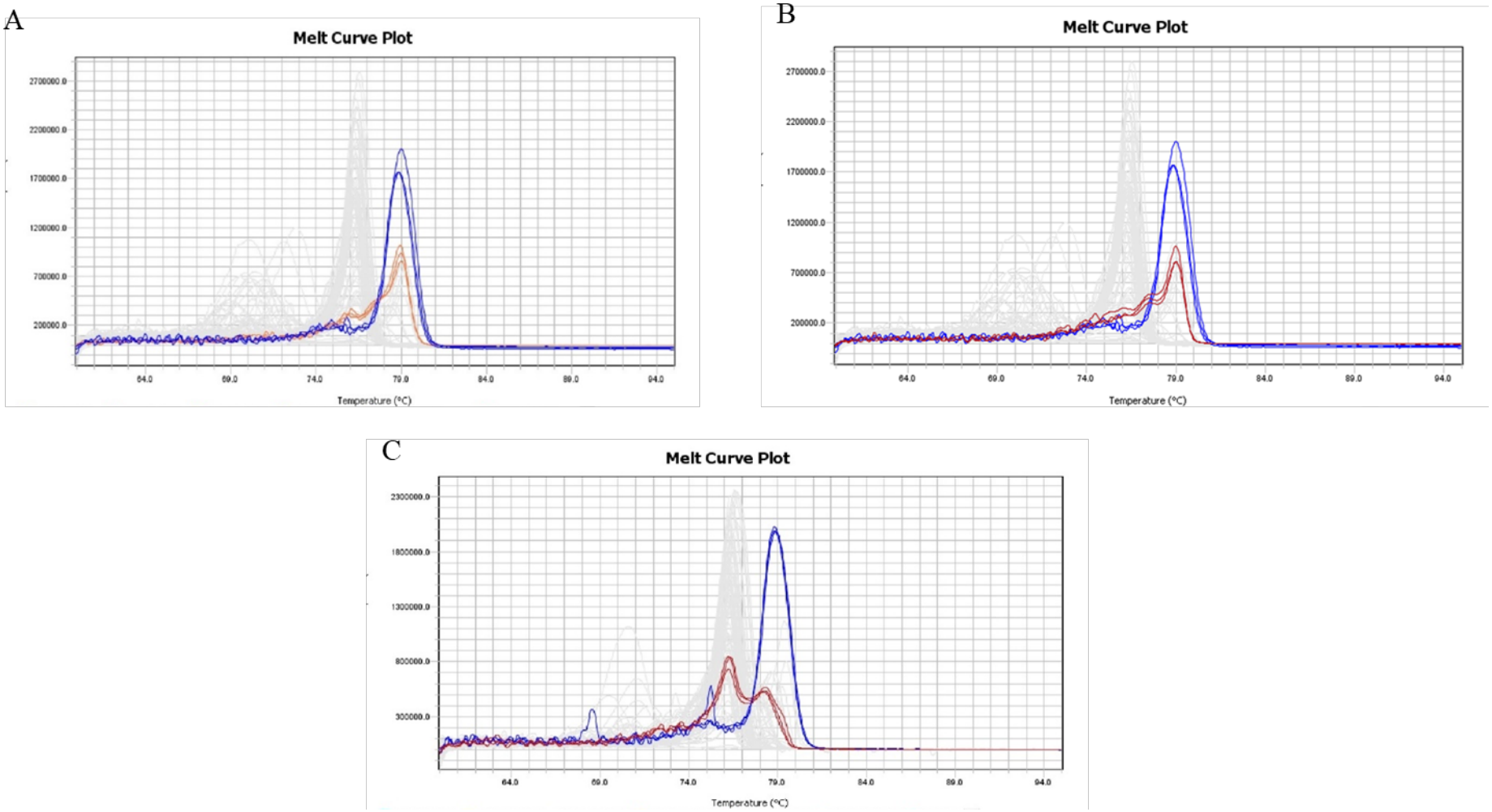

Figure 4.13: Melting profiles of heterogenous *CADM1* methylation. The methylation positive control for *CADM1* gene (blue) and the heterogenous methylation melting curves for specimens (A) MLMPath-014 (orange) (B) MLMPath-055 (red) and (C) MLMPath-024 (maroon) are presented.

4.7 Correlation of HR-HPV infection, p16^{INK4a} and Ki-67 co-expression and methylation status of *CADM1*, *MAL* and *miR-124-2* genes for prediction of cervical intraepithelial lesion progression

The results of this pilot study were to inform whether a larger study is warranted for the development of a tool to discern the likelihood of cervical intraepithelial lesions progressing to more severe disease to ultimately reduce unnecessary referrals for colposcopy. An algorithm including predictors such as HR-HPV, p16^{INK4a} and Ki-67 co-expression and the methylation status of *CADM1*, *MAL* and *miR-124-2* genes was evaluated.

The agreement between cytological and histological diagnosis were determined, for comparisons and correlation analysis. As data for cytology diagnosis are usually classified into 4 categories (LSIL, HSIL, ASC-US and ASC-H) and that of histology into only 2 (LSIL and HSIL), the LSIL and ASC-US were grouped together as low-grade cervical intraepithelial lesions. Similarly, the HSIL and ASC-H were grouped together as high grade cervical intraepithelial lesions (personal communication, Mrs Greta Neethling, Cytotechnologist). The Cohen Kappa coefficient was used to determine the agreement between cytological and histological diagnoses. The Cohen Kappa coefficient of 0.26 suggest that agreement between the two diagnosis types were fair (Landis and Koch (1977a,) with agreement at 64.9% compared to the expected agreement of 53.7%. When doing a Pearson chi square test, a significant relationship was seen between the cytology diagnosis and the histology diagnosis ($p<0.05$)

Receiver Operator Characteristic (ROC) analysis was used to assess the performance of our model for predicting cervical intraepithelial lesions at risk of progressing to more advance disease. The model included the following predictors: HR-HPV typing, p16^{INK4a}/Ki-67 co-expression and methylation markers (*CADM1*, *MAL* and *miR-124-2*). The gold standard for the analysis of LSIL/ASC-US samples were confirmed LSIL histology, whereas that of the HSIL/ASC-H samples was confirm HSIL histology. In addition, the (binary) logistic regression analysis was used to determine the correlation between the indicated predictors and lesion severity (LSIL vs HSIL).

Using a logistic regression model to evaluate the association between methylation markers (*CADM1*, *MAL* and *miR-124-2*), HR-HPV and $p16^{INK4a}$/Ki-67 co-expression and LSIL cytology it was found that the $p16^{INK4a}$/Ki-67 (CINtec result) were correlated with LSIL cytology result ($p<0.05$) (data not shown). The odds ratio was 0.19 (95% CI: -2.9 to -0.29), meaning that the odds of having a LSIL result with a positive $p16^{INK4a}$/Ki-67 (CINtec result) results decreased by 81% (Figure 4.14). No other predictors in the model were found to significantly associate with LSIL cytology. The model might be improved by removing or adding other predictors.

Figure 4.14 illustrates the ROC curves for different predictor tests and shows the sensitivity and specificity as well as the area under the curve values (AUC). LSIL histology was used as gold standard The AUC of the LSIL cytology diagnosis compared to LSIL histology diagnosis with AUC = 0.55. Our model has a sensitivity of 40% and a specificity of 83.8%. The positive predictive value was 57,14% while the negative predictive value was 72,09%. The algorithm may be improved if more participants are included in future studies to increase sensitivity of the developed tool.

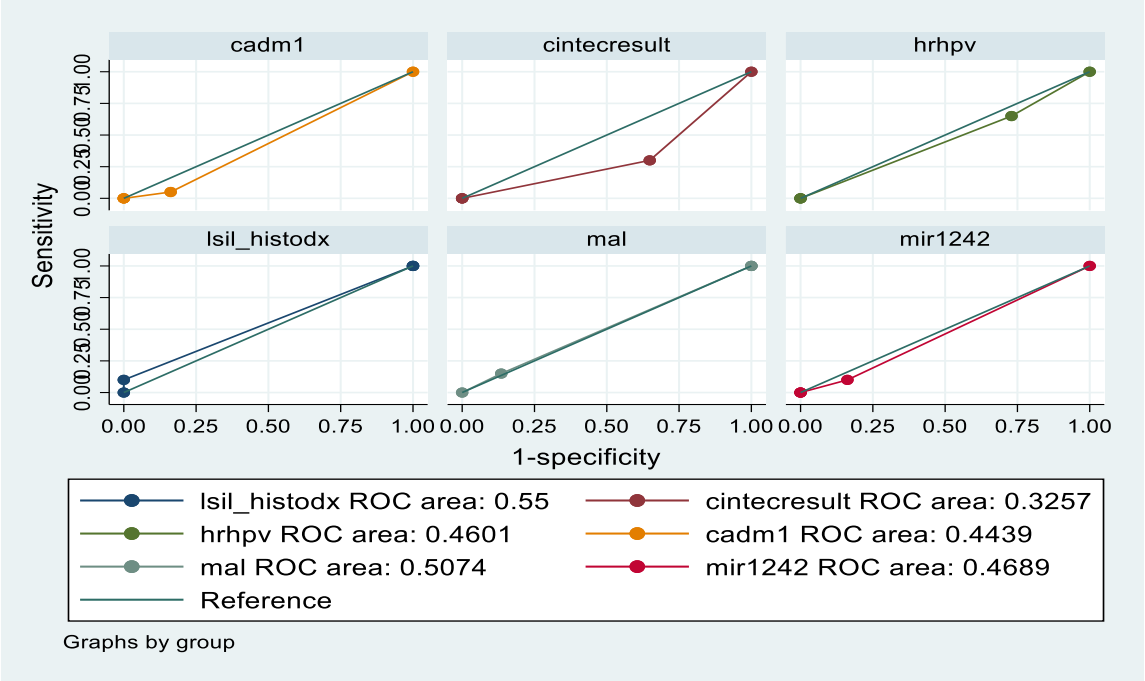

Figure 4.14: A ROC curve showing the relationship between LSIL cytology diagnosis and LSIL histology diagnosis for sensitivity and specificity relationship. The ROC curve is plotted with TPR (True Positive Rate) against the FPR (False Positive rate) where TPR is on the y-axis and FPR is on the x-axis.

The odds ratio for HSIL cytology diagnosis was 5.1 (95% CI: 1.3 to 19.3), meaning that the odds of receiving HSIL result with a positive $p16^{INK4a}$/Ki-67 (CINtec result) is 49.2 % higher than the odds of receiving an LSIL result. No other predictors in the model were found to significantly associate with HSIL cytology and by removing or adding other predictors the model could be improved. This can also be observed with the AUC values for the p16/ki67 (CINtec result) which was 67.43% (Fig 4.14). The HSIL model had sensitivity of 83,78% and a specificity of 40%, The positive predictive value was 72,09%, while the negative predictive value was 57,14 %.

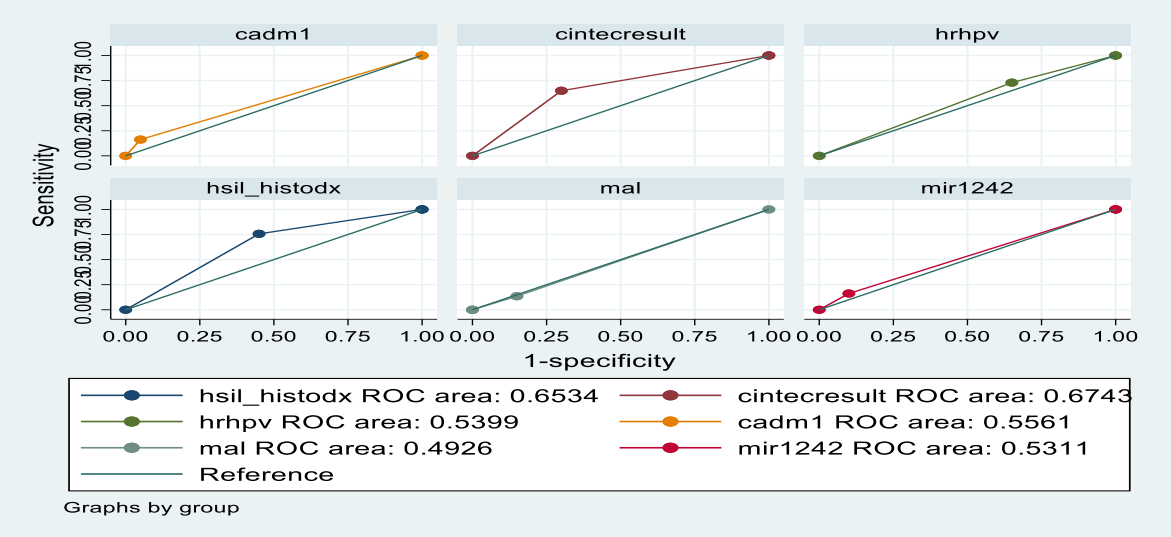

Figure 4.15: A ROC curve showing the relationship between HSIL cytology diagnosis and HSIL histology diagnosis for sensitivity and specificity relationship. The ROC curve is plotted with TPR (True Positive Rate) against the FPR (False Positive rate) where TPR is on the y-axis and FPR is on the x-axis.

Chapter 5

Discussion

Due to their weakened immune systems, WLWH require effective HPV infection prevention strategies as well as methods for early detection of cervical cancer and its precursor lesions. Significant risk factors for cervical cancer include the high incidence of HIV and the absence of structured cervical cancer screening programs (Musa *et al*., 2019). It remains challenging to distinguish between HPV infections which will progress to high-grade cervical lesions or cancer and which infections will regress. It is therefore essential to identify molecular assays that may aid in the early detection of cervical intraepithelial lesions with the potential to progress to higher grade lesions. The clinical usefulness of ancillary molecular tests such as HR-HPV typing, $p16^{INK4a}$/Ki-67 dual staining and DNA methylation (for example *CADM1*, *MAL* and *miR-124-2* genes) in combination with the gold standard Pap smear methods was evaluated. Moreover, it was evaluated whether the sensitivity and specificity of the standard Pap smear for the identification of abnormal cervical lesions can be improved when used in combination with such ancillary molecular tests.

Findings of this pilot study may be useful to design larger studies in future to better understand the clinical usefulness of HPV testing, $p16^{INK4a}$ /Ki-67 dual staining and gene methylation for the early identification and detection of cervical intraepithelial neoplasia and cancer. Results from larger studies may then be used to develop algorithms that may allow the early discernment of women at risk of oncogenic transformation of cervical epithelial cells into precancerous lesions or carcinoma. Such effective algorithms may eventually lead to fewer unnecessary referrals for colposcopy-guided biopsies in the clinic.

5.1 Sample acquisition

The required number of cervical swabs for some diagnosis group could not be collected. Reasons attributable include participants missing their appointments and some declining to participate in the research study. Another contributing factor for reduced recruitment is that some referrals to the colposcopy clinic, such as HIV negative women with an indication of

LSIL are not frequently seen at the clinic. In fact, none were seen during this study collection period. Similarly, few HIV negative patients with ASC-US and ASC-H were seen at the Colposcopy clinic during specimen collection timeline. A significant relationship between Pap cytology diagnosis and HIV status was found in our research study. However, this is a pilot study that does not have sufficient power to show the strength of association between Pap cytology diagnosis and HIV status. Therefore, a larger study is warranted to support this finding statistically.

5.2 Description of study Population

Age is an identified risk factor for the development of cervical intraepithelial neoplasia (Aykut and Tuncer, 2020). As previously indicated, early sexual debut, increased sexual partners and HIV co-infections are risk factors for HPV infection (Wencel-Wawrzeńczyk *et al*., 2022; Hariri *et al*., 2014). We investigated the relationship between age and HIV status. The age distribution for HSIL was discordant with the findings of the cross-sectional descriptive study by Omoyeni and Tsoka-Gwegweni, (2022) conducted in KwaZulu Natal, South Africa. In contrast to Omoyeni and Tsoka-Gwegweni (2022), the majority of our LSIL groups was older than 30 years and that of our HSIL was younger than 45 years. Although study by Omoyeni and Tsoka-Gwegweni, (2022) did not separate the ASC cytology group, most of their participants in this group were older than 45 whereas the participants in our ASC-H and ASC-US groups were younger than 45 years. Similarly, to our study, a study done in Thailand by Suntornlimsiri. (2008) showed a median age of 45 for participants diagnosed with ASC-US and ASC-H.

5.3 HPV types detected in our study population

There is a significant risk of HPV-related malignancies among people who are HIV-positive (Clark *et al.,* 2021; Stelzle *et al.,* 2020; Lekoane *et al.,* 2020). The association between HIV and HPV is thus clinically important. In our research study no significant association was seen between HIV status and HPV. A possible reason for this is due to the limited sample size for this pilot study. The sample size for this pilot study was not determined using statistical methods but based on a rule of thumb for pilot studies (Julious, 2005). Therefore, our study is not significantly statistically powered to detect the association between HIV status and HPV

infection. It is known in literature that HIV infection increases the risk of being infected with multiple HPV strains or vice versa (Lekoane *et al.,* 2020; Stelzle *et al.,* 2020). Even though this was a pilot study, similar findings were seen in our setting where most of our study participants had HPV and HIV co-infection.

The most common HR-HPV types detected in our study group in descending order was HPV16, HPV 35, HPV, 33, HPV 82 and HPV 58. This compares well with the most common HPV types in South Africa associated with the precancerous and cancerous lesions of the cervix uteri as reported by Bruni *et al*. (2021). The top 5 HR-HPV types in South Africa indicated in precancerous and cancerous lesions of the cervix uteri are HPV 16, HPV 18, HPV 31, HPV 33, and HPV 35 (Bruni *et al*., 2021). Another study done in the Eastern Cape by Mbulawa *et al.* (2018) found that HPV 16, HPV 58, HPV 51, HPV 66 and HPV 18 were the most prevalent HR-HPV types in that region. This variability of the HPV prevalence in different geographical regions is in accordance with Reade *et al*. (2014). Taken together, these findings suggest that HPV 16 is the most prevalent HR-HPV type associated with cervical pathogenesis in South Africa (Bruni *et al.,* 2021; Mbulawa *et al.,* 2018) and our findings were in concordance.

The detection of HPV infections in NILM samples (MLMPath-060, MLMPath-062 and MLMPath-064) points to coincidental findings. One reason could be that these are relatively new infections. No HPV-related cellular changes were yet observed upon cytological examination nor genital warts during visual examination. Only MLMPath-060 was co-infected with a HR-HPV type (HPV45), whereas the others were infected with LR-HPV (HPV60 and HPV 71). Another reason attributable to this outcome could be that swabs taken were inadequate and not truly representative of the cervical mucosa harbouring HPV, for Pap smear analysis. Therefore, HPV types could be detected using the more sensitive method of PCR-based HPV testing and not with the Pap smear.

5.4 The simultaneous expression of p16^{INK4a} /Ki-67 proteins in cervical epithelial cells

As previously mentioned, the simultaneous expression of p16^{INK4a} and Ki-67 in the same cell may be used as biomarker to identify cells undergoing HR-HPV associated carcinogenesis. Interest in using dual p16^{INK4a}/Ki-67 staining as ancillary molecular test for the diagnosis of cervical intraepithelial lesions has lately increased. In fact, in 2020, the FDA has approved the CINtec® Plus Cytology kit for p16^{INK4a}/Ki-67 dual staining from Roche for cytology screening and is currently used in the United States of America. The test can be used to determine the risk of a patients infected with a high-risk HPV type to develop pre-cancerous lesions or cancer.

Each sample was subjected to 3 rounds of p16^{INK4a} /Ki-67 staining to have triplicate technical replicates. Out of the 3 rounds done, discordance was found between Rounds 1, 2 and 3 where specimens with a positive p16^{INK4a} and Ki-67 dual staining result stained positive in round 1 but negative in the other two consecutive rounds. It was noticed that similar results were found in round 1 and first batch of round 2. However, specimens stained in round 2 (batch 2 to 5) showed faded staining for p16^{INK4a} (brown stain) and Ki-67 (red stain). The reference cells counterstained with Haematoxylin had a "ghost" cell appearance under an Olympus BX41 microscope, making scoring and interpretation of the staining difficult. The intensity of the stain was thus not optimal as was seen for round 1. We therefore decided to use round 1 p16^{INK4a} /Ki-67 staining result only.

Our results showed increased p16^{INK4a} /Ki-67 dual staining positivity for HSIL lesions rather than LSIL lesions. This result was in accordance with a research study by Secosan *et al.,* (2022) showing similar results indicating increased positive p16^{INK4a} /Ki-67 dual staining for HSIL lesions compared to LSIL lesions. These authors found that 6,4% (2/31) of LSIL was positive and 83,8% (26/31) of HSIL samples positive for p16^{INK4a} /Ki-67 using the CINtec plus kit Secosan *et al*. (2022). While another study by Sharma *et al*., (2021) showed 100% of p16^{INK4a} /Ki-67 positivity in HSIL samples. Thus, confirming that high-grade cervical lesions frequently display increased expression of p16^{INK4a} and Ki-67.

A significant relationship between $p16^{INK4a}$ /Ki-67 dual-staining and HPV status was demonstrated in this study. This finding is in concordance with a study by Ikenberg *et al*. (2013) showing significant association between $p16^{INK4a}$ /Ki-67 dual-staining and HPV infection. Moreover, Chen *et al*. (2022) also indicates a significant relationship between $p16^{INK4a}$ /Ki-67 dual-staining and HPV in high -grade lesions.

Different factors may be attributable to insufficient staining of the CINtec plus staining kit. It is recommended that the CINtec plus staining kit is utilised on a BenchMark XT automated slide stainer (Roche Tissue Diagnostics/Ventana Medical Systems, Inc., Tucson, AZ). Therefore, reagents and antibodies will be utilised within a short time period. When comparing our timeline used for the manual ICC staining of samples, we deduced that our time period was extended compared to what it would have been for the automated platform. Staining for round 1 was done within 5 consecutive days of opening the CINtec ® Plus kit, whereas the staining for rounds 2 and 3 occurred after a month of opening the kit as we were waiting on the delivery of the Haematoxylin stain. Even though reagents and antibodies were stored at the correct temperature as per manufacturer's recommendation, the extended time period since opening the kits and use for rounds 2 and 3 may have resulted in some oxidation of reagents. Another factor was that these kits were nearing their expiry dates, which could have led to instability of reagents and antibodies. This might have then reduced efficacy, leading to suboptimal performance.

5.5 Assessment of the DNA methylation with *CADM1, MAL* and *miR-124-2* genes.

Molecular-based test relying on epigenetic changes occurring during the progression to pre-cancer and cancer, such as DNA methylation that result in the activation or exclusion of certain genes and/or the hypermethylation of cytosine residues leading to the switching off genes are also being explored (Meršaková *et al.,* 2018). For instance, the hypermethylation of the *CADM1, MAL* and *miR-124-2* genes have been implicated in the progression of cervical intraepithelial neoplasia (Zhang *et al.,* 2022; De Strooper *et al.,* 2014).

As previously mentioned, PCR amplicons containing bisulfite-treated DNA are typically used to evaluate DNA methylation. There are two possible CpG positions for each template molecule: methylation or unmethylated. Heterogenous methylation has been described where alleles are methylated to different degrees, meaning methylated and unmethylated regions are different for those alleles (Mikeska, Candiloro and Dobrovic, 2010). For example, one allele could be completely methylated, while the other allele is unmethylated. Therefore, different ratios of methylation and unmethylated alleles may be present in a heterogeneous combination of cells (Mikeska, Candiloro and Dobrovic, 2010; Kerjean *et al.,* 2000).

It was observed that the melting temperatures for the controls of the various kits obtained in this study was slightly different to those indicated in the assay manuals of the methylation assays for the *CADM1*, *MAL* and *miR-124-2* genes. The difference in melting temperatures for the respective methylation positive, negative and assay controls ranged between 0.2 ^{o}C and 1.9 ^{o}C. The accuracy of the melting temperatures is important as they are used to discern the methylation status of samples (heterogenous and/or homogenous methylation versus unmethylated genes). The difference in control melting temperatures may be attributed to the different real-time PCR platforms used for the MS-HRM assays. The manufacturer used a Roche LightCycler® 480 platform for validation and quality control procedures, whereas a QuantStudio 5 real-time PCR machine was used in this study. Even though the control melting temperatures obtained with the QuantStudio 5 real-time PCR machine were different to that of the Roche platform, results received was comparable for controls and samples.

Out of a total of 57 samples subjected to DNA methylation, 17 indicated heterogenous methylation, with only 1 of these (MLMPath-055) showing heterogenous methylation of all three genes tested. The routine cytology diagnosis of this sample was ASC-US while the histological diagnosis was HSIL. This result agrees with Del Pino *et al*, (2019), who found that when all three genes (*CADM1*, *MAL* and *miR-124-2*) are methylated, the specificity for predicting an HSIL lesion is 100%.

Several studies demonstrated that HR-HPV together with methylation of *CADM1*, *MAL* and/or *miR-124-2* genes, either alone or in combination, are associated with higher grade lesions

(Meršaková *et al*., 2018; Del Pino *et al.*, 2019 and De Strooper *et al*., 2018). This was also observed in this pilot study where 13 HSIL samples also had HR-HPV present as well as heterogenous methylation of one or more of the genes tested (*CADM1*, *MAL* and/or *miR-124-2*). However, 4 samples deviated from this phenomenon (MLMPath-007, MLMPath-008, MLMPath-044) as these were infected with LR-HPV, whilst MLMPath-015 had no HPV detected. A plausible explanation could be that these samples could have been infected with a HR-HPV type not included in the Hybrispot HPV Direct Flow Chip kit used. However, this is unlikely as the Hybrispot HPV Direct Flow Chip Kit includes a universal control that covers HPV types not in included in the kit. Another reason may be that other viruses themselves (for example, HIV or Epstein-Barr Virus (EBV) or due to their interactions with HPV could have contributed to the heterogenous methylation of *CADM1* and *MAL* in MLMPath-007, MLMPath-008, MLMPath-015 and MLMPath-044 (Buch *et al.,*2018; de Lima *et al.,* 2018; Vranic *et al.,* 2018). Our results also showed samples that were HPV positive but had no gene methylation. A possible reason for no methylation in samples with HR-HPV positivity and high-grade cervical lesions, could be those genes other than *CADM1*, *MAL* and *miR-124-2*, such as *FAM19A4* (De Strooper *et al*., 2018) could possibly be involved. Another plausibility could be that a methylation-independent mechanism for the pathogenesis could be responsible for this phenomenon. However, as this is a pilot study with limited sample size, a larger cohort is required to evaluate these inferences.

5.6 Limitations

A major limitation in this research study was the inability to collect the required number of specimens for each diagnostic group, which may have impacted on the analysis and interpretation of some results such as the relationship between HIV and age. A technical issue was experienced with the intensity of the haematoxylin and $p16^{INK4a}$/Ki-67 staining of the CINtec plus Cytology kit. This might have been due to the extended duration of reagent and antibody storage after opening, where possible oxidation of solution compounds could have bene introduced, because of prolonged delivery of the haematoxylin stain. Additionally, these kits were nearing their expiry dates and reagents and antibodies could have become unstable, which might have reduced the efficacy and performance. Although staining was done in technical triplicates, due to these technical challenges, only the first round of $p16^{INK4a}$ /Ki-67 staining could be used and therefore the repeatability of the test was compromised. Another

limitation of the study was that the DNA concentration of some samples were too low for the DNA bisulfite conversion, resulting in higher volumes of sample required for 1 µg of input DNA. Such volumes exceeded the maximum allowable DNA volume for the bisulfite conversion reaction of 40 µl. Therefore, an Eppendorf concentrator was used to concentrate the samples, thereby reducing the volume required for 1 µg of input DNA to 40 µl or less.

Chapter 6

Conclusion

The aim of this pilot study was to identify the HR-HPV types as well as to investigate the simultaneous expression of $p16^{INK4a}$ and Ki-67 and the methylation of *CADM1, MAL* and *miR124-2* genes in cytology specimens collected from HIV positive and HIV negative women. Inclusion criteria was a diagnosis of LSIL, HSIL, ASC-US, and/or ASC-H, known HIV status and women aged between 18 years and 60 years.

Generally, the methods used for HPV testing, $p16^{INK4a}$ and Ki-67 dual-staining and the MS-HRM DNA methylation were successful. A test model for the early detection of lesions with oncogenic transformative potential using HPV testing, $p16^{INK4a}$ and Ki-67 dual-staining and the DNA methylation of the *CADM1, MAL* and *miR124-2* genes was evaluated. Overall, upon comparing the sensitivity and specificity of the test model using the histology diagnosis as gold standard, the sensitivity for predicting LSIL was low but the specificity was acceptable. Conversely, the sensitivity to predict HSIL was high whilst specificity was low. Due to the small sample size of this pilot study and therefore the lack of statistical power, no generalization to the wider population was made with the findings of the tested model. The model was merely used to test the feasibility of these ancillary test. However, a larger study cohort would provide sufficient statistical power to improve the test model to discern the likelihood of cervical intraepithelial lesions progressing to more severe disease, to ultimately reduce unnecessary referrals for colposcopy.

Future work entails comparing the HPV types observed in the exfoliated cervical intraepithelial cells with that of the corresponding FFPE cervical tissue biopsies to ascertain if the HPV types are the same. mRNA expression analysis of $p16^{INK4a}$ and Ki-67 transcripts could be investigated and compared to the protein expression in cases where $p16^{INK4a}$ and $p16^{INK4a}$/ Ki-67 dual staining was observed. Moreover, other DNA methylation biomarkers indicated in cervical cancer development could also be examined.

Chapter 7

References

The reference management software, Mendeley was used for in-text references as well as to compile the reference list. The Harvard referencing style was used.

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Chapter 8

Addendum

UNIVERSITEIT
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Approval Notice

New Application

09/06/2021

Project ID: 21888

HREC Reference No: S21/03/050

Project Title: Correlating p16/ki67 co-expression and gene methylation with high-risk HPV in abnormal cervical squamous intraepithelial cells

Dear Miss Meagan Louw

The **response to modifications** received on 11/05/2021 11:24 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 09/06/2021 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Date: 09 June 2021

Protocol Expiry Date: 08 June 2022

Please remember to use your Project ID 21888 and Ethics Reference Number S21/03/050 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/21888

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Ms Brightness Nxumalo
HREC 2 Coordinator

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects;the South African Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes andStructures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

Stellenbosch UNIVERSITY IYUNIVESITHI UNIVERSITEIT

forward together
sonke siya phambili
saam vorentoe

Approval Letter
Progress Report

27/05/2022

Project ID: 21888

Ethics Reference No: S21/03/050

Project Title: Correlating p16/ki67 co-expression and gene methylation with high-risk HPV in abnormal cervical squamous intraepithelial cells

Dear Miss M Louw

We refer to your request for an extension/annual renewal of ethics approval dated 18/05/2022 12:13.

The Health Research Ethics Committee reviewed and approved the annual progress report through an expedited review process.

The approval of this project is extended for a further year.

Approval date: 09 June 2022

Expiry date: 08 June 2023

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your Project Id 21888 and ethics reference number S21/03/050 on any documents or correspondence with the HREC concerning your research protocol.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Yours sincerely,

Ms Brightness Nxumalo
Coordinator: Health Research Ethics Committee 2 (HREC 2)

National Health Research Ethics Council (NHREC) Registration Number:
REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects; the South African Department of Health (2006).Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

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Addendum C

HPV Direct Flow Chip Kit

LOTS

PCR:	HPVP013L-2	7/31/2023
Chips:	HPVE071	7/31/2023
Reagent:	HPVH095E	1/31/2023

SAMPLE DETAILS

ID SAMPLE: poscontrol-1 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

HPV POSITIVE

Positive sample for:

High-Risk:

16, 18

The sample is negative for the rest of genotypes included in the HPV direct flow chip test.

PROTOCOL

Detection and genotyping of HPV viral DNA by PCR and reverse dot blot hybridization:

- High risk genotypes: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.
- Low risk genotypes: 6, 11, 40, 42, 43, 44/55, 54, 61, 62/81, 67, 69, 70, 71, 72, 84.

Sample preparation/DNA purification

Add cell suspension/purified DNA for PCR amplification:

- PCR protocol (standard): 1x 25°C 10 min, 1x 94°C 3min; 15x94-42-72°C (30”-30”-30”), 35x 94-60-72°C (30”-30”-30”), 1x 72°C 5 min.
- PCR protocol (lyophilized): 1x 25°C 10 min, 1x 94°C 3min; 15x94-47-72°C (30”-30”-30”), 35x 94-65-72°C (30”-30”-30”), 1x 72°C 5 min.

REVERSE-DOT BLOT protocol:

- Hybridization of the biotinilated PCR products to the HPV CHIP.
- Post-hybridization washes.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.

Automatic analysis of results

NOTES

FACULTATIVE: Default Doctor, doctor **Validated:** 3/1/2022

Performed by: Default Tech, tech **Processed:** 3/1/2022

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Addendum D

HPV Direct Flow Chip Kit

LOTS

PCR:	HPVP013L-2	7/31/2023
Chips:	HPVE071	7/31/2023
Reagent:	HPVH095E	1/31/2023

SAMPLE DETAILS

ID SAMPLE: negcontrol-1 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

BLANK

Inappropriate material.

Insufficient Material.

PCR inhibited.

PROTOCOL

Detection and genotyping of HPV viral DNA by PCR and reverse dot blot hybridization:

- High risk genotypes: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82.
- Low risk genotypes: 6, 11, 40, 42, 43, 44/55, 54, 61, 62/81, 67, 69, 70, 71, 72, 84.

Sample preparation/DNA purification

Add cell suspension/purified DNA for PCR amplification:

- PCR protocol (standard): 1x 25°C 10 min, 1x 94°C 3min; 15x94-42-72°C (30”-30”-30”), 35x 94-60-72°C (30”-30”-30”), 1x 72°C 5 min.
- PCR protocol (lyophilized): 1x 25°C 10 min, 1x 94°C 3min; 15x94-47-72°C (30”-30”-30”), 35x 94-65-72°C (30”-30”-30”), 1x 72°C 5 min.

REVERSE-DOT BLOT protocol:

- Hybridization of the biotinilated PCR products to the HPV CHIP.
- Post-hybridization washes.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.

Automatic analysis of results

NOTES

FACULTATIVE: Default Doctor, doctor **Validated:** 3/1/2022

Performed by: Default Tech, tech **Processed:** 3/1/2022

Addendum E

$p16^{INK4a}$ and Ki-67 co-expression results with HPV positivity

Study ID	p16/ki-67 dual staining	HR-HPV subtype
ML-Mpath-001	Pos	HPV31; HPV66; HPV68
ML-Mpath-002	Pos	HPV16; HPV18; HPV52
ML-Mpath-004	Pos	HPV82
ML-Mpath-006	Pos	HPV66
ML-Mpath-013	Pos	HPV33; HPV35; HPV58
ML-Mpath-014	Pos	HPV33
ML-Mpath-015	Pos	HPV Neg
ML-Mpath-016	Pos	HPV82
ML-Mpath-017	Pos	HPV16; HPV18; HPV31; HPV52; HPV53; HPV59
ML-Mpath-018	Pos	HPV45; HPV68; HPV82
ML-Mpath-022	Pos	HPV45; HPV56
ML-Mpath-023	Pos	HPV33; HPV45; HPV51; HPV59; HPV82
ML-Mpath-024	Pos	HPV16
ML-Mpath-026	Pos	HPV16
ML-Mpath-027	Pos	HPV16
ML-Mpath-028	Pos	HPV31; HPV45
ML-Mpath-029	Pos	HPV Neg
ML-Mpath-030	Pos	HPV18; HPV26; HPV33; HPV35; HPV39
ML-Mpath-031	Pos	HPV16; HPV18; HPV52; HPV58; HPV73
ML-Mpath-032	Pos	HPV16
ML-Mpath-038	Pos	HPV31; HPV82
ML-Mpath-041	Pos	HPV52; HPV82
ML-Mpath-048	Pos	HPV18; HPV33
ML-Mpath-050	Pos	HPV35; HPV58
ML-Mpath-054	Pos	HPV18; HPV35

Addendum F

Study I.D	Age	HIV status	Cytolology Diagnosis	Histology Diagnosis	Routine p16 IHC diagnosis	HPV result	HPV type	HR-HPV subtype	Low-Risk HPV subtype	p16/ki-67 dual staining	CADM1 methylation status	MAL methylation status	mir-124-2 methylation status
ML-Mpath-001	36	Pos	LSIL	HSIL	Not requested	Pos	Multiple HR	HPV31; HPV66; HPV68	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-002	36	Pos	LSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV16; HPV18; HPV52	HPV 43; HPV 54; HPV 61, HPV 67	Pos	Neg	Neg	Neg
ML-Mpath-003	42	Pos	LSIL	LSIL	Not requested	Pos	Mixed HR/LR	HPV16; HPV33; HPV73	HPV 54	Neg	Neg	Neg	Neg
ML-Mpath-004	42	Pos	LSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV82	HPV 6; HPV 54; HPV 62, HPV 81	Pos	Neg	Neg	Neg
ML-Mpath-005	35	Pos	LSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV58	HPV 62; HPV 81	Neg	Neg	Neg	Heterogenous
ML-Mpath-006	37	Pos	LSIL	LSIL	Pos	Pos	Single HR	HPV66	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-007	36	Pos	LSIL	HSIL	Not requested	Pos	Multiple LR	HPV Neg	HPV 6; HPV 44; HPV 55, HPV 62; HPV 81	Neg	Neg	Heterogenous	Neg
ML-Mpath-008	44	Pos	LSIL	LSIL	Not requested	Pos	Single LR	HPV Neg	HPV 11	Neg	Neg	Heterogenous	Neg
ML-Mpath-009	31	Pos	LSIL	LSIL	Not requested	Pos	Single LR	HPV Neg	HPV 70	Neg	Neg	Neg	Neg
ML-Mpath-010	44	Pos	LSIL	cervicitis	Not requested	Pos	Multiple LR	HPV Neg	HPV 6; HPV 42; HPV 62, HPV 81	Neg	Neg	Neg	Neg
ML-Mpath-011	46	Pos	LSIL	LSIL	Not requested	Pos	Mixed HR/LR	HPV53	HPV 43; HPV 67; HPV 70	Neg	Neg	Neg	Neg
ML-Mpath-012	32	Pos	LSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV16; HPV35; HPV51;HPV58; HPV73	HPV 11	Neg	Neg	Neg	Neg
ML-Mpath-013	47	Pos	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV33; HPV35; HPV58	HPV42; HPV43;	Pos	Neg	Neg	Neg
ML-Mpath-014	33	Pos	HSIL	HSIL	Not requested	Pos	Single HR	HPV33	HPV Neg	Pos	Heterogenous	Neg	Neg
ML-Mpath-015	35	Pos	HSIL	HSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Pos	Heterogenous	Neg	Neg
ML-Mpath-016	35	Pos	HSIL	HSIL	Not requested	Pos	Single HR	HPV82	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-017	27	Pos	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV16; HPV18; HPV31;HPV52; HPV53; HPV59	HPV6; HPV71	Pos	Neg	Neg	Neg
ML-Mpath-018	39	Pos	HSIL	HSIL	Not requested	Pos	Multiple HR	HPV45;HPV68;HPV82	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-019	31	Pos	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV18;HPV31;HPV33;HPV45	HPV6; HPV42; HPV44;HPV55; HPV62; HPV81	Neg	Neg	Heterogenous	Neg
ML-Mpath-020	39	Pos	HSIL	LSIL	Not requested	Pos	Mixed HR/LR	HPV58	HPV40	Neg	Neg	Heterogenous	Neg
ML-Mpath-021	35	Pos	HSIL	LSIL	Not requested	Pos	Mixed HR/LR	HPV16;HPV35;HPV82	HPV54; HPV70	Neg	Neg	Neg	Heterogenous
ML-Mpath-022	52	Pos	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV45;HPV56	HPV42; HPV61	Pos	Neg	Neg	Heterogenous
ML-Mpath-023	29	Pos	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV33; HPV45; HPV51;HPV59; HPV82	HPV61	Pos	Neg	Neg	Neg
ML-Mpath-024	49	Pos	HSIL	HSIL	Not requested	Pos	Single HR	HPV16	HPV Neg	Pos	Heterogenous	Heterogenous	Neg
ML-Mpath-025	45	Neg	HSIL	HSIL	Not requested	Pos	Multiple LR	HPV Neg	HPV62;HPV81	Neg	Neg	Neg	Neg
ML-Mpath-026	31	Neg	HSIL	ISCC	Not requested	Pos	Single HR	HPV16	HPV Neg	Pos	Heterogenous	Neg	Heterogenous
ML-Mpath-027	28	Neg	HSIL	HSIL	Not requested	Pos	Single HR	HPV16	HPV Neg	Pos	Heterogenous	Neg	Heterogenous
ML-Mpath-028	37	Neg	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV31; HPV45	HPV62; HPV67; HPV81	Pos	Neg	Neg	Neg
ML-Mpath-029	34	Neg	HSIL	HSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-030	27	Neg	HSIL	HSIL	Not requested	Pos	Multiple HR	HPV18; HPV26; HPV33;HPV35; HPV39	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-031	33	Neg	HSIL	HSIL	Not requested	Pos	Multiple HR	HPV16; HPV18; HPV52;HPV58;HPV73	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-032	60	Neg	HSIL	HSIL	Not requested	Pos	Single HR	HPV16	HPV Neg	Pos	Neg	Heterogenous	Neg
ML-Mpath-033	42	Neg	HSIL	LSIL	Not requested	Pos	Single HR	HPV52	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-034	30	Neg	HSIL	LSIL	Not requested	Pos	Single HR	HPV52	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-035	51	Neg	HSIL	HSIL	Not requested	Pos	Mixed HR/LR	HPV16	HPV70	Neg	Heterogenous	Neg	Neg
ML-Mpath-036	44	Neg	HSIL	HSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-037	34	Pos	ASC-H	LSIL	Neg	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-038	47	Pos	ASC-H	HSIL	Not requested	Pos	Mixed HR/LR	HPV31;HPV82	HPV54	Pos	Neg	Neg	Neg
ML-Mpath-039	21	Pos	ASC-H	LSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-040	44	Pos	ASC-H	LSIL	Not requested	Pos	Single HR	HPV35	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-041	42	Pos	ASC-H	HSIL	Not requested	Pos	Multiple HR	HPV52;HPV82	HPV Neg	Pos	Neg	Heterogenous	Neg
ML-Mpath-042	29	Pos	ASC-H	HSIL	Not requested	Pos	Mixed HR/LR	HPV16; HPV58; HPV82	HPV40; HPV70; HPV84	Neg	Neg	Neg	Heterogenous
ML-Mpath-043	31	Pos	ASC-H	HSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-044	34	Pos	ASC-H	LSIL	Not requested	Pos	Multiple LR	HPV Neg	HPV44; HPV55	Neg	Neg	Neg	Neg
ML-Mpath-045	41	Pos	ASC-H	HSIL	Not requested	Pos	Single HR	HPV16	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-046	38	Pos	ASC-H	LSIL	Not requested	Pos	Multiple HR	HPV16;HPV33	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-047	37	Pos	ASC-H	HSIL	Pos	Pos	Multiple LR	HPV neg	HPV44; HPV55;HPV67	Neg	Neg	Neg	Neg
ML-Mpath-048	41	Neg	ASC-H	HSIL	Pos	Pos	Multiple HR	HPV18;HPV33	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-049	32	Neg	ASC-H	HSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-050	37	Pos	ASC-US	HSIL	Not requested	Pos	Multiple HR	HPV35;HPV58	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-051	44	Pos	ASC-US	LSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-052	27	Pos	ASC-US	HSIL	Not requested	Pos	Single HR	HPV33	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-053	41	Pos	ASC-US	LSIL	Not requested	Pos	Single LR	HPV Neg	HPV67	Neg	Neg	Neg	Neg
ML-Mpath-054	33	Neg	ASC-US	HSIL	Pos	Pos	Multiple HR	HPV18;HPV35	HPV Neg	Pos	Neg	Neg	Neg
ML-Mpath-055	49	Neg	ASC-US	HSIL	Not requested	Pos	Multiple HR	HPV35;HPV52;HPV56;HPV73	HPV Neg	Neg	Heterogenous	Heterogenous	Heterogenous
ML-Mpath-056	52	Neg	ASC-US	LSIL	Not requested	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg	Neg	Neg
ML-Mpath-057	33	Neg	ASC-US	LSIL	Not requested	Pos	Mixed HR/LR	HPV58	HPV67	Neg	Neg	Neg	Neg
ML-Mpath-058	41	Pos	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)

ML-Mpath-059	45	Pos	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contr	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-060	45	Pos	NILM	N/A	N/A	Pos	Mixed HR/LR	HPV35	HPV40	Neg	Not included in analysis	Not included in ana	Not included in analysis
ML-Mpath-061	47	Pos	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-062	24	Pos	NILM	N/A	N/A	Pos	Single LR	HPV Neg	HPV61	Neg	Not included in analysis	Not included in ana	Not included in analysis
ML-Mpath-063	44	Pos	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-064	26	Neg	NILM	N/A	N/A	Pos	Multiple LR	HPV Neg	HPV61;HPV71	Neg	Not included in analysis	Not included in ana	Not included in analysis
ML-Mpath-065	39	Neg	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-066	35	Neg	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-067	36	Neg	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-068	37	Neg	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)
ML-Mpath-069	29	Neg	NILM	N/A	N/A	Neg	HPV Neg	HPV Neg	HPV Neg	Neg	Neg (pooled Neg contro	Neg (pooled Neg co	Neg (pooled Neg control)

Shortcut codes
Pos = Positive
Neg = Negative
N/A = Not applicable
HR = High Risk
LR = Low Risk