Exploring options for the secondary prevention of cervical cancer in South Africa

by

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Dissertation presented for the degree of Doctor of Philosophy in the Faculty of Medicine and Health Science at Stellenbosch University

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December 2024

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Abstract

Cervical cancer stands as a significant global health challenge, impacting millions of women annually. While primary prevention measures like human papilloma virus (HPV) vaccination show promise, the role of secondary prevention, including screening programmes and early detection methods, is paramount. This thesis aims to assess and elucidate the multifaceted nature of secondary prevention strategies for cervical cancer.

In Chapter 2, the evolution and current state of cervical cytology in South Africa is discussed, highlighting its successes and systemic challenges. The chapter emphasises the need for robust infrastructure, efficient follow-up systems, and the lessons learned from cytology that can inform the implementation of HPV DNA screening.

In Chapter 3, the study findings suggest that visual inspection methods perform better in women living with HIV (WLWH) compared to HIV-negative women. This implies the justification for utilizing visual inspection methods in cervical cancer screening for WLWH. However, it is noted that these methods exhibit satisfactory sensitivity only under rigorous quality assurance measures, which might pose challenges in non-academic settings, potentially leading to over-treatment due to reduced specificity.

Chapter 4 explores cervical cancer control guidelines in the Southern African Development Community (SADC) region, highlighting the reliance on visual inspection as a screening method, despite the World Health Organization (WHO) prioritizing HPV deoxyribose nucleic acid (DNA) testing in its guideline of 2021. HPV DNA testing offers high sensitivity and a negative predictive value approaching 100%, reducing the need for frequent follow-up visits and additional tests, thus enhancing cost-effectiveness. However, HPV DNA testing alone may not provide clinically relevant information without a triage test to identify women with clinically significant disease.

A pragmatic advantage of HPV testing is its compatibility with triage tests like host-cell DNA methylation assessment, streamlining the diagnostic process and reducing discomfort for individuals undergoing screening. In South Africa, HPV testing is increasingly available, with reflex cytology recommended as the triage test, though molecular biomarkers like DNA methylation show promise for further risk stratification. However, more evidence is needed,

particularly in low- and middle-income countries (LMICs), to support their inclusion in screening programmes.

Chapter 5 discusses the potential of DNA methylation assays as triage tools, citing studies showing high sensitivity for cervical cancer detection. However, conclusive evidence is lacking, especially in LMICs, necessitating further research and adaptation of methylation assays for cost-effective use. Longitudinal studies focusing on women living with HIV are particularly crucial due to limited existing research.

Chapter 6 outlines a study protocol comparing thermal ablation (TA) to large loop excision of the transformation zone (LLETZ) in treating cervical precancer, acknowledging South Africa's human immunodeficiency virus (HIV) burden and high-risk HPV prevalence. Understanding TA's efficacy including treatment failure rates is vital for adopting accessible treatments, considering the context of resource constraints.

In summary, secondary prevention of cervical cancer involves a diverse array of strategies, from screening methods to treatment modalities. While HPV DNA testing represents a significant advancement, challenges remain in implementation, especially in LMICs. Further research, particularly on triage methods and treatment efficacy, is essential for improving outcomes, especially in populations disproportionately affected by cervical cancer.

Opsomming

Servikale kanker bly 'n beduidende globale gesondheidsuitdaging, wat jaarliks miljoene vroue affekteer. Terwyl primêre voorkomingsmaatreëls soos menslike papilloomvirus (HPV)-inenting belofte toon, is die rol van sekondêre voorkoming, insluitend siftingsprogramme en vroeë opsporingsmetodes, van uiterste belang. Hierdie tesis het ten doel om die veelsydige aard van sekondêre voorkomingsstrategieë vir servikale kanker te assesseer en te verduidelik.

In Hoofstuk 2 word die evolusie en huidige stand van servikale sitologie in Suid-Afrika bespreek, met die klem op die suksesse en sistemiese uitdagings daarvan. Die hoofstuk beklemtoon die behoefte aan robuuste infrastruktuur, doeltreffende opvolgstelsels en die lesse wat uit sitologie geleer is, wat die implementering van HPV deoksiribonukleïensuur (DNS)-toetsing kan inlig.

In Hoofstuk 3 dui die studievindings daarop dat visuele inspeksiemetodes beter presteer in vroue wat met MIV leef in vergelyking met MIV-negatiewe vroue. Dit impliseer die regverdiging vir die gebruik van visuele inspeksiemetodes in servikale kankersifting vir vroue wat met MIV leef. Daar word getoon dat hierdie metodes slegs bevredigende sensitiwiteit toon onder streng gehalteversekeringsmaatreëls, wat moontlike uitdagings in nie-akademiese omgewings kan veroorsaak, moontlik lei tot oor behandeling as gevolg van verminderde spesifisiteit.

Hoofstuk 4 verken servikale kankerbeheerriglyne in die Suider-Afrikaanse Ontwikkelingsgemeenskap (SAOG)-streek en beklemtoon die belang van visuele inspeksie as 'n siftingsmetode, ten spyte van die prioritisering van HPV DNS-toetsing deur die Wêreldgesondheidsorganisasie (WGO) in sy 2021 riglyn. HPV DNS-toetsing bied 'n hoë sensitiwiteit en 'n negatiewe voorspelling waarde wat naby 100% kom, wat die nodigheid aan gereelde opvolgbesoeke en addisionele toetse verminder, en dus die kostedoeltreffendheid verbeter. Tog mag HPV DNS-toetsing alleen nie klinies relevante inligting verskaf sonder ’n bykomende triage-toets om vroue met progressiewe siekte te identifiseer nie.

'N Pragmatiese voordeel van HPV-toetsing is die verenigbaarheid daarvan met triage-toetse soos selmatriks-DNS-metilasie-assessering, wat die diagnostiese proses stroomlyn en ongemak vir individue wat sifting ondergaan, verminder. In Suid-Afrika word HPV-toetsing

toenemend beskikbaar, met refleks-sitologie wat as die triage-toets aanbeveel word, alhoewel molekulêre biomerkers soos DNS-metilasie belofte toon vir verdere risikostratifisering. Meer bewyse is egter nodig, veral in lae- en middelinkomstelande, om hul insluiting in siftingsprogramme te ondersteun.

Hoofstuk 5 bespreek die potensiaal van DNS-metilasie as triage-hulpmiddels en verwys na studies wat 'n hoë sensitiwiteit vir servikale kankerspore toon. Daar is egter onsekerheid, veral in lae- en middelinkomstelande, wat verdere navorsing en aanpassing van metilasie-assesseerders vir koste-doeltreffende gebruik noodsaaklik maak. Lang termyn studies wat fokus op vroue wat met MIV leef, is as gevolg van beperkte bestaande kennis.

Hoofstuk 6 skets 'n studieprotokol wat termiese ablasie (TA) met groot lus eksisie van die transformasiesone vergelyk in die behandeling van servikale pre-kanker, waarby die invloed van menslike immuniteitsgebrek-virus (MIV) in Suid-Afrika en die hoë voorkoms van hoë risiko-HPV erken word. Die begrip van die langtermyn doeltreffendheid van TA is van kardinale belang vir die ontwikkeling van toeganklike behandeling, gegewe die konteks van hulpbronbeperkings.

In opsomming, behels sekondêre voorkoming van servikale kanker 'n diverse reeks strategieë, van siftingsmetodes tot behandelingsmodaliteite. Terwyl HPV DNS-toetsing 'n beduidende vooruitgang verteenwoordig, bly uitdagings bestaan in implementering, veral in lae- en middelinkomstelande. Verdere navorsing, veral oor triage-metodes en behandelingseffektiwiteit, is noodsaaklik vir die verbetering van uitkomste, veral in populasies wat buitensporig deur servikale kanker geraak word.

Acknowledgments

Thank you, Prof Hennie Botha, for your unwavering guidance, support, and encouragement throughout my PhD journey. Your expertise, patience, and mentorship have been invaluable in shaping both my research and personal growth.

To my parents, sisters, family and friends, your love, understanding, and endless encouragement have sustained me through the challenges and triumphs of this doctoral pursuit. Your steadfast support has been the bedrock of my success. I am profoundly grateful for everything you have done for me.

Thank you, Gyn.org and the Department of Obstetrics and Gynaecology, for your belief in me. Your listening ears and support have made this journey more manageable and memorable. Your presence has been a source of strength and joy, and I am incredibly fortunate to have you in my life.

Thank you, Prof Greta Dreyer, and the VACCS research group. Your collaboration and expertise have enriched my research experience beyond measure. I am grateful for the stimulating discussions, shared insights, and collective efforts that have propelled my work forward.

Thank you to Renske Steenbergen, Lisanne Verhoef, Marianne Wolfaardt, Azwidowi Lukhwareni, and the Self-screen team. Your invaluable assistance and expertise have been instrumental in the success of my research endeavours. I deeply appreciate your support and commitment to advancing scientific knowledge in the field of DNA-methylation and cervical cancer prevention.

Thank you, Qiagen, for the provision of research materials and resources to complete the DNA-methylation section of this project.

To each and every individual who has contributed to my doctoral journey, in ways both seen and unseen, I extend my heartfelt thanks. Your support, encouragement, and belief in my abilities have made all the difference. I am profoundly grateful for the privilege of learning and growing alongside such remarkable individuals.

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List of Abbreviations

Abbreviation	Definition
AGUS	Atypical glandular cells of unknown significance
AIDS	Acquired Immunodeficiency Syndrome
AIS	Adenocarcinoma in situ
ART	Antiretroviral treatment
ASC-US	Atypical squamous cells of undetermined significance
BD	Becton Dickinson Italia
CAB	Community Advisory Board
cART	Combination antiretroviral therapy
CHAI	Clinton Health Access Initiative
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIN 1	Cervical intraepithelial neoplasia graded 1
CIN 2	Cervical intraepithelial neoplasia graded 2
CIN 3	Cervical intraepithelial neoplasia graded 3
CAB	Community Advisory Board
Ct	Cycle threshold
DHIS	District Health Information System
DiaVACCS	DIAgnosis in Vaccine And Cervical Cancer Screen
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
ESU	Electrosurgical unit
GCP	Good clinical practice
GIAHC	Global Initiative Against HPV and Cervical Cancer
GLOBOCAN	Global Cancer Observatory
HCW	Healthcare workers
HIC	High-income countries
HIV	Human immunodeficiency virus

HPV	Human papillomavirus
HREC	Health Research Ethics Committee
hrHPV	High-risk HPV
HSIL	High-grade squamous intraepithelial lesions
IARC	International Agency for Research on Cancer
ICF	Informed consent form
ICO/IARC	The Catalan Institute of Oncology/International Agency for Research on Cancer
IQR	Interquartile range
LBC	Liquid-based cytology
LEEP	Loop electrosurgical excision procedure
LLETZ	Large loop excision of the transformation zone
LMIC	Low- and Middle-income Countries
LSIL	Low-grade squamous intraepithelial lesions
MRC	Medical Research Council
mRNA	Messenger ribonucleic acid
NHLS	National Health Laboratory Service
NHRD	National Health Research Database
NPV	Negative predictive value
NTC	No template control
NCD	Noncommunicable Diseases
Pap	Papanicolaou
PCR	Polymerase Chain Reaction
PI	Principal investigator
PPV	Positive predictive value
qPCR	quantitative Polymerase chain reaction
Rb	Retinoblastoma
RBC	Rwanda Biomedical Centre
RCF	Relative centrifugal field
RCI	Reid's colposcopic index

Rpm	Rotations per minute
SADC	Southern African Development Community
SAHPRA	South African Health Products Regulatory Authority
SCC	Cervical squamous cell carcinoma
SCJ	Squamocolumnar junction
SD	Standard deviation
SMS	Short message service
TA	Thermal ablation
TALL Study	Cervical Precancer Thermal Ablation versus LLETZ Excision Comparative Efficacy Study
TB	Tuberculosis
TVUS	Transvaginal ultrasound
VIA	Visual inspection with acetic acid
VILI	Visual inspection with Lugol's iodine
VLPs	Virus-like particles
WHO	World Health Organization
WLWH	Women living with HIV

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CHAPTER 1

General introduction and dissertation outline

1.1 BACKGROUND

1.1.1 Cervical cancer statistics

The global cancer burden is a matter of significant concern, with an estimated 20 million incident cases and 9.7 million deaths reported in 2022. According to the GLOBOCAN estimates, these alarming statistics are projected to worsen, with the number of new cancer cases expected to exceed 35 million, by the year 2050. This increase can be attributed to the rising prevalence of lifestyle-related risk factors, population growth, aging, and socio-economic factors. [1]

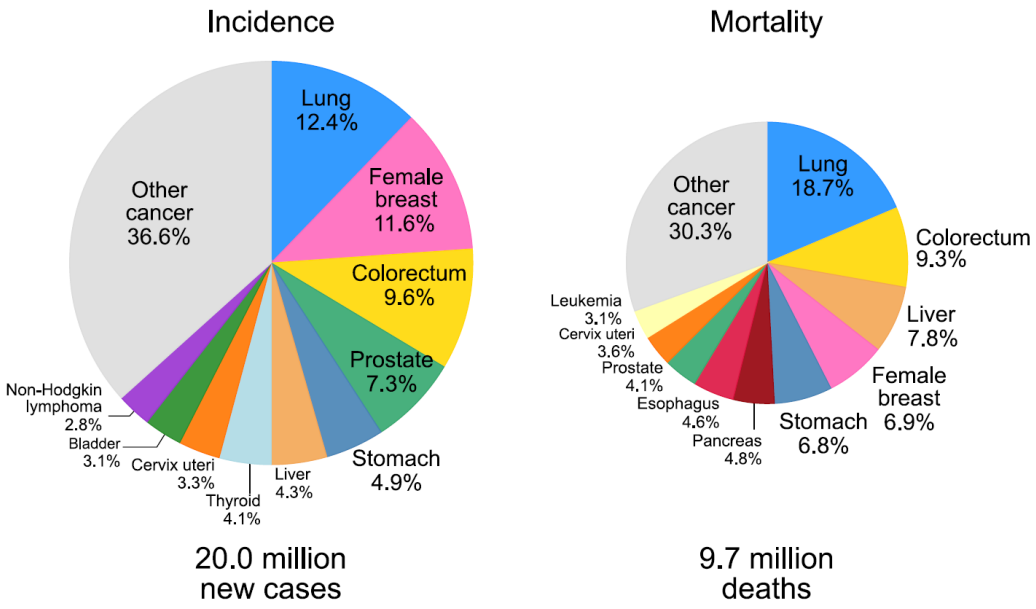


Figure 1.1 Breakdown of Global cancer incidence and mortality rates as reported by GLOBOCAN.[1]

While breast cancer remains the most prevalent cancer among women globally, followed by lung cancer and colorectal cancer, cervical cancer ranks as the fourth most common. According to GLOBOCAN estimates for 2022, approximately 661 021 women worldwide are diagnosed with cervical cancer annually, resulting in 348 189 deaths. [1] In South Africa, cervical cancer holds the position as the second most frequently diagnosed cancer among women, particularly impacting the reproductive age group of 15 to 49 years. Reported estimates for 2023 indicate 10 702 incident cases with an age-standardized incidence rate

of 35.3 per 100 000, and 5 870 deaths with an age-standardized incidence rate of 18.9 per 100 000. [1,2]

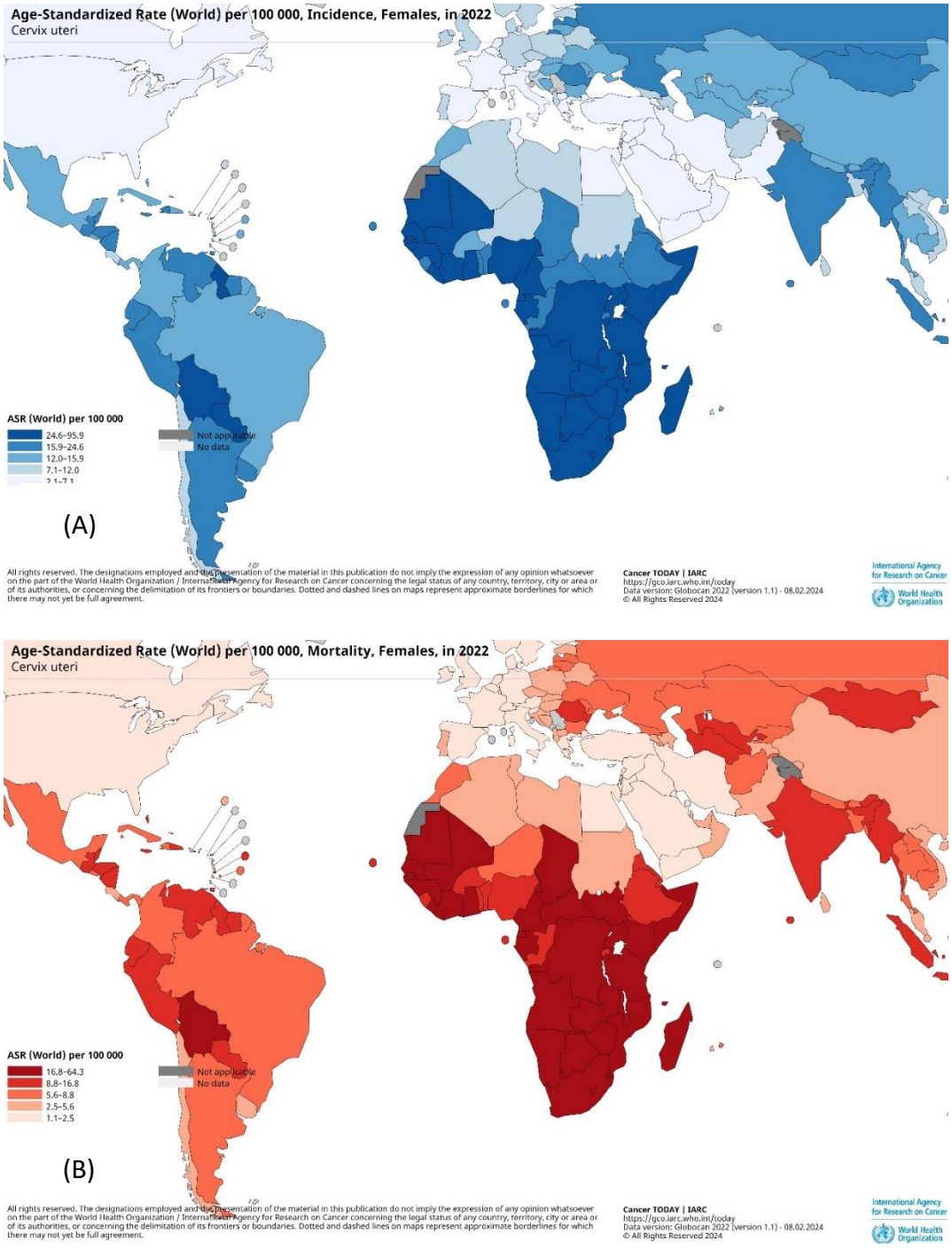


Figure 1.2 Age-standardised incidence (A) and mortality rates (B) of cervical cancer by country in 2022. Adapted from GLOBOCAN database.[1]

1.1.2 Cervical abnormalities and HPV

The primary etiological factor of cervical precancer and cancer is the persistent infection of the cervix with human papillomavirus (HPV). [3] HPV is recognized as one of the most prevalent sexually transmitted infections globally, affecting approximately 70-80% of sexually active women at some point in their lives with at least one strain of the virus. [4]

1.1.3 The virology and molecular pathogenesis of HPV

HPV is a non-enveloped capsid virus with a double-stranded deoxyribonucleic acid (DNA) genome, comprising approximately 8 000 nucleotides. It belongs to the Papillomaviridae family, a group of viruses known for their specific affinity to epithelial tissues. To date, researchers have characterized over 200 human HPV genotypes, which have been categorized into five major phylogenetic genera: Alpha, Beta, Gamma, Mu, and Nu papillomaviruses. [5]

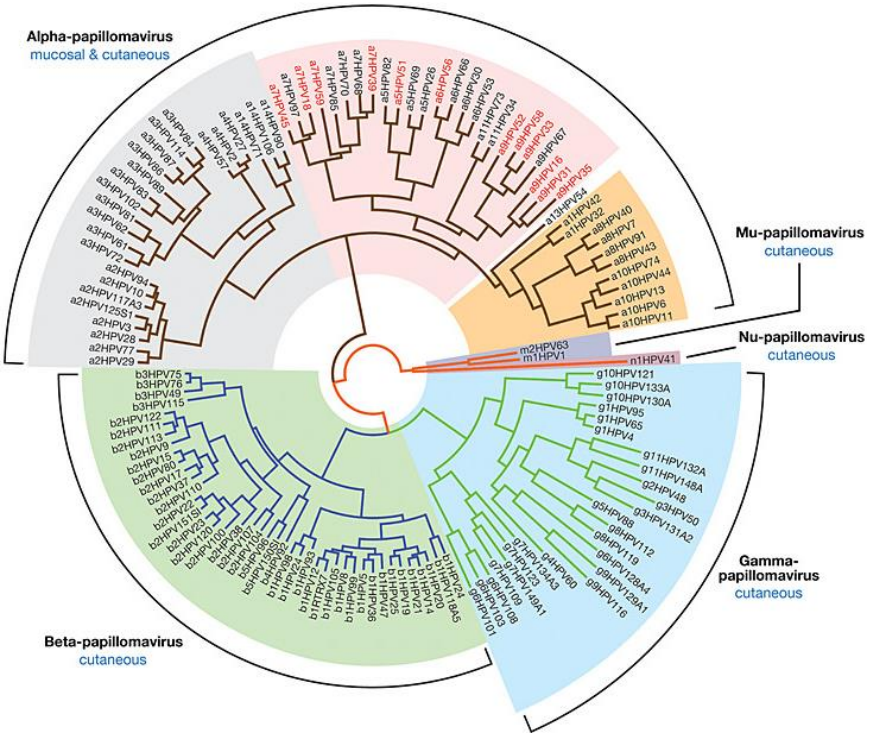


Figure 1.3 HPV found in humans are divided into five genera, with Alpha and Beta/Gamma being the largest groups. HPV types from the Alpha genus are categorized as low-risk cutaneous (grey), low-risk mucosal (orange), or high-risk (pink). The evolutionary tree is

constructed from the alignment of the E1, E2, L1, and L2 genes. Image adapted from Doorbar et.al. [6]

Members of the alpha genus, collectively known as high-risk HPV (hrHPV), serve as the primary etiological agents for nearly all cervical cancer cases. Among these, 13 specific genotypes, including alpha-5 HPV genotype 51, alpha-6 HPV genotypes 56 and 66, alpha-7 HPV genotypes 18, 45, 39, and 59, as well as alpha-9 HPV genotypes 16, 31, 33, 35, 52, and 58, are categorized as IARC group 1 cervical carcinogens. [5] Since 1995, HPV 16 and 18 have been recognized as cervical carcinogens, accounting for approximately 70% of cervical cancer cases worldwide. [4]

HPV infects epithelial cells exclusively. Replication of the virus occurs in the nuclei of the host infected cells and the production of mature virions occurs in the suprabasal epithelial cell layers. [3] The resultant alterations in infected cells manifest as cervical intraepithelial neoplasia (CIN), graded from CIN 1 to CIN 3 depending on the severity of disease. [7]

The virus is equipped with dual promoters encoding two distinct groups of proteins vital for viral survival: the early genes (E1, E2, E4, E5, E6, and E7) and the late genes (L1 - major capsid protein and L2 - minor capsid protein). Malignant transformation is predominantly driven by the E6 and E7 early genes. [8]

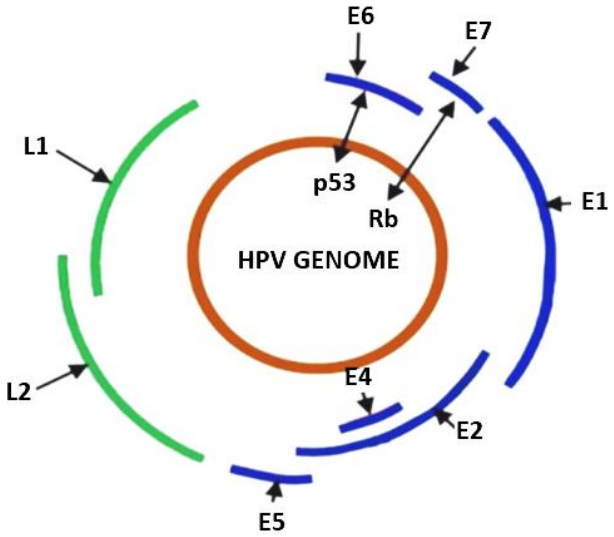


Figure 1.4 Image illustrating the genomic organization of HPV. Adapted from Tomaić. [8]

The E6 oncoprotein exerts its oncogenic effects by binding and degrading the tumour suppressor gene p53, thus inducing alterations in host DNA, leading to aberrant cell replication and heightened mutation rates. On the other hand, the E7 protein targets the tumour suppressor protein retinoblastoma (Rb), disrupting its inhibitory function, thereby resulting in uncontrolled cellular proliferation in response to growth stimulators. This dysregulation of key cellular mechanisms by E6 and E7 ultimately culminates in malignant transformation. [8]

1.1.4 Primary prevention of cervical cancer

The primary prevention of cervical cancer revolves around reducing exposure to cancer causing agents including mitigating the risk of HPV acquisition. This preventive approach encompasses several strategies, particularly measures to reduce exposure to the virus such as advocating for delayed sexual debut and promoting the consistent use of barrier contraception, health education addressing aspects of sexual behaviour and tobacco use and vaccination against HPV strains. [9]

The South African HPV vaccination programme was launched in 2014 under the Integrated School Health Programme, aiming to administer the bivalent vaccine in a two-dose schedule to girls aged nine to 12 years, spaced five to six months apart. Presently in South Africa, three prophylactic HPV vaccines are commercially available: the bivalent vaccine Cervarix® (GlaxoSmithKline), which includes virus-like particles (VLPs) for HPV 16 and HPV 18; the quadrivalent vaccine Gardasil® (Merk & Co., Inc.), containing VLPs for HPV 6, HPV 11, HPV 16, and HPV 18; and the nonavalent vaccine Gardasil®9 (Merk & Co., Inc.), offering additional protection against HPV types 31, 33, 45, 52, and 58. [10]

The HPV vaccine is most effective when administered prior to exposure to HPV, hence the recommendation to vaccinate individuals before sexual debut. However, it is crucial to recognize that prophylactic HPV vaccines offer benefits beyond HPV-naïve individuals. They also exhibit potential in preventing re-infections or new infections that could lead to HPV-related diseases in sexually active individuals, particularly post-treatment. [11,12] A clinical trial conducted by Giuliano et. al, in sexually active female participants with detectable HPV infections at baseline showed that Gardasil®9 (Merk & Co., Inc.) vaccination prevented new

persistent infections with other vaccine targeted HPV types and concluded that effective implementation of the nine-valent HPV vaccine may substantially reduce the burden of HPV-related diseases and related medical procedures. [13] The vaccines, however, are quite costly and adult vaccinations have not been integrated into the South African national cervical cancer screening programme.

1.1.5 Secondary prevention of cervical cancer – primary cervical cancer screening tests

Cervical cancer typically has a protracted precursor phase spanning from five to 20 years, during which screening can identify abnormalities and assess risk. During an active HPV infection, screening may detect low-grade cervical abnormalities known as low-grade squamous intraepithelial lesions (LSIL) or grade 1 CIN (CIN1), however, approximately 90% of newly acquired HPV infections are transient and typically clear up within one to two years due to the host's immune response, often without any intervention, leading only to asymptomatic infections. [14]

Only a minority of HPV infections persist beyond 12 months, increasing the risk of progression to cervical precancer, such as high-grade squamous intraepithelial lesions (HSIL) or grade 2 or 3 CIN lesions (CIN2/3), and potentially to invasive cancer if not addressed promptly.

Therefore, the primary aim of screening is to reduce mortality associated with cervical cancer by detecting and treating CIN lesions before they progress to invasive cancer.

1.1.5.1 Cervical cytology

In 2000, South Africa initiated a comprehensive national screening programme aimed at preventing cervical cancer. This programme involved providing three Papanicolaou (Pap) tests per lifetime for women, with screening commencement at the age of 30 for general population, and at 25 for women living with human immunodeficiency virus (HIV) (WLWH). For HIV-negative women, screening intervals were established at 10-year intervals, whereas WLWH were recommended to undergo screening every three years. As per national guidelines, women with atypical squamous cells of undetermined significance (ASC-US) or

higher cytology results, whether from conventional or liquid-based methods, are recommended to undergo colposcopic directed biopsy. Those with negative biopsy results or diagnosed with CIN 1 should undergo re-screening after three years. Women diagnosed with HSIL, or CIN2/3 are offered appropriate precancer treatment options, such as ablative or excisional methods. [15]

Despite the efficacy of cytology in reducing cervical cancer incidence, cases still arise due to the inherent variability in screening. The implication thereof is delayed diagnosis and treatment of precancer, increased risk of morbidity and mortality and an overall healthcare system burden. Thus, there is a need for further improvement of screening protocols to correctly identify women with disease and minimize unnecessary interventions while maximizing the programmes efficacy in reducing cervical cancer burden.

1.1.5.2 Visual inspection with stain

Visual inspection with acetic acid (VIA) is a screening method where the uterine cervix is examined visually after the application of a 3-5% acetic acid solution. This test works by acetic acid coagulating cellular proteins in the cervical epithelium if they are present in abnormal quantities. This process causes the affected area to appear opaque and white, indicating potential abnormalities.

Following the VIA test, visual inspection with Lugol's iodine (VILI) may be conducted. In this method, Lugol's iodine solution is applied to the cervix, reacting with glycogen to produce a brown or black colour. Normal mature squamous epithelium, which contains glycogen, will turn dark brown upon application of Lugol's iodine. However, precancerous lesions and cancerous areas lack sufficient glycogen, causing them to turn yellow or remain unchanged, providing a visual contrast for further examination.

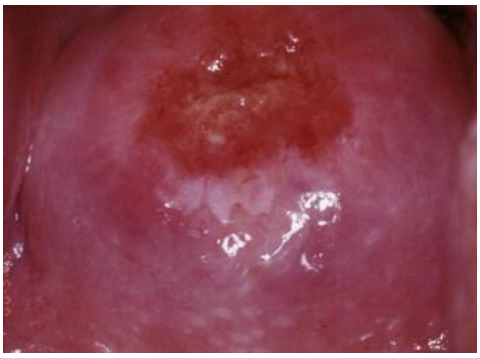
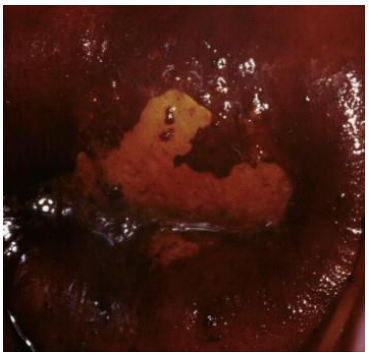
(A)  (B) 

Figure 1.5 Positive visual inspection with acetic acid test (A); positive visual inspection with Lugol's iodine test (B). Image from Sankaranarayanan et.al. [16]

In low-resource settings, where the implementation of conventional or liquid-based cytology is challenging due to limited infrastructure and specialized training, VIA and/or VILI has been recommended as a suitable alternative for primary cervical cancer screening. VIA/VILI is cost-effective, relatively straightforward, and can be performed by non-medical healthcare workers, providing immediate results that enable a screen-and-treat approach. [17,18] Despite these benefits, the reliance on user perception and interpretation can lead to a high rate of false positives, potentially resulting in overdiagnosis and overtreatment. [19–22] Additionally, VIA/VILI is not appropriate for use in women when the transformation zone is not visible or after menopause. Due to these challenges, the WHO advises current programmes using VIA/VILI as the primary screening test to transition rapidly to more reliable and consistent screening methods.

1.1.5.3 HPV DNA testing

The direct detection of HPV in cervical specimens through target DNA amplification using Polymerase Chain Reaction (PCR) provides an alternative to population-based cytological screening. HPV DNA testing is the most sensitive method for cervical cancer screening, [23,24] with a negative predictive value (NPV) close to 100%. This high NPV allows for extended screening intervals, reducing the frequency of tests and thus making the process more cost-effective than traditional cytological testing. [25–27]

HPV testing can also be performed on self-collected samples, which can lead to higher participation rates in screening programmes. [28] Moreover, the future development of point-of-care tests that provide instant results has the potential to enhance treatment rates. [29,30]

Screening with HPV testing poses a challenge because transient HPV infections are prevalent, especially among young women, where most infections resolve on their own without intervention.[31] Even in women over 30 years of age, HPV infections often resolve without progressing, and only a small subset of women with persistent infections may develop cervical precancer or cancer.[14] Therefore, a key issue in cervical cancer screening is determining which tests or strategies can effectively identify HPV-positive women who are at higher risk of developing significant disease, so they can receive appropriate evaluation and treatment.

1.1.5.4 Insights from the DiaVACCS study

In addition to the primary cervical cancer screening methods discussed, the DIAgnosis in Vaccine And Cervical Cancer Screen (DiaVACCS) study, a multicentric screening trial, has provided significant insights into the prevalence of cervical abnormalities among South African women. This study, as discussed in Chapters 3 and 5, was designed to evaluate the effectiveness of various screening tests and strategies. The study included 1 104 women aged 25 - 65 years eligible for screening, of whom 465 were WLWH and 639 were HIV-negative. Screening methods involved visual inspection, molecular tests, and cytology on samples collected both by participants themselves and healthcare workers. Participants who screened positive and 49.1% of those who screened negative were invited for further colposcopy and biopsy, with those requiring treatment undergoing LLETZ.

The findings revealed a high prevalence of HPV-related cervical pathology among the HIV-negative women, with significantly higher prevalence rates among WLWH. The study concluded a need for effective screening programmes targeting these populations.

1.1.6 Secondary prevention of cervical cancer - triage testing

Secondary or triage testing is aimed at avoiding overtreatment of women with abnormal screening results which may not confer a high risk for severe dysplasia. It is hence a way to manage intermediate risk results.

As mentioned earlier, the current screening methods involve cytology, HPV DNA testing, or VIA/VILI, depending on what is suitable for the facility. Triage typically uses a different test

than the initial screening test but repeat cytology after a defined time interval is suitable if medium-risk cytology results are obtained. [32]

1.1.6.1 HPV mRNA for triage of screen positive women

The use of HPV messenger ribonucleic acid (mRNA) testing for the triage of screen-positive women represents a significant advancement in cervical cancer screening strategies. Unlike HPV DNA testing, which identifies the presence of the virus regardless of its activity, HPV mRNA testing specifically detects the expression of E6 and E7 oncogenes from hrHPV types, which are directly involved in the process of carcinogenesis. This targeted approach provides a more precise identification of women at higher risk of developing cervical lesions or cervical cancer, as it indicates that the virus is actively contributing to cellular changes. Consequently, mRNA testing can help clinicians prioritize patients who require more immediate and intensive follow-up, such as colposcopy or biopsy, while reducing unnecessary procedures for those with transient infections that are unlikely to progress.[33 - 36]

1.1.6.2 DNA-methylation markers for triage of screen positive women

DNA-methylation is an epigenetic mechanism that occurs by the addition of a methyl group (CH_3) to cytosine in the dinucleotide 5'-CpG-3'. This mechanism is mediated by a family of methyltransferases. Most CpG dinucleotides in the human genome are methylated, however, unmethylated clusters of CpGs are found in the promoter region of many genes. These are termed CpG islands. DNA-methylation plays an essential role in biological processes including gene expression and cell differentiation, amongst others. [37] Abnormal methylation of CpG islands of promoter regions of tumour suppressor genes is common in various cancers.

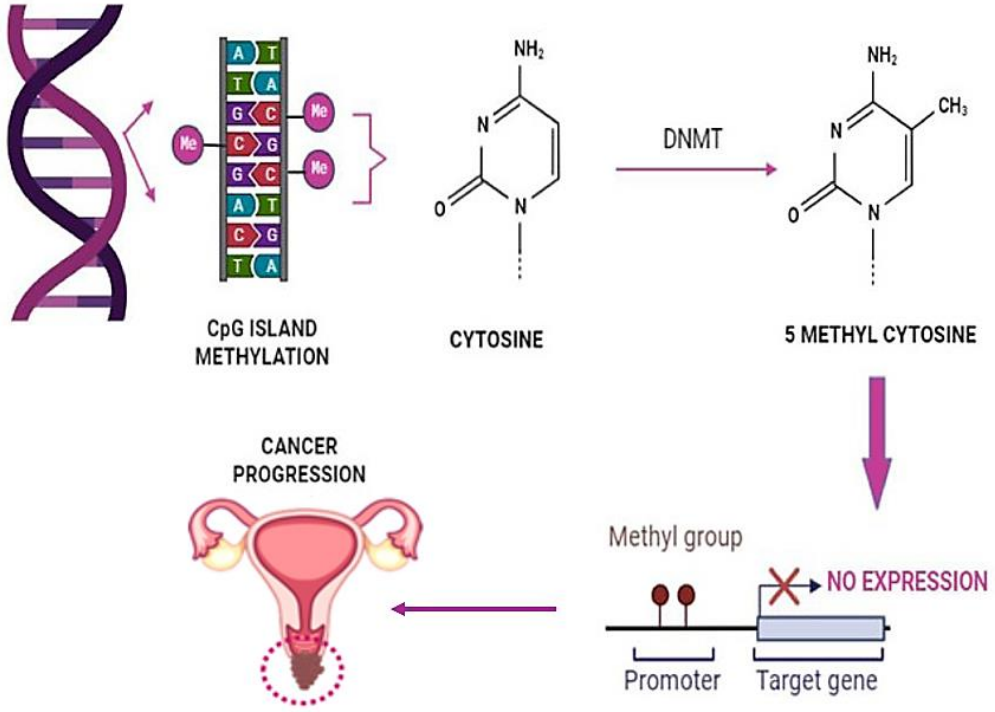


Figure 1.6 DNA methylation-mediated silencing of tumour suppressor genes. Figure adapted from Ragavi et.al. [38]

Analysis of host cell DNA methylation patterns could thus be considered a candidate cervical precancer triage test since it is able to identify clinically meaningful HPV associated cervical lesions. More than 100 human methylation biomarker genes have been evaluated and identified in cervical tissue. Approximately 10 have been repeatedly shown to have elevated methylation in cervical cancers and CIN2+, specifically CADM1, EPB41L3, FAM19A4, MAL, miR-124, PAX1 and SOX1. [39]

Figure 1.7 Relationship between hypermethylation of promotor regions in tumour suppressor genes and cervical intraepithelial lesions. Adapted from Saavedra et al. [40]

DNA methylation testing has several advantages over cytology and immunohistochemical p16INK4a/Ki-67 staining. The molecular basis of DNA methylation makes the test automatable, reducing the likelihood of interpretational errors that are more common with morphological tests. [41] Additionally, DNA methylation testing can be performed on the same sample used for HPV DNA testing, whether collected by a healthcare worker or self-collected, which simplifies the sample collection process. [41] This test identifies epigenetic changes directly associated with carcinogenesis, offering higher specificity and predictive value for identifying women at risk of developing cervical cancer.

Moreover, there is currently no recommendation for using HPV mRNA testing in WLWH due to a lack of evidence on its effectiveness in this population. The second edition of the WHO guidelines for screening and treatment of cervical pre-cancer lesions highlights the very low-certainty evidence comparing the performance of HPV mRNA tests between health-care provider-collected and self-collected samples. Current data suggest that HPV mRNA testing is less reliable with self-collected samples. Although these guidelines address screening specifically, the same principles can be applied to triage. Given these considerations, DNA methylation testing emerges as a preferred method due to its consistent performance across different sample collection methods and populations.[42,43]

1.2 AIMS AND OUTLINE OF DISSERTATION

The aim of this PhD project is to describe the multi-faceted journey of the secondary prevention of cervical cancer. This was a broad-based PhD project with components of different research methodologies built into it around a central theme. This included components such as widely implemented primary screening tests with a focus on Low- and Middle-income Countries (LMIC), newer generation screening tests, triage tests for HPV DNA screen positive women, and the implementation of thermal ablation for the treatment of cervical precancer.

Chapter 1 is a narrative literature review focusing on the epidemiology and aetiology of cervical cancer, as well as the primary and secondary prevention of the disease. Chapter 2 is a comprehensive review that provides an in-depth examination of the development and present status of cervical cytology in South Africa. It outlines the achievements and systemic

obstacles encountered. This chapter stresses the essential need for a robust infrastructure and effective follow-up mechanisms. Moreover, it draws attention to the valuable insights gained from cytology that can guide and enhance the adoption of HPV DNA screening in South Africa. Chapter 3 focuses on the performance of older screening technologies in the South African setting, specifically VIA and VILI. Chapter 4 is a focused narrative review on the implementation of HPV DNA testing as a primary screening tool in South Africa as well as other countries in the Southern African Development Community (SADC) region and addressed the possible barriers of implementation into national screening programmes. In chapter 5, we evaluate the triage capability of the QIAsure assay for the detection of biomarkers FAM19A4 and hsa-mir124-2 in hrHPV positive women (HPV 16 and HPV 18 negative). Chapter 6 is a study protocol for a randomized trial comparing the cure rate and effects of thermal ablation to those of LLETZ in treating cervical precancer. Chapter 7 is a general discussion based on the findings of the PhD project. This chapter also examines the then-current South African cervical cancer screening policy and recommended evidence-based amendments for the screening pathway.

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CHAPTER 2

Cervical cytology in South Africa: Aiming for the impossible?

2.1 INTRODUCTION

2.1.1 The burden of cervical cancer in South Africa

Cervical cancer remains a significant public health challenge in South Africa, where it is the leading cause of cancer-related deaths among women. Despite advances in global cancer prevention and screening, South Africa continues to face disproportionately high cervical cancer incidence and mortality rates. In 2023, an estimated 10 702 women were diagnosed with cervical cancer in South Africa, with 5 870 women dying from the disease.[1] This is particularly concerning given that cervical cancer is largely preventable through effective screening and vaccination. In 2022, the age-standardized incidence rate of cervical cancer in South Africa was reported at 35.3 per 100 000 women, with an age-standardized mortality rate of 18.9 per 100 000. [2] These statistics highlight the ongoing struggle to achieve a significant reduction in cervical cancer through cytology-based screening alone.

Cervical cytology, primarily through the Papanicolaou (Pap) smear test, has been a cornerstone in the prevention and early detection of cervical cancer. Despite its proven efficacy in reducing cervical cancer incidence and mortality in high-income countries, [3–6] the same success, however, has not been uniformly replicated in low- and middle-income countries (LMICs), such as South Africa. [7]

2.1.2 Cervical cancer screening coverage in South Africa

The South African government, responding to the World Health Organization (WHO) Noncommunicable Diseases (NCD) Country Capacity Survey in 2019, indicated that the national cervical cancer screening programme had a coverage of "more than 50% but less than 70%".[8] Despite these seemingly positive figures, a deeper look reveals significant gaps. Denny reports that over the past 5 years, approximately 50% of women over the age of 30 have been screened with conventional and liquid-based cytology (LBC) across South Africa's nine provinces, with coverage ranging widely from around 32% to 64%.[9]

However, the effectiveness of these efforts is undermined by critical challenges. One major issue is the inability to assess the proportion of screened women who have received appropriate diagnosis and management according to national guidelines. This is largely due

to the National Health Laboratory Services' (NHLS) current inability to link cytology results with histology, which is essential for confirming diagnoses and guiding treatment.[9] Without this linkage, the screening programme cannot fully ensure that women who test positive receive the necessary follow-up care, potentially diminishing the impact of the screening efforts.

2.2 CHALLENGES IN SOUTH AFRICA'S NATIONAL SCREENING PROGRAMME

2.2.1 The multifaceted challenges of cervical cancer screening in South Africa: a story informed by real experiences.

Meet Laura, a 30-year-old woman preparing for her first Pap smear at Hilltop Community Clinic. At this age, she qualifies for the national cervical cancer screening programme initiated by the South African National Department of Health in 2000, which aims to provide the general population of women with three Pap tests per lifetime at 10-year intervals and women living with human immunodeficiency virus (HIV) (WLWH) with three Pap smears per lifetime at three-year intervals, starting at age 25. Knowing about the long queues at the clinic, she sets her alarm for 03h00, hoping to arrive early and be first in line. Despite leaving home at 04h00 for the 50-minute trek, she finds about 50 people already ahead of her when she arrives.

Laura waits anxiously as the hours pass by, sharing a sense of unease and frustration with others in the clinic, many of whom are grappling with a range of health issues including HIV/Acquired Immunodeficiency Syndrome (AIDS), tuberculosis (TB), hypertension, cardiovascular diseases, and diabetes. The clinic is overcrowded, with worn-out chairs and no relief from the uncomfortable heat due to the lack of air conditioning. On the walls are faded, outdated health posters, and the noise of crying children heightens the already tense atmosphere.

By the time Laura's name is called by Sr. Curie, it is well past noon. Sr. Curie, already drained from a challenging morning and running on only a few hours of sleep, tries to smile and be encouraging. Despite her best efforts, the strain is evident in her voice and demeanour.

Laura is ushered into a small examination room and observes that the equipment appears dated, with the examination beds covering showing signs of wear. Sr. Curie explains the procedure, but Laura struggles to understand due to her limited medical knowledge and Sr. Curie's rushed explanation. Intimidated and embarrassed, Laura does not ask the questions she had prepared.

Before the examination begins, Sr. Curie ensures that the specimen collection materials are accurately labelled, and the laboratory request form is thoroughly completed (this step is crucial for the smooth processing of the sample).

During the examination, Sr. Curie works quickly but gently. Still, Laura feels vulnerable, exposed, and uncomfortable. After the procedure, Sr. Curie tells Laura to expect the results in a few weeks, but Laura has heard stories from other women about delays.

The journey of Laura's specimen to the laboratory presents some challenges. Specimen collection intervals vary between clinics, which can sometimes delay the initial transport. When the specimen arrives at the laboratory, the cytotechnologists often face a backlog of tests. Despite the laboratory being well-equipped, a shortage of specialized staff means that each specimen may not always receive the thorough attention it requires, occasionally leading to errors in labelling and processing.

After months of waiting, Laura receives her results; to her despair, she has a high-grade lesion. She does not fully understand what it means, but the fear of it potentially being cancer overwhelms her. She needs an assessment at a colposcopy clinic at a referral hospital for further management and treatment. However, the long waiting lists leave her feeling anxious and uncertain as she faces prolonged delays in an overburdened healthcare system.

Despite good intentions, the cervical cytology screening programme struggles with systemic issues. Clinics are understaffed and underfunded, resulting in long waiting times and overworked healthcare providers. The laboratory system, while doing its best under difficult conditions, still faces challenges that can lead to delays and occasional errors. Additionally, patient factors such as fear, lack of knowledge about cervical cancer, and limited understanding of the importance of regular screening contribute to low screening uptake

and follow-up. These challenges, combined with gaps in communication, often result in women not following up or missing the care they need.

2.2.2 Programme related issues

2.2.2.1 *Laboratory*

A study conducted by Makura et al. revealed significant gaps in the implementation of South Africa's national cervical cancer screening programme. Analysing 791 067 cytology slides submitted by 4 562 facilities between April 2013 and March 2014, the study found that 44% of Pap smears were from WLWH, and 53% were from the general population of women aged 30 and older. The median Pap smear coverage rate for women aged 30 and older was only 33%, with 44 out of 52 districts falling below 50% coverage and only three districts achieving at least 70% coverage. For WLWH aged 25 and older, the median coverage was 31%, with 44 out of 52 districts below 50% coverage and only five districts reaching the 70% target. Additionally, the median adequacy rate of the smears was only 47%, due to factors such as missing endocervical cells, broken slides, unreadable or missing labels, and specimens that were bloody or obscured.[10]

2.2.2.2 Programme organisation

The Cervical Health Implementation Project, conducted by Moodley et al. from January 2001 to May 2003, aimed to improve public sector cervical cancer screening services in South Africa. The study involved developing and evaluating health system interventions, such as health worker training, health system tools and protocols, and a community awareness programme. Despite improvements in service organization, availability of screening equipment, and education materials, cytology turnaround times remained lengthy, and only 50% of women with high-grade squamous intraepithelial lesions (HSIL's) received colposcopies and biopsies within six months of a Pap smear. While the number of new smears performed increased from 1 544 in 2001 to 2 801 in 2002, overall coverage remained below 4%.[11]

A retrospective study by Blanckenberg et al. examined the uptake of colposcopy in a rural South African sub-district using data from 2007 and 2009. The study found that in 2009, 14% (43/310) of women who had cervical smears were not informed of their results,

primarily because they were not seen again. In some cases, women were seen again, but healthcare workers failed to note the results due to them either not being in the file or being overlooked. Notably, women with HSIL's or suspected cancer on cytology were not scheduled for colposcopy. Most of these patients were either loss to follow-up (LTFU) (15/23) or known to have died (3/23). However, five patients remained in care with cytology results that were either never actively sought by the staff or were in the file but not acted upon.[12] The District Health Information System (DHIS) is intended to monitor and evaluate clinic activities, but inadequate data management and follow-up systems hinder effective tracking and management of screened individuals.

A 2016 review article by Jordaan et al. on the evolution of cervical cancer screening in South Africa highlights several factors contributing to the failure of the national screening programme, including a shortage of healthcare workers, lack of equipment, LTFU of patients with abnormal cytology, and insufficient awareness programmes. Additionally, the scarcity of colposcopy and large loop excision of the transformation zone (LLETZ) facilities has resulted in prolonged waiting lists for patients with abnormal Pap smear results, sometimes extending up to 18 months. The authors emphasize that for screening programmes to be truly successful, it is essential not only to achieve adequate coverage but also to ensure that effective treatment is provided for women who test positive.[7]

2.2.2.3 Patient related: Access and awareness

Despite progress in improving healthcare access in South Africa, significant disparities persist, especially in rural areas. Many women in these regions face challenges in accessing regular healthcare services, which hampers routine screening efforts. Dr. Nwabisa Giyose, Head of the Gynaecology Oncology Unit at Nelson Mandela Academic Hospital in Mthatha, highlights that women in rural areas often need to travel long distances to reach the nearest primary healthcare facility. This journey incurs costs, which can be burdensome in poverty-stricken communities where financial resources are already stretched thin due to other pressing needs, such as purchasing food and caring for children.[13]

Lack of awareness and education about cervical cancer and its screening remains a significant barrier to effective prevention. Cultural beliefs and stigma further discourage

women from seeking screening. A hospital-based cross-sectional study conducted by Godfrey et al. investigated knowledge, attitudes, and adherence to Pap smear screening, analysing 234 responses with a mean age of 29 years (SD = 8.3 years). The study revealed that only 32.5% of women had previously undergone a Pap smear, with 33.3% being WLWH and 53.0% HIV-negative. Alarmingly, only 19.2% of women recognized that a Pap smear is used for cervical cancer screening. The findings highlight a widespread lack of understanding about cervical screening, contributing to the low uptake of Pap smears, especially among WLWH who are at greater risk for cervical cancer. [14] Furthermore, research has shown that health education programmes can significantly enhance knowledge about cervical cancer and improve screening uptake. Women with prior knowledge of cervical screening are more likely to seek screening services compared to those without such knowledge. [15–17]

2.2.3 The effect of high HIV Prevalence

In South Africa, the high prevalence of HIV significantly complicates the management of cervical cancer. Women living with HIV face an elevated risk of cervical cancer due to their immunocompromised status, which can influence both the progression of the disease and the effectiveness of treatment interventions. [18,19] The intersection of HIV and cervical cancer introduces additional challenges, including inconsistent follow-up care and increased risk of treatment failure. Even when abnormalities are detected through screening, delays in accessing essential follow-up services such as colposcopy, can lead to disease progression. For instance, waiting times for colposcopy appointments can vary widely, ranging from one to eight months. [7,12,20] These delays exacerbate the challenges faced by WLWH, underscoring the need for a more efficient and responsive screening and treatment infrastructure. Addressing these systemic issues is crucial for improving outcomes and ensuring that WLWH receive timely and effective care.

2.2.4 Limitations of cervical cytology in screening (test characteristics)

Cervical cytology suffers from relatively low sensitivity, resulting in false negatives. This limitation means that many women with precancerous lesions might not be detected in time for effective intervention. A meta-analysis by Nanda et al., which reviewed 97 studies,

found that cervical cytology, even when performed under optimal conditions, has only moderate accuracy. The study revealed that the average sensitivity for detecting cervical cancer or precancerous lesions was notably lower than commonly perceived, approximately 51% (with a range of 30% to 87%). [21] A 2006 study reviewing European and North American research on human papillomavirus (HPV) testing in primary cervical cancer screening, which included data from over 60 000 women, found an overall sensitivity of cytology of 53.0% (ranging from 48.6% to 57.4%) for detecting cervical intraepithelial neoplasia grade 2 (CIN2+). However, sensitivity varied significantly, from as low as 18.6% in a German study to as high as 76.7% in a British study. [22] In a South African study involving 903 women, Snyman et al. reported that the sensitivity of cytology was 48.6% for detecting CIN2+ and 62.9% for CIN3+. [23] Given that cytology has only moderate sensitivity, the observed reduction in cervical cancer risk in developed countries is likely attributed to frequent screening. Cervical neoplasia progresses slowly, with many low-grade precancerous lesions either regressing spontaneously or remaining stable. High-grade lesions that might be missed in one screening cycle are more likely to be detected in subsequent cycles if screening is performed regularly.[24] While more frequent cytology screenings can enhance sensitivity and improve detection rates, implementing such programmes is challenging in low-frequency screening environments.

2.2.5 Improvements in cytology test characteristics

2.2.5.1 Liquid-based cytology

Liquid-based cytology has been developed as an alternative for conventional cervical cytology and involves collecting cells from the cervix and suspending them in a liquid medium. This method improves sample preservation and reduces the presence of obscuring elements such as blood and mucus, leading to clearer and more representative samples. Despite numerous studies and systematic reviews, there is still debate about its diagnostic accuracy.

A cluster randomized controlled trial by Siebers et.al., involving 89 784 women aged 30 to 60 years within the Dutch cervical screening programme was conducted across 246 family practices. Of these, 122 practices used LBC to screen 49 222 patients, while 124 practices

used the conventional Pap test to screen 40 562 patients between April 2004 and July 2006. Follow-up continued for 18 months until 31 January 2008. The adjusted detection rate ratios were 1.01 (95% confidence interval [CI], 0.85-1.19) for CIN1+, 1.00 (95% CI, 0.84-1.20) for CIN2+, 1.05 (95% CI, 0.86-1.29) for CIN3+, and 1.69 (95% CI, 0.96-2.99) for carcinoma. The adjusted positive predictive value (PPV) ratios, evaluated at different cytological thresholds and for various CIN outcomes, did not show significant differences. The study concluded that LBC does not offer superior performance compared to conventional Pap tests in terms of relative sensitivity and PPV for detecting cervical cancer precursors. [25]

In a randomized study conducted in South Africa, where all women underwent colposcopy at the time of screening, no significant differences were found between LBC and conventional cytology regarding sensitivity or PPV for identifying women with CIN2+. At an atypical squamous cells of undetermined significance (ASC-US) cutoff, LBC had a sensitivity of 71% (95% CI, 58%-81%), while conventional cytology had a sensitivity of 84% (95% CI, 71%-92%). The PPV for LBC was 9.4% (95% CI, 7%-12.3%), compared to 11.4% (95% CI, 8.5%-15.0%) for conventional cytology. [26]

However, LBC has logistical and operational advantages (interpretation at higher speed, lower rate of unsatisfactory smears and possibility of HPV co-testing using remnant fluid) but is more expensive and is neither more sensitive nor more specific than conventional cytology with respect to detection of histologically confirmed high-grade CIN. Liquid-based cytology techniques, such as ThinPrep® and BD SurePath™, improve the preservation and uniformity of cell samples, reducing the number of unsatisfactory slides.

2.2.5.2 Automated cytology systems

Systems like the ThinPrep Imaging System (Hologic Inc.) and FocalPoint GS imaging system (FPGS; BD Diagnostics, Burlington, NC) use algorithms to pre-screen slides and highlight areas of interest. This technology helps cytopathologists identify abnormal cells and can reduce human error, potentially enhancing both sensitivity and specificity in detecting cervical abnormalities.

Studies have investigated the use of these AI technologies in cervical cancer screening.[27–30] However, their implementation is primarily seen in high-income countries due to factors

such as cost, infrastructure, and training requirements. The use of such advanced technologies in South Africa and similar LMIC's is limited. This limitation is often due to budget constraints, lack of infrastructure, and other logistical challenges that make it difficult to adopt and maintain these technologies in resource-constrained settings.

2.2.5.3 p16/Ki67 dual staining in cervical cytology

The combination of p16, a marker of HPV-related transformation, and Ki-67, a proliferation marker, in the same cell has been shown to improve the sensitivity of cytology. The presence of both markers in a single cell suggests higher risk of significant cervical pathology, enhancing the predictive value of cytology.

A large prospective study involving 27 349 women undergoing routine cervical cancer screening across five European countries was conducted. All participants underwent Pap cytology, HPV testing, and p16/Ki67 dual staining cytology. Women with ASC-US or higher on Pap cytology, positive p16/Ki67 dual staining, and/or a positive HPV test were referred for colposcopy. The diagnostic performance of these tests for detecting CIN2+ during colposcopy was assessed. The sensitivity of dual staining was found to be higher than that of the Pap test, while its specificity was similar. However, in women younger than 30 years, the sensitivity of dual staining was lower compared to HPV testing. Additionally, the study showed that dual staining had 50% fewer false-positive results than HPV testing, suggesting that it provides a more effective triage for colposcopy. [31]

2.3 FROM CYTOLOGY TO HPV DNA SCREENING: WILL THIS SHIFT ACTUALLY MAKE A DIFFERENCE?

With South Africa set to implement HPV deoxyribonucleic acid (DNA) testing as the primary method for cervical cancer screening, it is essential to assess whether the objectives are feasible within the country's unique context. HPV DNA testing represents a significant advancement over traditional cytology due to its superior sensitivity,[32–35] making it a more effective tool for the early detection of high-grade lesions and cervical cancer. This method has a high negative predictive value (NPV) nearing 100%, which allows for safe longer intervals between screenings for those with negative results. [36] As a result, HPV testing

reduces the need for frequent follow-up visits and additional tests, ultimately proving to be more cost-effective compared to cytological screening.[37,38]

Self-sampling is a significant advantage of HPV testing, especially in underserved areas. By allowing individuals to collect their own samples, this method eliminates the need for a speculum examination, which is often a barrier to participation due to discomfort or logistical challenges. Self-sampling can also reduce the long waiting lines at clinics, as individuals can perform the sample collection at their convenience and without the need for an immediate clinical visit. This approach has the potential to boost participation rates when accompanied by effective public education and easy access to self-sampling kits.[39]

Automation and high-throughput HPV testing improve efficiency by processing large volumes of samples at once. This technology also speeds up turnaround times, providing faster results. Though initial investments are high, the long-term benefits include lower human resources costs and the ability to manage many samples at once. Additionally, the development of point-of-care testing options can further streamline the process, providing immediate results and facilitating on-site diagnosis and treatment, which is especially beneficial in remote or underserved areas.[40]

Using HPV DNA testing as a primary screening method often requires a follow-up triage test to identify clinically relevant infections, especially when positive results are for non-16/18 HPV types. Many HPV infections are transient and only a small percentage are linked to cervical abnormalities, so HPV DNA testing alone may not provide sufficient clinical information. In such instances, reflex cytology could be offered to increase the specificity of screening for women with non-16/18 HPV types. This approach enhances programme effectiveness compared to treating all positive cases or following all women with positive results.[41,42]

Robust monitoring and evaluation systems are essential to ensure the effectiveness of the screening programme. Implementing these systems requires investment in data management, continuous feedback mechanisms, and enhanced call and recall systems to track and follow up with patients effectively. Utilizing a multi-channel communication approach, such as short message service (SMS), phone calls, emails, and postal notifications,

can improve patient engagement and ensure they receive their results. Involving Community Advisory Boards (CAB) can also enhance outreach efforts and ensure that communication strategies are culturally appropriate and effective. With appropriate support and strategic planning, these components are achievable and crucial for maintaining the quality and impact of the screening programme.

2.4 CONCLUSION

The extensive experience and insights gained from the implementation of cervical cytology in South Africa have provided valuable lessons that can significantly inform and enhance the adoption of HPV DNA screening in the country. The challenges encountered, such as systemic obstacles, long waiting times, and low screening uptake, have highlighted the need for a more effective and efficient screening approach. As South Africa transitions to HPV DNA screening, these lessons are crucial in shaping a strategy that addresses existing gaps and maximizes the potential of the new technology.

To optimize the implementation of HPV DNA screening in South Africa, it is essential to apply the key lessons learned from the cytology-based programme. Ensuring that *healthcare staff are well-educated and motivated* is vital for maintaining high standards and effective programme delivery. Developing *robust laboratory quality control management systems*, *establishing clear results pathways*, and *maintaining good turnaround times* are critical for ensuring accuracy and reliability. Strong *links to care*, including *effective referral systems* and *timely follow-up*, are necessary to address the needs of patients. Additionally, enhancing *public education* about the benefits and importance of cervical cancer screening will help increase participation and compliance. By integrating these elements, South Africa can address the challenges faced in the previous screening programme and create a more effective and comprehensive HPV DNA screening strategy.

While HPV DNA testing represents a significant advancement, cervical cytology still has a role to play in South Africa's screening framework. Cytology can be utilized as a reflex triage test for women who test negative for HPV16/18 but have other oncogenic HPV types. This approach allows for continued use of cytology in conjunction with HPV testing, ensuring comprehensive screening and follow-up.

By leveraging our understanding of the limitations and successes of cytology, South Africa can better design and implement an HPV DNA screening programme that overcome previous barriers, improve patient participation, and ensure timely follow-up and treatment. This knowledge not only guides the refinement of screening processes but also highlights the importance of robust infrastructure, effective communication, and community involvement in achieving a more impactful and equitable cervical cancer prevention strategy in South Africa.

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CHAPTER 3

Visual inspection using naked eye and colposcopy as predictor of high-grade lesions on final histology in HIV-positive and negative South African women.

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https://doi.org/10.36303/SAJGO.374

3.1 ABSTRACT

Background: Although potentially preventable, cervical cancer is the fourth most common cancer among women globally and a leading cause of cancer-related deaths. Women living in resource-limited countries are especially at risk due to poor access to cervical cancer screening and treatment. Alternative cervical cancer screening methodologies have been investigated where cytology-based screening is not feasible. This study aimed to assess the test performance of naked eye visual inspection analysis, in addition to the comparative performance of physician/colposcopist clinical impression to the RCI grading system for histopathology, in the South African setting.

Methods: WLWH and HIV negative women aged 25 to 65 were recruited from three sites in South Africa. A cross-sectional study which assessed VIA, VILI, colposcopic impression and RCI for the detection of histologically confirmed CIN2+ and CIN3+ was performed. Test positivity rates, sensitivity, specificity, and predictive values were calculated.

Results: 344 WLWH and 409 HIV negative women, with a median age of 40 years were included in this analysis. 38.51% of women had a histologically confirmed CIN2+ and 18.99% CIN3+. Overall, positive test rates for VIA were 42.76%; VILI, 45.68%; colposcopic impression, 48.26% and RCI, 46.65%. Overall sensitivities/specificities for VIA and VILI for CIN3+ were 76.92%/65.25% and 75.52%/61.31%, respectively. The sensitivities, however, increased for WLWH (VIA, 82.61%; VILI, 80.43%) and decreased in HIV negative women (VIA, 66.67%; VILI, 66.67%). Colposcopic impression/RCI performed better in WLWH (PPV, 37.96%/37.74%) than in HIV negative women (PPV, 25.63%/26.80%).

Conclusion: The current study demonstrates that visual inspection methods perform better in WLWH than in HIV negative women. VIA and VILI performed similarly within each sub-population, as did colposcopic impression and RCI. The use of visual inspection methods in cervical cancer screening in WLWH is warranted.

Keywords: cervical cancer, visual inspection with acetic acid (VIA), visual inspection with Lugol's iodine (VILI), colposcopic impression, Reid's colposcopic index (RCI)

3.2 BACKGROUND

Cervical cancer is the fourth most frequently diagnosed cancer among women globally and the fourth leading cause of death. The GLOBOCAN estimates for 2020 indicate that approximately 604 127 women are diagnosed with cervical cancer annually, while 341 831 die from the disease.[1] The highest regional incidence and mortality is in Sub-Saharan Africa, with rates elevated in Eastern Africa, Southern Africa, and Middle Africa. In South Africa, cervical cancer is the second most common cancer diagnosed among women and the commonest cancer among women in the reproductive age group 15 to 49 years. [2] Estimates for 2020 were reported at 12 333 incident cases (age standardized incidence rate 36.4/100 000) and 6 867 deaths (age standardized incidence rate 20.6/100 000). These alarming figures are particularly tragic given cervical cancer is a potentially preventable disease. Successfully organised national cervical cancer prevention programmes have not yet been implemented in most developing countries, due to reasons such as poverty, competing funding priorities, low prioritization of cervical cancer and cultural practices.[3, 4]

South Africa launched a national screening programme for cervical cancer prevention in 2000, offering three Papanicolaou (Pap) smears per lifetime starting after the age of 30 with 10-year intervals for HIV negative women and three-year intervals for women living with HIV (WLWH). This national screening programme however has not been implemented widely in the public sector. Screening programmes based on Pap smears require technical capabilities and systems for training, effective communication, follow-up visits and transportation that are sometimes beyond the capacity of healthcare infrastructure in parts of South Africa. While cytology has reduced the cervical cancer incidence, cases of cervical cancer still occur due to the wide variability of the sensitivity of the screening method.[5,6] Consequently, over referrals and over treatment often occurs. Thus, other methods of cervical-cancer screening provision have been investigated.

Several other screening tests are currently available for detecting pre-invasive cervical lesions. The most recent option is the direct detection of the human papillomavirus (HPV) in cervical specimens by target DNA amplification using Polymerase Chain Reaction (PCR). The known cause of cervical precancer and cancer is persistent infection with HPV.[7] HPV is one of the most prevalent sexually transmitted infections worldwide, with infection with at least one

strain of HPV occurring in approximately 70 - 80 % of sexually active women during their lifetime.[8]

HPV DNA testing is the most sensitive test for cervical cancer screening and has a negative predictive value (NPV) approaching 100% allowing for an increase in the screening interval for negative tests. As a result, fewer follow-up visits and additional tests are required, making it more cost effective than cytological testing. [9] The use of HPV DNA testing as a primary screening method, however, requires the use of a triage test to identify women with clinically relevant infections, as many of the detected infections are transient, and only a minority are associated with cervical abnormalities. HPV DNA testing in isolation may therefore not be clinically meaningful.[10]

In South Africa, HPV testing is only offered in the private sector and is not yet widely available in the public sector as part of the national screening programme. This method of screening also requires specialised equipment.[5] Women who test positive for HPV16 and HPV18 (often referred to as highest risk HPV) are referred for a colposcopy and biopsy. Women who test positive for "non-highest risk" HPV or a non-discriminate test are considered medium or intermediate risk. A test is thus needed to determine the need for referral to colposcopy or other follow-up procedures.[11]

Another screening option involves searching visually for macroscopic cervical precancerous lesions with the application of diluted acetic acid (3 – 5%) (VIA) and/or Lugol's iodine (VILI). The purpose of this screening technique is to identify acetowhite areas for VIA and areas of iodine non-uptake (non-staining) (areas in the form of pale or yellowish-white areas, particularly in the transformation zone, close to the squamocolumnar junction) for VILI, which may indicate tissue undergoing precancerous changes. Screening with VIA and/or VILI is advantageous given the procedure is inexpensive, relatively straightforward, can be performed by non-physician healthcare workers and results are available immediately allowing for the implementation of a screen-and-treat approach. It is however important to note that VIA and/or VILI may require rigorous, standardized training and continuous quality assurance since it is subjective and dependent on expertise. While many researchers question the relevance of visual inspection in the cervical cancer screening paradigm, the South African Cervical Cancer Prevention and Control Policy proposes to offer VIA as the screening approach in resource constrained regions pending the national scale up of liquid-based cytology and

the roll out of HPV testing.[5] Additionally, many non-governmental organizations and governments around the world continue to support VIA and/or VILI because of the low cost and low technological demand and the fact that results are available immediately making screen-and-treat possible.[12–14]

A large cluster-randomized trial by R. Sankaranarayanan *et al.,* assessing the effect of visual screening on cervical cancer incidence and mortality in India, reported a significant 25% reduction in cervical cancer incidence (hazard ratio 0·75 [95% CI 0·55 – 0·95]) and a significant 35% reduction in cervical cancer mortality (hazard ratio 0·65 [0·47 – 0·89]) in 49 311 women screened with a single round of VIA. Their findings indicate that VIA is a simple, feasible, and effective method to prevent cervical cancer and death among deprived populations in developing and developed countries.[15]

Colposcopy is an advanced method of visual inspection that allows a detailed assessment of the cervix, following a positive screening test result. As described in the manual for Colposcopy and Treatment by the International Agency for Research on Cancer (IARC), a colposcopic examination includes: 1) the assessment of the cervix with low- and high magnification of at least 6 – 15 ×, 2) the assessment with acetic acid and/or Lugol's iodine and 3) the assessment with white and/or green light. Various quantitative scoring systems are available. For the purpose of this study, we chose to evaluate colposcopic impression and the Reid's Colposcopic Index (RCI). The RCI is a systematic, objective method of colposcopically grading the severity of premalignant cervical lesions. The index considers four colposcopic signs: lesion margin, colour of acetowhitening, blood vessels, and iodine staining.[16]

This study aimed to assess the test performance of naked eye visual inspection analysis in the South African setting where data is limited, in addition to the comparative performance of physician/colposcopist clinical impression to the RCI grading system for histopathology.

3.3 MATERIALS AND METHODS

3.3.1 Design, setting and population.

The DiaVACCS study (ethics approval ref no 196/2014) was a cross-sectional cohort study in which women between the ages of 25 and 65, unscreened in the preceding five years, were recruited from three sites in South Africa from December 2016 to March 2020. The study

design, methodology and basic descriptive data have previously been described. [17] The aim of this study was to evaluate the performance of screening tests in general female and HIV-infected populations.

The current study comprised 753 women, of which 344 were HIV positive. Women with unknown HIV status were recruited from the general population, and HIV positive women from adult antiretroviral treatment (ART) clinics. All women were included in the VIA and VILI analysis. Of the 753 women, seven had missing colposcopy data and were excluded from the colposcopic impression and RCI analysis.

3.3.2 Visual inspection without magnification

During the speculum exam, 3 – 5% acetic acid was applied to the cervix using forceps and a cotton ball. Lesions were described based on size in quadrants, grade per colour and transformation zone seen. The presence of other lesions, such as warts and ulcers were also noted. Sharp, distinct, and well defined acetowhite areas were considered a test positive. Following acetic acid application, Lugol's iodine was liberally and gently applied to the cervix using forceps and a cotton ball. Non-staining areas were considered a test positive.

3.3.3 Colposcopy

A colposcopy was performed after the application of 3 – 5% acetic acid and before and after the application of Lugol’s iodine. The colposcopist recorded a clinical impression of the character of the lesion based on size in quadrant, acetowhitening, iodine uptake, vessels, and margin/surface of the lesion. The clinical impression was reported as either negative, low-grade, or high-grade, with both low-grade and high-grade considered test positives. The RCI was then formally calculated based on the character of the lesion. An RCI score of 5 and above was considered positive.

3.3.4 Biopsy and histology processing

All visually detected lesions were biopsied. If no lesions were seen in a cervical quadrant, a random biopsy was obtained at the squamocolumnar junction in that quadrant. Two biopsies were obtained per participant. Histology examination was performed by an experienced histopathologist. In women who underwent large loop excision of the transformation zone

(LLETZ) treatment, the worst histology between the biopsy and LLETZ was taken as final diagnosis.

3.3.5 Statistical analysis

Microsoft Excel was used as the primary software for the data analysis process. VIA, VILI, and colposcopy results were calculated by age group, HIV status and overall. The performance on the sensitivity, specificity, negative and positive predictive values were calculated with a gold standard of histology CIN2+ and CIN3+. Missing histology data were imputed based on age, HIV status, ARV use and other screening test results. Continuous variables were summarized using means and standard deviations. Categorical variables were summarized using percentages. Results were summarised in tables with two-sided confidence intervals calculated based on a 95% t-distribution. Significance level was set at 5%.

3.4 RESULTS

The median age of women was 40 years (IQR 34 – 48). A total of 344 (45.68%) were WLWH and 409 (54.32%) were HIV negative. CIN1+ was diagnosed in 504 (66.93%) women, CIN2+ in 290 (38.51%) women, CIN3+ in 143 (18.99%) women and 15 (2%) were screen-detect cervical cancers (Table 3.1). Overall, the positivity rate for VIA was 322 (42.76%) and for VILI was 344 (45.68%). The positivity rates for both visual inspection screening tests were significantly higher in WLWH than in HIV negative women (P < 0.00001), with the highest observed in VILI (62.21%). The rate of screen positives in WLWH was almost double than that of HIV negative women (Table 3.2).

Table 3.1 Histology results amongst WLWH and HIV negative women.

	WLWH n = 344		HIV neg n = 409		Total N = 753		p value
Histology	Count	%	Count	%	Count	%	
Negative	95	28.02%	141	36.53%	236	31.34	P = 0.01309
CIN2+	170	50.15%	120	31.09%	290	38.51	P < 0.00001
CIN3+	92	27.14%	51	13.21%	143	18.99	P < 0.00001
Cervical Cancer	9	2.65%	6	1.55%	15	1.99	P = 0.25281
Other	1	0.29%	1	0.26%	2	0.27	

Table 3.2 Percentage screen positives for visual inspection cervical cancer screening methods.

Screening Visual Inspection Method	WLWH n = 344		HIV neg n = 409		Total N = 753		p value
	n	%	n	%	n	%	
VIA	204	59.30	118	28.85	322	42.76	P < 0.00001
VILI	214	62.21	130	31.78	344	45.68	P < 0.00001

Overall, positive test rates for colposcopic impression and RCI were 360 (48.26%) and 348 (46.65%) respectively. The highest observed positivity rates were in WLWH for both colposcopic impression and RCI. The positivity rate for RCI in WLWH was 24.56% higher in absolute terms in comparison to HIV negative women (Table 3.3).

Table 3.3 Percentage abnormal colposcopic findings.

Diagnostic (Visual Inspection) Method	WLWH n = 344		HIV neg n = 402		Total N = 753		p value
	n	%	n	%	n	%	
Colposcopic impression	210	61.05	150	37.31	360	47.81	P < 0.00001
RCI	206	59.88	142	35.32	348	46.22	P < 0.00001

The overall sensitivity, specificity, PPV and NPV of VIA for CIN2+ was 65.17%, 71.27%, 58.70% and 76.57%, respectively. Similarly, the overall sensitivity, specificity, PPV and NPV of VILI for CIN2+ was 64.48%, 66.09%, 54.36% and 74.82%, respectively. The sensitivity of VIA to detect CIN2+ was higher in WLWH compared to HIV negative women; 76.47% and 49.17%, respectively. A similar trend was observed for the sensitivity of VILI to detect CIN2+ in WLWH, 75.88% and in HIV negative women, 48.33%.

Table 3.4 Comparative performance of cervical cancer visual inspection screening tests for histologic outcome CIN3+.

	Characteristic	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Total	VIA	76.92 [69.96 – 83.89]	65.25 [61.46 – 69.03]	34.16 [28.96 – 39.36]	92.34 [89.83 – 94.86]
	VILI	75.52 [68.42 – 82.63]	61.31 [57.44 – 65.18]	31.40 [26.47 – 36.32]	91.44 [88.72 – 94.16]
WLWH	VIA	82.61 [74.76 – 90.46]	49.21 [43.00 – 55.41]	37.25 [30.58 – 43.93]	88.57 [83.25 – 93.89]

	VILI	80.43 [72.22 – 88.65]	44.44 [38.28 – 50.61]	34.58 [28.17 – 40.99]	86.15 [80.16 – 92.15]
HIV -	VIA	66.67 [53.41 – 79.93]	76.54 [72.13 – 80.94]	28.81 [20.56 – 37.07]	94.16 [91.45 – 96.86]
	VILI	66.67 [53.41 – 79.93]	73.18 [68.58 – 77.79]	26.15 [18.53 – 33.78]	93.91 [91.09 – 96.73]

Both visual screening methods performed equally within each sub-population and are thus equally suited methods for the detection of CIN3+. The sensitivity is above 75% overall. The sensitivity of VIA to detect CIN3+ was higher in WLWH compared to HIV negative women; 82.61% and 66.67% respectively (Table 3.4).

The lowest specificity was observed for VILI in WLWH, 44.44% and the highest for VIA in the HIV negative cohort, 76.54%. The lowest PPV was observed in the HIV negative cohort, with VIA and VILI having comparative results, 28.81% and 26.15%, respectively (Table 3.4).

The overall PPV and NPV for colposcopic impression for CIN2+ was 57.45%, and 80.22%, respectively. RCI performed similarly with a PPV of 58.08% and a NPV of 79.79%. Colposcopic impression and RCI performed similarly in both sub-populations for CIN2+. The PPVs of both colposcopic tests for CIN2+ (colposcopic impression, 64.81%; RCI, 64.62%) in WLWH were significantly higher than for CIN3+ (colposcopic impression, 37.96%; RCI, 37.74 %).

Table 3.5 Comparative performance of colposcopy to Reid Colposcopic Index for histologic outcome CIN3+.

	Characteristic	PPV, % (95% CI)	NPV, % (95% CI)
Total	Colposcopic Impression	32.71 [27.96 – 37.47]	94.58 [92.26 – 96.90]
	RCI	33.15 [28.31 – 38.00]	94.23 [91.88 – 96.58]
WLWH	Colposcopic Impression	37.96 [31.45 – 44.47]	92.19 [87.49 – 96.88]
	RCI	37.74 [31.17 – 44.30]	90.91 [85.96 – 95.86]
HIV -	Colposcopic Impression	25.63 [18.81 – 32.44]	95.98 [93.53 – 98.43]
	RCI	26.80 [19.72 – 33.87]	95.98 [93.53 – 98.43]

Both methods performed equally within each sub-population. The PPVs of both colposcopic tests in WLWH were higher than for HIV negative women. Expectably, a reverse trend was observed for the NPVs, with the strongest rule-in combinations performing slightly less well in ruling out disease. All testing combinations yielded NPVs above 90%.

3.5 DISCUSSION

3.5.1 Overall test positivity rates

The current study evaluated the test performance of VIA and VILI *screening* methods and the *diagnostic* accuracy of colposcopic impression and RCI for CIN2+ and CIN3+ detection in South African women. Study findings were analysed overall and by HIV status.

Overall, we observed a notably high rate of positive test results including CIN2+, 38.51%; VIA, 42.76%; VILI, 45.68%; colposcopic impression, 47.81% and RCI, 46.22%. Test positivity rates are consistent with findings in other studies conducted in South Africa and is potentially due to a high prevalence of HPV infection, high HIV burden and an unscreened general population.[18–20]

3.5.2 Overall performance of VIA and VILI as screening tools

Overall, both VIA and VILI performed adequately at CIN2+. Performance improved at a higher disease threshold. Comparable sensitivities were observed for both tests at different disease thresholds. Our results are in line with several studies which demonstrate relatively high sensitivities, a range of specificities and low PPVs. A cross-sectional study conducted in Angola by Muwonge *et al*., evaluating the feasibility of cervical screening using VIA or VILI to detect and treat CIN found VILI to be more sensitive in detecting CIN2+ with a sensitivity and specificity of 88.0% (95% CI, 78.4% – 94.4%) and 68.9% (95% CI, 67.9% – 69.9%), respectively. The sensitivity of VIA was 70.7% (95% CI, 59.0% – 80.6%) and specificity 94.5% (95% CI, 94.0% –95.0%).[21] The clinical significance of the screening tests can be described by evaluating the predictive values. The PPVs for both screening tests were approximately 20% higher for CIN2+ than CIN3+ meaning only 34.16% and 31.40% of women screened positive with VIA and VILI respectively, truly had a confirmed CIN3+ on histology. The high NPV for CIN3+ observed for both VIA and VILI mean that 92.34% and 91.44% of women who screened negative with VIA and VILI respectively, in fact did not have a confirmed CIN3+.

3.5.3 Performance of VIA and VILI as screening tools in WLWH and HIV negative women

Positive test results were especially high in WLWH including 50.15% CIN2+, 27.14% CIN3+, 59.30% VIA, 62.21% VILI, 61.05% colposcopic impression and 59.88% RCI. Numerous studies have reported the association of HPV infection with increasing immunosuppression. WLWH have a high prevalence of HPV infection and are infected with a broader range of HPV genotypes than HIV negative women, as described by Mbulawa *et al*., in which the prevalence of HPV in South African men and women according to age and HIV status was examined. The study included 486 women and demonstrated a high HPV prevalence of 74.0 % (205/277; 95 % CI: 68.5 – 78.8 %) among WLWH.[22] A prospective cohort study conducted in Cape Town by Zeier *et al*. found that immune restoration of WLWH by the initiation of combination antiretroviral therapy (cART) significantly reduced the risk for detection of HPV by 77% [OR 0.23, 95% CI 0.15 – 0.37].[23]

Both screening methods had sensitivities higher than 75% for both CIN2+ and CIN3+ in WLWH with specificities ranging from 45% to 58%. Our HIV-positive sensitivity results appear consistent with other studies in Africa with variable specificities reported. A study conducted in Kenya by Akinwuntan *et al*., assessing the correlation of cervical cytology and VIA in 150 WLWH found VIA to have a sensitivity of 76.0% (95% CI 52.0% – 91.0%); specificity 83.0% (95% CI 77.0% – 88.0%), PPV of 34.0% (95% CI 21.0% - 49.0%), and a NPV of 97.0% (95% CI 92.0% - 99.0%).[24] In a randomized clinical trial by Kuhn *et al*., assessing two screen-and-treat strategies among 6 555 women in Cape Town, South Africa, among whom 956 were HIV-positive, the sensitivity of VIA was 63.9% (95% CI 46.2% - 79.2%) for CIN2+ and 58.3% (95% CI 27.7% - 84.8%) for CIN3+.[25]

3.5.4 Overall performance of colposcopic impression and RCI as diagnostic tools

Both colposcopy methods had comparable PPVs for CIN2+ and CIN3+ with the highest observed PPVs for CIN2+. A study by Durdi *et al*., aimed at estimating the diagnostic efficacy of colposcopy and determining the strength of correlation between colposcopic impression using RCI and histopathology, found the PPV and NPV of colposcopy with CIN1 as a disease threshold was 77% and 93.5% respectively and with CIN2 as a disease threshold 95.8% and 98.3% respectively, quite high in comparison to our findings. No results were reported for CIN

3. The degree of correlation between colposcopic impression using RCI and histopathology was substantial (k=0.73).[26]

The difference observed in their study in comparison to ours could be explained by the disease prevalence of their population. As described by Power *et al*., PPVs and NPVs are attractive because they are clinically insightful. But, because they are dependent on prevalence, predictive values may not be applicable in practice.[27] As disease prevalence increases, the PPV of the test increases, and reciprocally, the NPV of the test decreases. As prevalence decreases, the opposite occurs: the NPV of the same test increases while the PPV decreases. Additionally, the design of our study differed in that all women in our study underwent colposcopy, regardless of screening test result.

3.5.5 Performance of colposcopic impression and RCI as diagnostic tools in WLWH and HIV negative women

The PPV of colposcopy for both disease thresholds were higher in WLWH than in HIV negative women, which is expected given the disease prevalence of WLWH compared to that of HIV negative women. Colposcopy has previously been demonstrated to correlate with histological diagnosis in WLWH in comparison to HIV negative women. A study conducted in Cape Town by Batra *et al*., assessing the utilisation and outcomes of cervical cancer prevention services among HIV-infected women found that in WLWH (N=897) the PPV was 83.0%, and in HIV negative women (N=537) it was 81.1%.[28]

3.6 STRENGTHS AND LIMITATIONS

The current study reports the cervical cancer screening among women in South Africa, a country with one of the highest HIV prevalence; and included a representative number of women attending adult ART clinics. The study included a high proportion of participants with confirmed histology.

3.7 CONCLUSION

Cervical cancer screening strategies requiring multiple visits for diagnosis and treatment have proven difficult to implement in low-resourced and most medium-resourced countries. Visual screening methods as an alternative to cytology-based screening programmes are

advantageous given it is affordable with immediate results, can be performed by non-physician practitioners, allows for point of care testing, and allows for immediate treatment meaning no loss to follow up.

While the current study demonstrates that visual inspection methods could be useful in cervical cancer screening, particularly in WLWH, it is important to consider that in most studies, VIA and VILI demonstrates adequate sensitivity, only if intensive quality assurance is undertaken. This, however, may not always be possible to implement in non-academic environments and may result in over-treatment due to relative lack of specificity.

The WHO guideline for screening and treatment of cervical precancer lesions for cervical cancer prevention, recommends the rapid transition of existing screening programmes using VIA/VILI as a primary screening tool in the general population and in WLWH because of the inherent challenges with quality assurance. If VIA/VILI is the only screening tool feasible in the screening setting, it should be used in a screen-and-treat approach, with immediate treatment after a positive test result. In a screening paradigm where HPV DNA testing is available, VIA/VILI can be used as a triage tool in a screen, triage and treat approach.

Colposcopy, whether formally or informally scored had a relatively poor PPV for CIN3+, regardless of HIV status, and for CIN2+ in HIV negative women. The main finding is that clinician impression (of experienced colposcopists) did similar to RCI.

3.8 CANDIDATE CONTRIBUTION

As a research assistant for the DiaVACCS study, I played a role in specimen collection and processing, as well as data capturing and management. For the current manuscript, I was responsible for conducting the formal analysis, creating visualizations, and writing the original draft.

3.9 DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

3.10 REFERENCES

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3.11 ADDENDUM A

Table A1. Distribution of age (years) and percentage WLWH and HIV negative women screened for cervical cancer.

Characteristic	No. (%)
Age group, y	
25 – 29	96 (12.75)
30 – 34	110 (14.61)
35 – 39	160 (21.25)
40 – 44	137 (19.07)
45 – 49	98 (18.19)
50 – 54	86 (11.42)
55 – 59	45 (5.98)
60 – 64	21 (2.79)
HIV status	
HIV positive	344 (45.68)
HIV negative	409 (54.32)

Table A2. Comparison of screen positives for visual inspection cervical cancer screening methods among different age groups in WLWH and HIV negative women.

Age groups	VIA n = 753				VILI n = 753				p value
	WLWH n = 344		HIV neg n = 409		WLWH n = 344		HIV neg n = 409		
	n	%	n	%	n	%	n	%	
25-44	153	44.48	73	17.85	156	45.35	73	17.85	P < 0.00001
45-64	51	14.83	45	11.00	58	16.86	57	13.94	P = 0.04904

Table A3. Comparison of abnormal colposcopic findings among different age categories in WLWH and HIV negative women.

Age groups	Colposcopic impression n = 746				RCI n = 746				p value
	WLWH n = 344		HIV neg n = 402		WLWH n = 344		HIV neg n = 402		
	n	%	n	%	n	%	n	%	
25-44	158	45.93	89	22.14	157	45.64	85	21.14	P < 0.00001
45-64	52	15.12	61	15.17	49	14.24	57	14.18	P = 0.98697

Table A4. Comparative performance of cervical cancer visual inspection screening tests for histologic outcome CIN2+.

	Characteristic	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Total	VIA	65.17 [59.67 – 70.68]	71.27 [67.14 – 75.41]	58.70 [53.30 – 64.09]	76.57 [72.56 – 80.58]
	VILI	64.48 [58.95 – 70.01]	66.09 [61.77 – 70.41]	54.36 [49.08 – 59.64]	74.82 [70.21 – 79.42]
WLWH	VIA	76.47 [70.05 – 82.89]	57.47 [50.07 – 64.87]	63.73 [57.09 – 70.36]	71.43 [63.88 – 78.98]
	VILI	75.88 [69.41 – 82.36]	51.15 [43.67 – 58.63]	60.28 [53.69 – 66.87]	68.46 [62.20 – 74.72]
HIV -	VIA	49.17 [40.13 – 58.20]	79.58 [74.92 – 84.25]	50.00 [40.88 – 59.12]	79.04 [74.34 – 83.73]
	VILI	48.33 [39.30 – 57.37]	75.09 [70.08 – 80.09]	44.62 [35.99 – 53.24]	77.78 [70.56 – 84.99]

Table A5. Comparative performance of cervical cancer visual inspection screening tests for histologic outcome CIN2+ stratified by age.

Age group	Characteristic	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
25 - 44	VIA	67.30 [60.93 – 73.67]	71.23 [66.02 – 76.45]	62.83 [56.50 – 69.17]	75.09 [69.97 – 80.21]
	VILI	65.88 [59.44 – 72.31]	69.18 [63.86 – 74.50]	60.70 [54.34 – 67.06]	73.72 [67.99 – 79.45]
45 - 65	VIA	59.49 [48.50 – 70.49]	71.35 [64.52 – 78.17]	48.96 [38.83 – 59.09]	79.22 [72.76 – 85.68]
	VILI	60.76 [49.82 -71.70]	60.82 [53.45 – 68.19]	41.74 [32.63 – 50.85]	77.04 [69.27 – 84.81]

Table A6. Comparative performance of cervical cancer visual inspection screening tests for histologic outcome CIN3+ stratified by age.

Age group	Characteristic	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
25 - 44	VIA	79.81 [72.00 – 87.61]	64.16 [59.44 – 68.88]	36.73 [30.41 – 43.04]	92.42 [89.29 – 95.55]
	VILI	78.85 [70.90 – 86.79]	63.16 [58.41 – 67.91]	35.81 [29.57 – 42.05]	91.97 [88.74 – 95.20]
45 - 65	VIA	69.23 [54.27 – 84.19]	67.30 [60.93 – 73.67]	28.13 [19.02 – 37.23]	92.21 [87.94 – 96.48]
	VILI	66.67 [51.39 – 81.95]	57.82 [51.12 – 64.52]	22.61 [14.88 – 30.34]	90.37 [85.35 – 95.39]

Table A7. Comparative performance of colposcopy to Reid Colposcopic Index for histologic outcome CIN2+.

	Characteristic	PPV, % (95% CI)	NPV, % (95% CI)
Total	Colposcopic Impression	57.45 [52.43 – 62.46]	80.22 [76.18 – 84.26]
	RCI	58.08 [53.00 – 63.13]	79.79 [75.66 – 83.92]
WLWH	Colposcopic Impression	64.81 [58.41 – 71.22]	76.56 [70.88 – 82.24]
	RCI	64.62 [58.15 – 71.10]	75.00 [69.14 – 80.86]
HIV -	Colposcopic Impression	47.50 [39.70 – 55.30]	82.16 [76.18 – 88.14]
	RCI	49.02 [41.03 – 57.00]	82.33 [76.24 – 88.42]

Table A8. Comparative performance of colposcopy to Reid Colposcopic Index for histologic outcome CIN2+ stratified by age category.

Age groups	Characteristic	CIN 2+	
		PPV, % (95% CI)	NPV, % (95% CI)
25 - 44	Colposcopic Impression	62.45 [56.55 – 68.35]	80.17 [75.31 – 85.03]
	RCI	63.28 [57.35 – 69.21]	80.17 [75.26 – 85.07]
45 - 65	Colposcopic Impression	46.09 [36.88 – 55.30]	80.30 [72.96 – 87.65]
	RCI	45.87 [36.41 – 55.33]	79.14 [71.42 – 86.85]

CHAPTER 4

Cervical cancer prevention in Southern Africa: a review of national cervical cancer screening guidelines in the Southern African development community.

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Journal of Cancer Policy

https://doi.org/10.1016/j.jcpo.2024.100477

4.1 ABSTRACT

Background: Cervical cancer poses a significant burden, particularly in low-and-middle income countries (LMIC) with limited access to healthcare. High-income countries have made progress in prevention, while LMIC face unacceptably high incidence and mortality rates, often lacking official screening recommendations. We analysed the presence and content of cervical cancer screening guidelines for the secondary prevention of cervical cancer in the Southern African Development Community (SADC) and compared it to the current World Health Organization (WHO) guidelines for screening and treatment of cervical precancer lesions for cervical cancer prevention.

Methods: A review of national cervical cancer guidelines across the SADC region was conducted. Data was obtained from government websites, international cancer control platforms, and WHO resources. Search terms included "cervical cancer" and "cervical cancer control guidelines", amongst others. There were no limitations on publication years, and the most recent versions of the guidelines were analysed, regardless of language. Each guideline was assessed for specific screening and treatment recommendations, in relation to the current WHO guidelines. Points were assigned for each data element.

Results: While most countries contributed data to this analysis there was a notable absence of adherence to the WHO guidelines. The most common screening method was naked eye visual inspection. There was a consensus on the age of screening initiation. Most countries recommended treatment by cryotherapy and loop excision.

Conclusion: Effective cervical cancer screening programmes, guided by evidence-based recommendations, can enhance early intervention and outcomes. This study highlights the need for standardized and evidence-based cervical cancer screening guidelines in the SADC region, to reduce the burden of cervical cancer and improve the health outcomes of women in these areas.

Keywords: SADC, cervical cancer prevention, screening guidelines, WHO

4.2 INTRODUCTION

4.2.1 The burden of cervical cancer

Cervical cancer disproportionately affects women in resource-limited settings and underserved populations who have limited access to healthcare services. The disparity between low-and-middle income countries (LMIC) and high-income countries (HIC) is stark. Cervical cancer remains the fourth most frequently diagnosed cancer among women globally and the fourth leading cause of death. The GLOBOCAN estimates for 2020 indicate that approximately 604 127 women are diagnosed with cervical cancer annually, while 341 831 die from the disease and of that, approximately 84% new cases and 87 – 90% deaths, occurred in LMIC. [1,2]

While HIC have made considerable progress towards elimination, attributable to the implementation of effective prevention strategies; the incidence, and mortality in LMIC is unacceptably high. Many LMIC do not have any documented official cervical cancer screening recommendations in place, which is crucial to achieve elimination. A recent worldwide review by Bruni *et al*., revealed that overall, only 69% (139 of 202) of countries had official recommendations for cervical cancer screening while 60% (83 of 138) LMIC and 33% (18 of 54) African countries had screening recommendations. [3] Additionally, a 2019 World Health Organization (WHO) report of the global survey assessing national capacity for the prevention and control of noncommunicable diseases reported that only 20% of the countries in the African region reported having a national cervical cancer screening programme, with almost all these programmes being opportunistic.[4]

4.2.2 The WHO guidelines

The WHO guideline for screening and treatment of cervical precancer lesions for cervical cancer prevention, is an international evidence-based guide that provides a comprehensive approach to cervical cancer prevention and control for different resource settings. [5]

The WHO recognizes that women living with human immunodeficiency virus (HIV) (WLWH) have unique considerations when it comes to cervical cancer screening. Several factors contribute to the differences in screening recommendations for WLWH compared to the general population of women.

There is an increased risk for developing cervical cancer in WLWH compared to the general population of women. This increased risk is primarily attributed to the weakened immune system caused by HIV infection, which can lead to persistent infection with high-risk types of human papillomavirus (HPV), the known cause of cervical precancer and cancer.

Infection with HIV increases the oncogenic potential of HPV, especially in immunosuppressed women. WLWH have a higher prevalence of persistent HPV infection and multiple HPV types and may therefore experience a more rapid progression from HPV infection to precancerous cervical lesions and subsequently to cervical cancer. [6]

Considering these factors, the WHO has developed specific recommendations for cervical cancer screening in WLWH to ensure early detection and timely intervention. These recommendations involve more frequent and intensive screening strategies, such as shorter screening intervals and additional screening methods, to address the higher risk and faster disease progression observed in this population.

The WHO guideline specifically focuses on two approaches: the “screen-and-treat approach”, where the decision to treat is based on a positive primary screening test only; and the “screen, triage and treat approach”, where the decision to treat is based on a positive primary screening test followed by a positive triage test with or without histologically confirmed diagnosis.

Briefly, the second edition of the WHO guideline prioritizes HPV deoxyribonucleic acid (DNA) detection as the preferred screening test, with or without triage for the general population of women. In a screen-and-treat approach using HPV DNA detection as the primary screening test, the WHO suggests treating women who test positive for HPV DNA among the general population of women. In a screen, triage and treat approach using HPV DNA detection as the primary screening test among the general population of women, the WHO suggests using partial genotyping, colposcopy, visual inspection with acetic acid (VIA) or cytology to triage women.

For WLWH, the WHO suggests using an HPV DNA primary screening test with triage rather than without triage. In a screen, triage and treat approach, the WHO suggests using partial genotyping, colposcopy, VIA, or cytology to triage women after a positive HPV DNA test.

HPV samples for both populations can be self-collected (including at home), in addition to traditional collection by healthcare workers (HCW) during a speculum examination.

Screening with quality-assured cytology can continue until HPV DNA testing is available, though programmes with VIA should transition rapidly.

Screening initiation for WLWH is 25 years and 30 years for the general population. The guideline further recommends WLWH be screened every three to five years and every five to 10 years for the general population. The recommended screening exit age, regardless of HIV status, is 65 years. The choice of triage test depends on feasibility, training, programme quality assurance and available resources in countries. [5]

4.2.3 National cervical cancer screening guidelines

National-level decision-makers are encouraged to make use of the WHO guideline as a basis for updating their own guidelines as this will 1) establish standardized practices for the country, ultimately aiding in minimizing variations in screening practices and facilitating effective monitoring and evaluation of screening programmes; 2) allow for cost-effective resource allocation since by providing recommendations on the most effective screening methods, target populations, and screening intervals, guidelines assist healthcare systems in utilizing their resources efficiently. [7]

While many countries may have cancer control policies, the existence of cervical cancer specific guidelines is lacking. A cancer control policy outlines the overarching principles, objectives, strategies, and priorities of government in relation to cancer prevention, treatment, research, and overall cancer control. Cancer policies often set out the broad goals of a cancer programme, establish the allocation of resources, and define the roles and responsibilities of different stakeholders. These policies provide a strategic framework for decision-making and resource allocation related to cancer care and research at a higher level.

A cervical cancer guideline, on the other hand, provides specific recommendations, best practices, and evidence-based protocols for the screening, diagnosis, treatment, and management of cervical precancer and cancer. These guidelines are developed by expert groups and offer healthcare professionals clear, standardized recommendations for delivering high-quality care to patients.

The development and implementation of evidence-based screening guidelines have proven to reduce disease burden, as can be seen in most HIC, such as the United States, where the number of deaths from cervical cancer decreased substantially after the implementation of widespread screening. Death due to cervical cancer declined from 2.8 to 2.3 per 100 000 women from 2000 to 2015. [8]

4.2.4 Objective

Review and compare cervical cancer control guidelines in the 16 member states of the SADC region, to the current WHO guideline for screening and treatment of cervical precancer lesions for cervical cancer prevention (second edition).

4.3 METHODS

4.3.1 Search strategy and data collection.

We conducted a review of national cervical cancer guideline documents for Angola, Botswana, Comoros, Democratic Republic of Congo (DRC), Eswatini, Lesotho, Madagascar, Malawi, Mauritius, Mozambique, Namibia, Seychelles, South Africa, United Republic Tanzania, Zambia, and Zimbabwe. The review methodology broadly followed the steps proposed by Akanda *et. al.* [9]

Data was obtained from health ministry websites, national/state government websites, national cancer institute sites, the international cancer control partnership portal, the HPV centre, the WHO Noncommunicable Disease Surveillance, Monitoring and Reporting country profiles and the WHO non-communicable disease document repository.

Search terms included the country name; cervical cancer; cervical cancer control guideline; cervical cancer screening or cervical cancer prevention.

There were no restrictions based on publication year and we analysed the most recent version of each guideline identified. There were no language restrictions, and non-English language documents were translated for analysis.

4.3.2 Data extraction and analysis

Each guideline document was reviewed for information related to clinical requirements for secondary prevention. Data elements included 1) recommended differentiation between

guidelines for WLWH and HIV negative women, 2) primary screening test, 3) age at screening initiation, 4) recommended age at screening programme exit, 5) screening frequency, 6) recommended triage test, 7) screening setting (organized or opportunistic), and 8) recommended precancerous lesion treatment method. Points were assigned per data element (Table 4.1).

Table 4.1 Data extraction and analysis approach.

Categories		Point assignment	Maximum points
1. Recommended screening tool	HPV DNA testing	3	3
	Cytology	0	
	Visual inspection	0	
	Other	0	
2. Differentiation between guidelines for WLWH and HIV negative women	Yes	1	1
	No	0	
3. Age at screening initiation (WLWH)	25 years	1	1
	Overall (if no differentiation) 25 – 30 years	1	
	No age mentioned	0	
	Other	0	
4. Age at screening initiation (HIV negative)	30 years	1	1
	Overall (if no differentiation) 25 – 30 years	1	
	No age mentioned	0	
	Other	0	
5. Age at screening exit (WLWH)	65 years	1	1
	Overall (if no differentiation) 65 years	1	
	No age mentioned	0	
	Other	0	
6. Age at screening exit (HIV negative)	65 years	1	1
	Overall (if no differentiation) 65 years	1	
	No age mentioned	0	
	Other	0	
7. Screening frequency (WLWH)	3 – 5 years	1	1
	Other	0	
8. Screening frequency (HIV negative)	5 – 10 years	1	1
	Other	0	
9. Triage test	Anything mentioned	1	1
	Nothing mentioned	0	
10. Precancer treatment options	Thermal ablation	1	1
	Cryotherapy	1	
	LLETZ/LEEP	1	
	Cold knife conization	1	
	Other	0	
11. Screening setting	Organised	1	1
	Opportunistic	0	
Total possible points		*13*	

4.4 RESULTS

Overall, since four countries had no official national recommendations, nor was any mention made of cervical cancer screening guidelines in any of the other national policies, 12 of the 16 SADC countries contributed data to this analysis (Table 4.2). There were 11 countries with recent (2015 or later) policies. Some countries had no official national cervical cancer screening guidelines, but mention was made of cervical cancer secondary prevention in the national cancer control guidelines.

Table 4.2 List of policy documents by countries, title, and year of publication.

Country	Title of policy	Type	Year of publication
Botswana	National cervical cancer prevention programme. Five-year comprehensive prevention and control strategy (2021-2025) [10,11]	Strategy	2021
Democratic Republic of Congo*	National strategy to combat cervical and breast cancers in Democratic Republic of the Congo [12,13]	Strategy	2015
Lesotho	Guidelines for screening and treatment of cervical precancer in Lesotho [14,15]	Guideline	2020
Madagascar*	Technical guide for the prevention of cervical cancer using the visual screening – cryotherapy method [16,17]	Technical guide	2016
Malawi	National cervical cancer control strategy 2016-2020 [18,19]	Strategy	2017
Mozambique*	National programme for the prevention and control of cervical and breast cancer - National guideline for cervical cancer prevention [20,21]	Guideline	2019
Mauritius	National Cancer Control Programme [22,23]	Action plan	2015
Namibia	National Cervical Cancer Prevention Guidelines [24,25]	Guideline	2018
South Africa	Cervical Cancer Prevention and Control Policy [26–29]	Policy	2017
Tanzania	National Cervical Cancer Prevention and Control Strategic Plan 2011 – 2015 [30,31]	Strategic plan	2011
Zambia	National Cancer Control Strategic Plan 2016 – 2021 [32,33]	Strategic plan	2016
Zimbabwe	The Zimbabwe cervical cancer prevention and control strategy (ZCCPCS) 2016-2020 [34–36]	Strategy	2015

**Translated to English*

Table 4.3 Recommended screening characteristics in the SADC member states.

SADC	Primary screening tool	Age at screening initiation (years)		Age at screening exit (years)		Screening frequency (years)		Triage Test	Precancer treatment options	Screening setting
		WLWH	HIV -	WLWH	HIV -	WLWH	HIV -			
Botswana	VIA, cytology, and HPV	25	25	65	65	3	3	AVE	Cryotherapy, thermo-coagulation and LEEP	Opportunistic
Democratic Republic of Congo	Conventional cytology, VIA/VILI, and HPV DNA	25	25	65	65	<50 – 3 >50 – yearly	<50 – 3 >50 – yearly	VIA/VILI	Cold conization and cryotherapy	Opportunistic
Lesotho	VIA/VILI, AVE, HPV NAT, and cytology	25	25	49	49	2	25-49 – 3 ≥50 – 5	-	Thermo-coagulation, cryotherapy and LEEP	Opportunistic
Madagascar	VIA/VILI	25	25	50	50	5	5	-	-	Opportunistic
Malawi	VIA/VILI	25	25	49	49	yearly	3 - 5	Screen-and-treat	Thermocoagulation and LLETZ	Opportunistic
Mozambique	VIA/VILI	30	30	54	54	-	-	-	-	Opportunistic
Mauritius	HPV DNA and cytology	At diagnosis	30	49	49	Cytology - 3 – 5 HPV - 5	Cytology - 3 – 5 HPV - 5	-	Cryotherapy and LEEP	Opportunistic
Namibia	Cytology, VIA, and HPV DNA	20	25	50	50	VIA – 3 Cytology - yearly	VIA – 5 Cytology - 3	VIA	Cryotherapy and LLETZ	Opportunistic
South Africa	Cytology, VIA/VILI, and HPV DNA	At diagnosis	30	75	50	3	10	Colposcopic biopsy	Cryotherapy and LLETZ	Opportunistic
Tanzania	VIA/VILI	30	30	49	49	-	-	-	Cryotherapy and LEEP	Opportunistic
Zimbabwe	VIA	20	30	49	49	yearly	3	Screen-and-treat	Cryotherapy and LEEP	Opportunistic
Zambia	VIA/VILI	-	-	-	-	-	-	Screen-and-treat	Cryotherapy and LEEP	Opportunistic

Abbreviations: VIA, visual inspection with acetic acid; HPV, human papillomavirus; VILI, visual inspection with Lugol's iodine; AVE, automated visual evaluation; NAT, nucleic acid amplification tests; LEEP, loop electrosurgical excision procedure; LLETZ, large loop excision of the transformation zone

Table 4.4 Points assigned to each country's cervical cancer screening policies with reference to the WHO guidelines.

Guidelines	Primary screening tool	Differentiation between WLWH and HIV -	Age at screening initiation (years)		Age at screening exit (years)		Screening frequency (years)		Triage Test	Precancer treatment options	Screening setting	TOTAL /13
			WLWH	HIV -	WLWH	HIV -	WLWH	HIV -				
Botswana	3	1	1	1	1	1	1	0	1	1	0	11
Democratic Republic of Congo	3	0	1	1	1	1	1	0	1	1	0	10
Lesotho	3	1	1	1	1	1	0	1	0	1	0	10
Madagascar	0	0	1	1	1	1	1	1	0	0	0	6
Malawi	0	0	1	1	1	1	0	1	1	1	0	7
Mozambique	0	0	1	1	1	1	0	0	0	0	0	4
Mauritius	3	1	0	1	1	1	1	1	0	1	0	10
Namibia	3	1	0	1	1	1	1	1	1	1	0	11
South Africa	3	1	0	1	0	1	1	1	1	1	0	10
Tanzania	0	0	1	1	1	1	0	0	0	1	0	5
Zimbabwe	0	1	0	1	1	1	0	0	1	1	0	6
Zambia	0	0	0	0	0	0	0	0	1	1	0	2

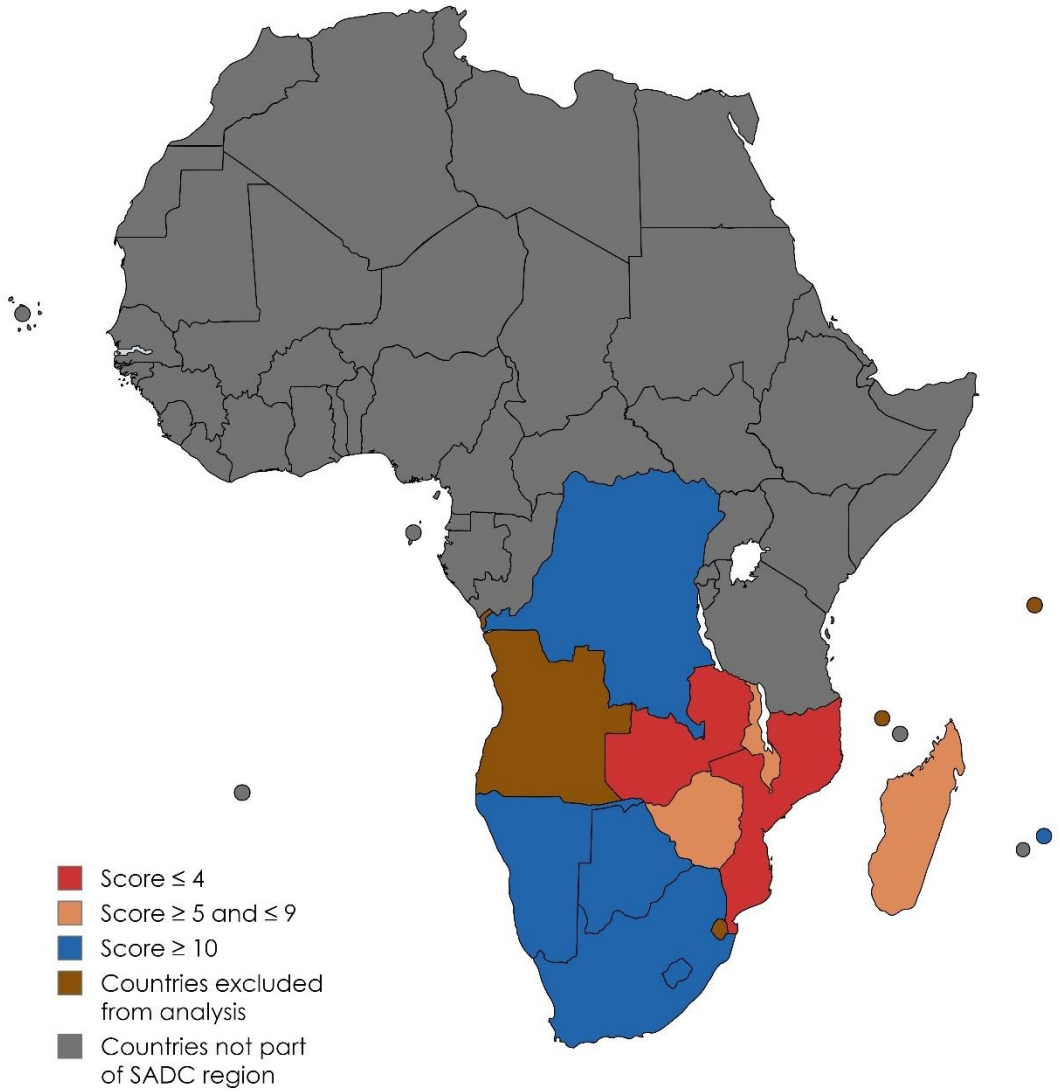

Figure 4.1 Cervical cancer guideline comprehensiveness across SADC member states.

The average score for compliance with the WHO guidelines was 8 (maximum possible score: 13). Zambia scored the lowest, 2, followed by Mozambique, 4, and Tanzania, 5, with Namibia and Botswana having the highest score, 11.

Guidelines that mentioned multiple screening approaches did so based on health facility resource capacity and low versus high-volume facilities. The most common recommended screening method was naked eye visual inspection. This included visual inspection with acetic acid (VIA), as well as visual inspection with Lugol's iodine (VILI). Only six countries recommended HPV DNA testing as the primary screening tool or made mention of the planned transition to HPV DNA testing as the primary screening tool, as recommended by the WHO.

Six of the countries had special considerations for high-risk groups, such as WLWH. These countries differentiated between screening target ages and screening frequencies. There was no apparent link between recommended screening test and age at screening initiation, with most countries recommending screening from the age of 25 years. Namibia and Zimbabwe recommended WLWH start screening at the age of 20 years while Zambia made no mention of age at all. South Africa was the only country that recommended screening WLWH after the age of 65, which is the WHO suggested screening exit age.

Three countries made no mention of screening frequencies. South Africa recommended 10 yearly screening for the general population of women regardless of screening test used, and three yearly screening for WLWH. Screening frequencies for WLWH is generally shorter regardless of recommended screening test. Three countries that recommended VIA/VILI as a primary screening tool, recommended a screen-and-treat approach. The most recommended cervical precancer treatment techniques were cryotherapy and large loop excision of the transformation zone/ loop electrosurgical excision procedure (LLETZ/LEEP).

4.5 DISCUSSION

The purpose of this analysis was to provide an overview of the scope and focus of national cervical cancer screening guidelines in the SADC region, and to compare these guidelines for consistency with the WHO guidelines.

While most countries included in the current review have national cervical cancer screening recommendations, it is interesting to note that the incidence and mortality rates are of the highest in the global context. Malawi, for instance, scored seven out of 15 points for compliance with the WHO guidelines, yet in 2020, the country had the second highest global cervical cancer incidence and mortality rates. [1] This could primarily be due to low screening coverage combined with an imperfect screening strategy.

Most countries in the SADC region recommend visual inspection as the preferred screening method. It has, however, become common knowledge that visual inspection screening methods is highly subjective and demonstrates adequate sensitivity, only if intensive quality assurance is in place [37,38], hence the WHO's recommendation for programmes with VIA to transition rapidly to HPV DNA testing.

Over the last few years, several HIC have successfully adjusted their cervical cancer screening paradigm to include HPV DNA testing as the primary screening method. The inclusion and rollout of this primary screening method in LMIC, however, has not been as widespread due to a multitude of factors including poorly developed healthcare services, budgetary limitations for human resource development, a lack of infrastructure, poor political support and competing public health priorities, amongst others. [39–42]

There are several factors to consider when planning and implementing a cervical cancer screening programme based on HPV testing as a primary screening test. [43] There are many commercially available HPV assays, and the development of new assays is ongoing. Public health decision-makers and programme managers should give careful consideration when selecting an assay, using analysis on the basis of documented information about the clinical validation for the required purpose. Arbyn *et al.,* published an updated 2020 list of clinically validated HPV assays suitable for primary cervical cancer screening according to the Meijer guideline. [44] This comprehensive list offers valuable guidance for public health decision-makers and programme managers tasked with selecting an appropriate assay for their specific screening initiatives.

South Africa, an upper middle-income country, part of the SADC region, launched a national screening programme for cervical cancer prevention in the year 2000, offering three cervical cytology tests per lifetime starting after the age of 30 years with 10-year intervals for HIV negative women and three-year intervals for WLWH. [26] The implementation of cervical cytology, however, has faced challenges in the public sector, particularly in parts of South Africa where healthcare infrastructure may lack the necessary technical capabilities and resources for effective execution. [45–47]

Cervical cancer is the second most common cancer diagnosed among South African women and the commonest cancer among women in the reproductive age group 15 to 44 years. Estimates for 2020 were reported at 10 702 incident cases (age standardized incidence rate 35.3/100 000) and 5 870 deaths (age standardized incidence rate 19.6/100 000). [48] Screening coverage remains suboptimal. Data from the WHO's cervical cancer country profiles (2019) indicate that in South Africa, 52% of women aged 30 – 49 years, were ever screened for cervical cancer, and that four in 10 women have been screened in the last five years. [49] A systematic review conducted by Mantula et.al., on the barriers to cervical cancer screening in

Africa, identified primary barriers as poor access to screening services, lack of awareness and knowledge on cervical cancer and screening, and socio-cultural influences. [50] Petersen et. al., identified the lack of knowledge and awareness of cervical cancer, and cervical cancer screening as the most frequent individual level barriers to the uptake of cervical cancer screening services in low-and-middle-income countries. [51]

These complexities, combined with the limitations of cytology in detecting high-grade cervical lesions and cancers [52,53], suggest that even with improved quality control, its effectiveness in low-resource settings would likely be modest.

HPV testing offers significant advantages as it is the most sensitive method for screening cervical cancer. With a negative predictive value (NPV) nearly reaching 100%, it allows for longer intervals between screenings for negative results, reducing the need for follow-up visits and additional tests. [54] This makes it more cost-effective compared to cytological testing. Moreover, HPV testing can be conducted using self-collected samples, eliminating the need for a gynaecological examination. Introducing self-sampling has the potential to overcome barriers to cervical cancer screening uptake, leading to better screening coverage. Additionally, HPV self-sampling can streamline cervical screening in healthcare centres with limited screening capacity. [55]

HPV testing is only offered in the private sector and is not yet widely available in the public sector as part of the national screening programme, although the most recent cervical cancer prevention and control policy guideline outlines a path towards complete transition to HPV based screening over the next decade. [26,28]

The South African HPV Board, in a 2023 guideline recommended primary HPV screening for WLWH at 25 years, followed by five to 10-yearly screening, regardless of resource setting. Similarly, for HIV-negative women, screening initiation is recommended at 25 years (although low-risk sub-populations first screening at age 30 may be more appropriate and will reduce false positive screen results and overtreatment) followed by five to 10-yearly screening regardless of resource setting. [56]

Although not part of the SADC region, Rwanda, an East African country has reached great heights in the journey towards cervical cancer elimination. According to the Rwanda Biomedical Centre (RBC), as of October 2021, Rwanda had HPV based screening at 89 of its

510 health centres, 10 of its 50 district hospitals and six of its 30 districts. The country's 2024 goal is to have HPV DNA based cervical cancer screening services available in all health facilities, at least 70% of eligible women screened for cervical cancer and at least 90% of women with cervical precancerous lesions treated, in keeping with the WHO's global strategy for the elimination of cervical cancer as a public health problem. [57]

This was made possible by collaboration among governments, international organizations, healthcare providers and researchers. In 2011, Rwanda, Merck and QIAGEN launched Africa's first comprehensive cervical cancer prevention programme incorporating both HPV vaccination and HPV testing. Merck donated two million doses of GARDASIL® and QIAGEN donated 250 000 HPV screening tests along with all necessary equipment and training to successfully perform the tests. Additionally, Unitaid and the Clinton Health Access Initiative (CHAI) are currently supporting the RBC to roll out the country's comprehensive national cervical cancer programme by scaling up the use of HPV testing, increasing the provision of treatment services, integrating cervical cancer screening with other health services, implementing patient tracking systems to ensure continuity of women's care, and generating demand for screening through district-wide awareness campaigns.[58] Furthermore, the Global Initiative Against HPV and Cervical Cancer (GIAHC) is supporting the work of the RBC and CHAI to attain the goals of cervical cancer elimination in Rwanda.

The collaboration between Rwanda and multiple partner organizations has demonstrated the potential for partnerships to facilitate knowledge exchange, resource mobilization, and advocacy endeavours aimed at prioritizing cervical cancer prevention and control. Rwanda's experience can serve as a valuable model for informing the development and implementation of guidelines in other countries within the SADC region. Our analysis has underscored the existence of notable gaps, making Rwanda's success a compelling source of guidance for the region's cervical cancer guideline development and implementation.

4.6 CONCLUSION

Implementing effective screening programmes based on evidence-based guidelines and promoting awareness of the importance of screening can lead to early intervention and improved outcomes. The ideal cervical cancer screening guideline should at least include information on the recommended primary screening test, age at screening initiation,

recommended age at screening programme exit, screening frequency, recommended triage test and recommended precancerous lesion treatment method. Furthermore, where HPV DNA testing is the recommended primary screening method, the guideline should specify the technical details of the recommended assay e.g., the use of non-discriminatory assays, which gives a “negative” or “positive” answer for the detection of any of the included high-risk HPV types or the use of discriminatory assays, which gives a result of “negative”, “positive for the highest risk viral types” (usually 16, 18, sometimes also 45) or “positive for other high-risk HPV types”. Additionally, guidelines should differentiate between WLWH and the general population of women.

To achieve elimination, there is a need for serious political commitment by national leaders of the SADC region to implement HPV DNA testing in cervical cancer screening programmes. National leaders could seek help from the WHO expertise to achieve uniformity with guidelines. Innovative strategies are needed to improve coverage of cervical cancer screening. Furthermore, governments need to address educational, and training needs amongst health workers and the wider population. Lastly, consistent, and complete collection of data on screening participation and outcomes is essential to monitor and optimize the performance of cervical cancer screening.

4.7 CANDIDATE CONTRIBUTION

I contributed to the analysis, investigation, and writing of the original draft of the research article. Additionally, I was involved in the conceptualization and methodology development for the research.

4.8 FUNDING SOURCES

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

4.9 DECLARATION OF INTEREST

The authors report no declarations of interest.

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CHAPTER 5

DNA-methylation for the detection of clinically relevant cervical lesions: the clinical performance of DNA-methylation as a molecular triage test for South African women who are screen positive for hrHPV excluding HPV 16 and HPV 18.

5.1 INTRODUCTION

5.1.1 The burden of cervical cancer

Cervical cancer is the fourth most common cancer among women globally, with approximately 661 021 new cases and 348 189 deaths reported annually. It disproportionately affects women in developing countries, where about 90% of cervical cancer deaths occur. [1] High rates of incidence and mortality are attributed to limited access to screening programmes, lack of human papillomavirus (HPV) vaccination, and inadequate treatment options.[2,3]

5.1.2 Association between high-risk HPV infection and cervical dysplasia

Persistent infection with high-risk HPV (hrHPV) types, particularly HPV 16 and 18, is the primary risk factor for cervical cancer. These high-risk types of HPV have the potential to induce cellular changes that can progress from low-grade cervical intraepithelial neoplasia (LSIL or CIN 1) to high-grade cervical intraepithelial neoplasia (HSIL or CIN 2/3) and eventually to invasive cervical cancer. The oncogenic proteins E6 and E7, encoded by HPV, play a significant role in disrupting cellular pathways involved in cell cycle regulation and DNA repair. Specifically, E6 promotes the degradation of the tumour suppressor protein p53, while E7 interacts with proteins such as retinoblastoma, leading to cell cycle dysregulation and genomic instability. [4,5] Other risk factors for cervical cancer include early sexual initiation, multiple sexual partners, immunosuppression, smoking, and lack of access to HPV vaccination and cancer screening programmes. [6–8]

5.1.3 HPV prevalence in South Africa

Although HPV 16 and 18 are intricately linked to cervical cancer, HPV 35 is found in approximately 10% of cervical cancer cases in women of African descent, compared to about 2% globally. Research conducted in Sub-Saharan Africa have indicated an HPV 35 prevalence reaching up to 40% among women diagnosed with CIN or cervical cancer.[9,10] Mbulawa et. al., in a recent hospital-based project among women with high-grade cervical lesions in the Eastern Cape, South Africa, found the six most dominant HPV types were HPV 16 (34.7%), followed by HPV 35 (17.4%), HPV 58 (12.1%), HPV 45 (11.6%), HPV 18 (11.4%) and HPV 52

(9.7%). When focusing on dominant HPV types that were detected as a single infection, HPV 16 remained the most dominant type, followed by HPV 35 and HPV 52. [11]

Other hrHPV types linked to high-grade cervical lesions and cervical cancer in South African women include HPV 35, 52, 31, 58, 51, 33, 45, and 39 for high-grade lesions, and HPV 33, 35, 45, 31, 52, 58, 51, and 56 for cervical cancer.[12]

5.1.4 Cervical cancer screening

Cytology-based testing has been the gold standard for cervical cancer screening primarily due to its high specificity. Nevertheless, several limitations have been reported, including low sensitivity and poor reproducibility. Moreover, it requires well-trained personnel and expensive infrastructure. Despite alternative methods such as visual inspection using acetic acid (VIA) or Lugol iodine (VILI), their sensitivities remain suboptimal. [13–20]

The implementation of HPV-based testing represents a major step forward in cervical cancer screening technology. In South Africa, HPV testing is now almost routinely offered in the private sector and may become more widely available in the public sector as part of the national screening programme. Current screening algorithms focuses specifically on HPV 16 and 18, however, consideration should be given to other hrHPV types, as listed above, given their association with high-grade lesions and cervical cancer.

Women testing positive for HPV 16 and HPV 18 typically receive direct referral for treatment. On the other hand, those screening positive for other high-risk HPV types are categorized as having intermediate or medium risk, depending on their age. If they are older than 40 years, they are referred directly for treatment; otherwise, they require a triage test for appropriate risk stratification.[21] Triage is essential for identifying women with clinically relevant infections, as many of the detected infections are transient, and only a minority are associated with cervical abnormalities. Therefore, HPV DNA testing in isolation may not be specific enough and therefore lack clinical significance.[22,23]

5.1.5 DNA-methylation as a biomarker

Methylation of host-cell DNA has been proposed as a promising biomarker for triage of HPV positive women at screening.[24–27] DNA-methylation is an epigenetic mechanism that occurs by the addition of a methyl group (CH3) to cytosine in the dinucleotide 5’-CpG-3’. This

mechanism is mediated by a family of methyltransferases. Most CpG dinucleotides in the human genome are methylated, however, unmethylated clusters of CpGs are found in the promoter region of many genes. These are termed CpG islands. This modification plays a crucial role in gene expression regulation by influencing chromatin structure and accessibility to transcriptional machinery. Hypermethylation of CpG islands in gene promoter regions often leads to transcriptional silencing, while hypomethylation can result in increased gene expression. [28]

5.1.6 DNA methylation as a triage test

Analysis of host-cell DNA methylation patterns could be considered a candidate cervical precancer triage test since it is able to identify clinically meaningful HPV associated cervical lesions. More than 100 human methylation biomarker genes have been evaluated and identified in cervical tissue. Approximately 10 have been repeatedly shown to have elevated methylation in cervical cancers and CIN2+, specifically CADM1, EPB41L3, FAM19A4, MAL, miR-124, PAX1 and SOX1. [25] Several recent studies have demonstrated that hypermethylation of FAM19A4 and miR124-2 promoters is associated with the presence and severity of cervical dysplasia, including HSIL and cervical cancer.[29–31] Vink et. al., in a retrospective cross-sectional worldwide study assessing the consistency of FAM19A4/miR124-2 methylation analysis in the detection of cervical cancer in a series of 519 invasive cervical carcinomas from over 25 countries, concluded that a negative FAM19A4/miR124-2 methylation assay result is likely to rule out the presence of cervical cancer.[32] In a post hoc analysis within the POBASCAM trial, De Strooper et al. examined the longitudinal outcome of FAM19A4/miR124-2 methylation analysis in an HPV-positive screening cohort over a 14-year follow-up period. Their findings suggest that a negative FAM19A4/miR124-2 methylation test is associated with a low cervical cancer risk in HPV-positive women aged 30 years and older. These results support the consideration of FAM19A4/miR124-2 methylation testing as an objective triage test in HPV-based cervical screening programmes.[33]

DNA methylation compared to cytology and immunohistochemical p16INK4a/Ki-67 staining is advantageous since the molecular basis of the test makes it automatable, resulting in less interpretational error than the morphological tests. Testing can also be done on the same

sample used for HPV DNA testing (healthcare worker-collected or self-collected) thereby simplifying the sample collection process.[27]

5.1.7 Objective

Assess the triage capability of the QIAsure assay for the detection of biomarkers FAM19A4 and mir124-2 in other hrHPV positive women (excluding HPV 16 and HPV 18). Compare DNA methylation results to increasing severity of histopathology results. Calculate the sensitivity, specificity, and predictive values of DNA-methylation.

5.2 METHODS

5.2.1 Study population

This study is a sub-study of the DIAgnosis in Vaccine And Cervical Cancer Screen (DiaVACCS) screening trial. The DiaVACCS trial was a cross-sectional cohort study in which women between the ages of 25 and 65, unscreened in the preceding five years, were recruited from three sites in South Africa from December 2016 to March 2020. The study was approved by the Health Research Ethics committees of Pretoria (196/2014) and Stellenbosch Universities (reciprocal approval 2015).

The study design, methodology and basic descriptive data have previously been described.[34] The DiaVACCS trial was aimed at evaluating the performance of screening tests in general female and HIV-infected populations, and included a total of 1 104 women, 639 HIV negative and 465 HIV positive.

Overall, 33.8% (373/1 104) of the total cohort tested positive for HPV using the Roche Cobas® HPV test. Of these, 221 (48.5%) were women living with HIV (WLWH) and 152 (23.5) were HIV negative. Of the total cohort, 246 women met the inclusion criteria for the present sub-study. Of these, 25 were excluded due to insufficient left-over LBC material. Furthermore, only 207 women had valid histology results, of which 146 (WLWH, n = 92, HIV negative and unknown, n = 54) had valid DNA methylation results and were included in the final analysis (Figure 5.1).

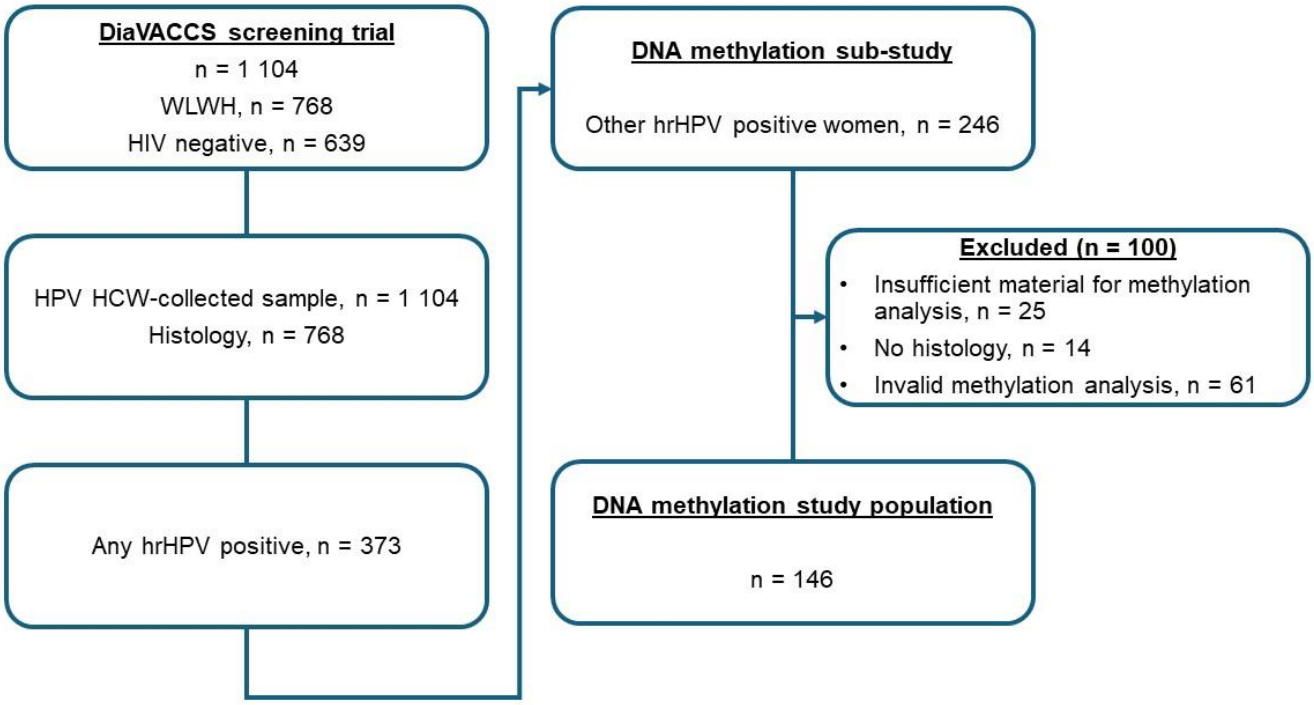

Figure 5.1 DNA methylation specimen acquisition flowchart.

5.2.2 Clinical and laboratory techniques

5.2.2.1 Specimen collection and storage

Cervical specimens were collected by trained healthcare workers during speculum examinations at the Gynaecology Oncology Research sites at Tygerberg Hospital, Kalafong Provincial Tertiary Hospital, and the Tshwane District Hospital. The specimens for the molecular analysis were collected using an endocervical brush and were transferred into a ThinPrep® (Hologic Inc, Marlborough, Massachusetts, USA) vial containing PreservCyt preservative and transport medium.

Specimens were transported at 4°C to the University of Pretoria, Department of Medical Virology, National Health Laboratory Service (NHLS), Tshwane Academic Division for processing. After processing, the remaining specimens were stored at 4°C until further analysis.

For the DNA-methylation assay, samples were aliquoted into 2 mL cryovials and transported at 4°C to the Division of Medical Virology, Department of Pathology, Stellenbosch University

for storage prior to processing. Initially, the plan was to conduct the DNA methylation laboratory procedures at Stellenbosch University. However, none of the facilities possessed a suitable centrifuge necessary for the "cleanup of bisulfite converted DNA using a centrifuge" procedure outlined below. Samples were thus transported at 4°C to the Lancet Laboratories, Richmond, Johannesburg.

5.2.2.2 Biopsy and histology processing

Each participant underwent two colposcopic-directed biopsies upon detection of any abnormality via HPV test or cytology within six months of receiving an abnormal result. However, due to loss-to-follow-up, not all participants with a screening abnormality underwent biopsies. A sample of women with negative screening results was also selected for colposcopy and biopsy to form a negative control group. For those who underwent large loop excision of the transformation zone (LLETZ) treatment, the most severe histological finding between the biopsy and LLETZ was considered the final diagnosis. Histological examination was conducted by an experienced histopathologist.

5.2.2.3 The Cobas® HPV test

The Cobas® HPV test is a real-time PCR assay that detects the presence of 14 HPV types, with HPV 16 and HPV 18 detected individually and a pool of 12 other HPV types (HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66, and HPV 68).

The Cobas® HPV test was performed by an experienced board-certified pathologist at the Lancet Laboratories. Briefly, the x480 sample preparation module was used to prepare samples for amplification and detection. Twenty-five microliter (25 µl) of specimen was added to 25 µl of the working master mix in a 96-well microwell plate. The plate was sealed according to the Cobas® Operators Manual. The plate was centrifuged using the Centrifuge 5415D (Eppendorf, Hamburg, Germany), for five seconds at 1 500 relative centrifugal field (RCF) and then transported to the Cobas® z 480 analyser (Roche Molecular Systems, Pleasanton, California, USA) for amplification and detection. Results are displayed as either positive/negative for HPV 16, positive/negative for HPV 18 and positive/negative for other hrHPV.

5.2.2.4 The QIAsure methylation test (Qiagen, Hilden, Germany)

The QIAsure Methylation Test was performed at Lancet Laboratories, Richmond, Johannesburg. Exfoliated cervical cells were used to measure methylation levels of the FAM19A4 and miR124-2 genes using quantitative methylation-specific polymerase chain reaction (qPCR). Methylation levels were summarized as cycle threshold (Ct) ratios compared with the β-actin gene.

The Rotor-Gene Q MDx 5plex HRM system (Qiagen) was used for running the test and the Rotor-Gene AssayManager (Qiagen) was used for results interpretation. The various approaches used to obtain valid results are described below.

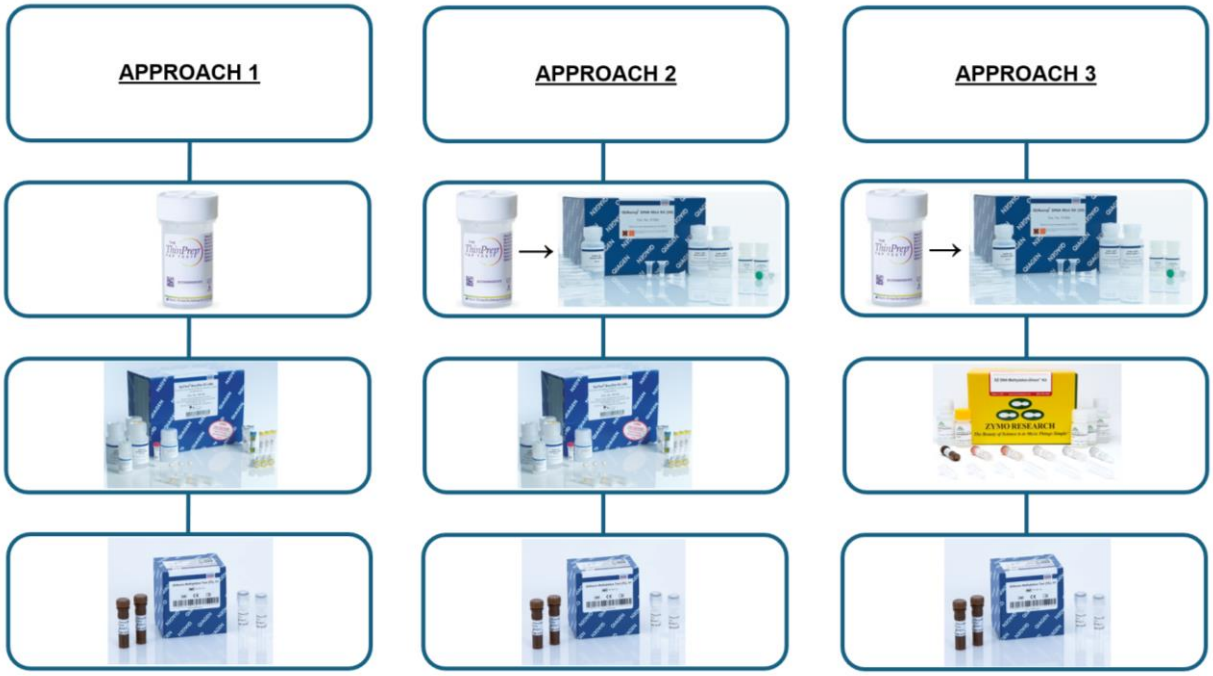

Figure 5.2 Overview of approaches used to obtain valid results.

Approach 1: Bisulfite-conversion directly on cervical specimens collected in PreservCyt® medium with the EpiTect Fast 96 Bisulfite Kit (Qiagen, Hilden, Germany)

a. Sample preparation

According to the manufacturer's instructions, for each specimen, 500 µl was aliquoted and centrifuged at minimal 3390 x g. The supernatant was discarded leaving the cell pellet at a maximum 20 µl PreservCyt collection medium. The cell pellet was used for the bisulfite-conversion reaction.

b. Bisulfite conversion preparation

85 µl Bisulfite Solution was added to the 20 µl DNA and mixed by pipetting. The DNA–Bisulfite Solution mixture was then transferred to the EpiTect 96 Conversion Plate, after which 35 µl DNA Protect Buffer was added, and mixed. The DNA Protect Buffer turned from green to blue after addition to the DNA–Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the DNA bisulfite conversion reaction.

The EpiTect 96 Conversion Plate was then sealed using the EpiTect 96 Cover Foil and mixed by vortexing. The plate was then centrifuged briefly at 650 x g (approximately 2000 rpm) to collect the reactions in the bottom of the wells. The bisulfite DNA conversion was performed using a thermal cycler. The thermal cycler was programmed according to Table 4.1. The programme took 30 minutes until completion.[35]

Table 5.1 Bisulfite conversion thermal cycler conditions.

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	10 min	60°C
Denaturation	5 min	95°C
Incubation	10 min	60°C
Hold	Indefinite	20°C

c. Cleanup of bisulfite converted DNA using a centrifuge

Once completed, the EpiTect 96 Conversion plate was briefly centrifuged at 650 x g and placed on top of an S-Block. 310 µl of Buffer BL was dispensed into the required EpiTect 96 Plate wells. The complete bisulfite reactions were transferred to the EpiTect 96 Plate and mixed with Buffer BL by pipetting up and down four times. 250 µl 96% molecular grade ethanol was added to each sample and mixed by pipetting up and down four times. The EpiTect 96 Plate was sealed with an AirPore Tape Sheet and loaded into the centrifuge plate holder then placed into the rotor bucket and centrifuged at 5800 x g for 1 min. The AirPore Tape Sheet was then removed and 500 µl Buffer BW added to each sample. The plate was sealed with a new AirPore Tape Sheet and centrifuged at 5800 x g for 1 min. The contents of the S-Block were discarded. The AirPore Tape Sheet was then removed and 250 µl Buffer BD was added to each sample. The plate was then sealed using a Tape Pad and was left to incubate for 15

min at room temperature. Following incubation, the plate was centrifuged at 5800 x g for 1 min. 500 µl Buffer BW was added to each sample. The plate with sealed with a new AirPore Tape Sheet and centrifuged at 5800 x g for 1 min. The contents of the S-Block were discarded. 500 µl Buffer BW was added to each sample. The plate was sealed with a new AirPore Tape Sheet and centrifuged at 5800 x g for 1 min. 250 µl 96% ethanol was added to each sample and sealed with a new AirPore Tape Sheet and centrifuge at 5800 x g for 1 min. The contents of the S-Block were disposed of. The AirPore Tape Sheet was removed and placed on top of an EpiTect Elution Plate and centrifuge at 5800 x g for 15 min. The EpiTect 96 Plate was then placed on top of a new EpiTect Elution Plate. To elute DNA, 70 µl Buffer EB was added to each sample using a multichannel pipette and centrifuged at 5800 x g for 1 min.

Figure 5.3 Images of centrifuge used for cleanup of bisulfite converted DNA.

d. QIAsure DNA-methylation sample processing on Rotor-Gene Q MDx instrument

A new work list was created according to the manufacturer's instructions.[36] The QIAsure Master Mix and QIAsure Calibrator were thawed in a dark room. The tubes were gently inverted 10 times before use. 17.5 µl of the ready-to-use QIAsure Master Mix was dispensed into the appropriate strip tubes. In a separate area, 2.5 µl water was added to position two, as the no template control (NTC). 2.5 µl of QIAsure Calibrator was added to position 1 and 2.5 µl of bisulfite-converted DNA to corresponding tubes.

A 72-well rotor was placed on the rotor holder. The rotor was filled with tubes in previously assigned positions and the experiment run initiated. Results were analysed by the Rotor-Gene AssayManager v1.0.

Approach 2: DNA extraction prior to bisulfite-conversion with the EpiTect Fast 96 Bisulfite Kit (Qiagen, Hilden, Germany)

a. DNA extraction

20 µl of proteinase K was pipetted into a 1.5 mL microcentrifuge tube. The ThinPrep vial was vortexed at maximum speed for five seconds. Then, 200 µl of the vortexed specimen was pipetted to the microcentrifuge tube. After that, 200 µl of Buffer marked "AL" was added to the sample, and it was mixed by pulse-vortexing for 15 seconds. The microcentrifuge was incubated for 10 minutes at 56 °C. The microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. 200 µl of ethanol (96–100%) was added to the sample and mixed again by pulse-vortexing for 15 seconds. After mixing, the microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. The mixture was carefully applied to the QIAamp Mini spin column. It was centrifuged at 8000 rotations per minute (rpm) for one minute. The QIAamp Mini spin column was placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. The QIAamp Mini spin column was opened, and 500 µl of the provided Buffer AW1 was added. It was centrifuged at 8000 rpm for one minute. The QIAamp Mini spin column was placed in a clean 2 mL collection tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was opened, and 500 µl of Buffer AW2 was added. It was centrifuged at 14,000 rpm for three minutes. The QIAamp Mini spin column was placed in a new 2 mL collection tube, and the old collection tube with the filtrate was discarded. It was centrifuged at 14,000 rpm for one minute. The QIAamp Mini spin column was placed in a clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was opened, and 50 µl of Buffer AE was added. It was incubated at room temperature (15–25°C) for one minute, and then centrifuged at 8000 rpm for one minute.[37]

b. DNA quantification

Upon the completion of DNA extraction, the purity of DNA was measured using the Qubit Fluorometer, with dsDNA BR assay kit (Thermo Fisher Scientific, USA), according to manufacturer's instructions. [38]

c. Bisulfite conversion preparation

The bisulfite conversion preparation process for approach 2, was the same as described for approach 1.

d. Cleanup of bisulfite converted DNA using a centrifuge

For the most part, the cleanup of bisulfite converted DNA using a centrifuge process for approach 2, was the same as described for approach 1. The following assay optimization steps were applied:

1. If DNA concentrations were high enough (>5,5 ng/ µl) we modified 250 ng and elute in 12,5 µl.
2. If concentrations were low (5,5 – 3 ng/ µl), we modified 45 µl and elute in 12,5 µl.
3. If concentrations are <3 ng/ µl, we modify 45 µl and elute in 6,25 µl.

e. QIAsure DNA-methylation sample processing on Rotor-Gene Q MDx instrument

QIAsure DNA-methylation sample processing on Rotor-Gene Q MDx instrument, as described in approach 1.

Approach 3: DNA extraction prior to bisulfite-conversion with the EZ-96 DNA Methylation™ Kit (Zymo Research, Irvine, California, USA)

a. DNA purification QIAamp® DNA Mini kit (Qiagen, Hilden, Germany)

DNA purification QIAamp® DNA Mini kit, as described in approach 2.

b. DNA quantification

DNA quantification as described in approach 2.

c. Bisulfite treatment of purified DNA EZ-96 DNA Methylation™ Kit (Zymo Research, Irvine, California, USA)

In the conducted experiment, 5 µl of M-Dilution Buffer was added to each DNA sample, and the total volume was adjusted to 50 µl with water. Each sample was mixed by pipetting up and down. Subsequently, the samples were incubated at 37°C for 15 minutes. Following this incubation, 100 µl of the prepared CT Conversion Reagent was added to each sample, and the mixture was thoroughly mixed. The samples were then incubated in the dark at 50°C for 12-16 hours. Afterward, the samples were incubated at 0-4°C for 10 minutes.

For the purification step, 400 µl of M-Binding Buffer was added to a Zymo-Spin™ IC Column, and the column was placed into a provided Collection Tube. The samples were loaded into the Zymo-Spin™ IC Column containing the M-Binding Buffer. The cap was closed, and the column was mixed by inverting it several times. Subsequently, the samples were centrifuged at 5000 x g for five minutes, and the flow-through was discarded.

Following the centrifugation step, 100 µl of M-Wash Buffer was added to each column, and centrifugation was carried out at full speed for 30 seconds. Then, 200 µl of M-Desulphonation Buffer was added to each well and allowed to stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifugation was performed at 5000 x g for five minutes.

To further wash the column, 200 µl of M-Wash Buffer was added, and centrifugation was carried out at full speed for 30 seconds. Another 200 µl of M-Wash Buffer was added, followed by centrifugation for an additional 30 seconds. The column was then placed into a 1.5 ml microcentrifuge tube.

Finally, 6.5 µl of M-Elution Buffer was added directly to the column matrix for samples with DNA concentration below 3ng/µl, and 12.5 µl M-Elution Buffer for samples with DNA concentration above 3ng/µl. Centrifugation was conducted for 30 seconds at full speed to elute the DNA.[39]

d. QIAsure DNA-methylation sample processing on Rotor-Gene Q MDx instrument

QIAsure DNA-methylation sample processing on Rotor-Gene Q MDx instrument, as described in approach 1.

5.3 RESULTS

Mean age of women was 39,3 years (25 – 64 years). Cytology data were the following: 59 women with NILM, 15 women with ASC-US, six women with ASC-H, 17 women with LSIL, and 44 women with HSIL, two women with atypical glandular cells of unknown significance (AGUS) and two suspicious of malignancy. One woman had an inadequate cytology.

In total, 36 women had no CIN, 25 women had CIN1, 36 women had CIN2, 44 women had CIN3, one woman had adenocarcinoma in situ (AIS) and four women had cervical squamous cell carcinoma (SCC). The overall positivity rate for FAM19A4/hsa-mir124-2 methylation was 123 (84,2%). The positivity rates for FAM19A4/hsa-mir124-2 methylation were significantly higher in WLWH than in HIV negative women (P = 0.0023).

Table 5.2 Cytology results amongst WLWH and HIV negative women.

	WLWH n = 92		HIV neg n = 54		Total N = 146		p value
Cytology	Count	%	Count	%	Count	%	
Negative	31	33,7	28	51,9	59	40,4	P = 0.0311
ASC-US	4	4,3	11	20,4	15	10,3	P = 0.0020
ASC-H	3	3,3	3	5,6	6	4,1	P = 0.5026
LSIL	14	15,2	3	5,6	17	11,6	P = 0.0819
HSIL	35	30,0	9	16,7	44	30,1	P = 0.0745
AGUS	2	2,2	0	0	2	1,4	P = 0.2740
Ca/suspicion/malignant cells	2	2,2	0	0	2	1,4	P = 0.2740
Inadequate	1	1,1	0	0	1	0,7	P = 0.4409

Table 5.3 Histology results amongst WLWH and HIV negative women.

	WLWH n = 92		HIV neg n = 54		Total N = 146		p value
Histology	Count	%	Count	%	Count	%	
Negative	21	22,8	15	27,8	36	24,7	P = 0.5000
CIN1	11	12,0	14	25,9	25	17,1	P = 0.0320
CIN2	25	27,2	11	20,4	36	24,7	P = 0.3592
CIN3	31	33,7	13	24,0	44	30,1	P = 0.0877
SCC	3	3,3	1	1,9	4	2,7	P = 0.6207
AIS	1	1,1	0	0	1	0,7	P = 0.4409

Table 5.4 Methylation detection amongst WLWH and HIV negative women.

	WLWH n = 92		HIV neg n = 54		Total N = 146		p value
Methylation positive	Count	%	Count	%	Count	%	
FAM19A4/hsa-mir124-2	84	91,3	39	72,2	123	84,2	P = 0.0023

The performance of FAM19A4/hsa-mir124-2 methylation in detecting CIN2 and CIN3 lesions was evaluated, with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) calculated across different cohorts.

For CIN2 lesions, the overall sensitivity, specificity, PPV, and NPV of FAM19A4/hsa-mir124-2 methylation were 83,33%, 16,19%, 25,42%, and 73,91%, respectively. Notably, the sensitivity of FAM19A4/hsa-mir124-2 methylation in detecting CIN2 was substantially higher in WLWH compared to HIV-negative women, at 92,00% and 63,64%, respectively. However, when the disease threshold increased to CIN3, the sensitivity for HIV-negative women improved to 84,62%.

In terms of specificity, the lowest value was observed in WLWH (8,96% for CIN2), while the highest specificity was noted in the HIV-negative cohort (31,71% for CIN3). Interestingly, the NPV was consistently high in the WLWH cohort, with comparable results for both CIN2 and CIN3 (75.00% [95% CI 39.31 – 93.29] and 75.00% [95% CI 39.12 – 93.34], respectively).

For CIN3 lesions, the overall sensitivity, specificity, PPV, and NPV of FAM19A4/hsa-mir124-2 methylation were 90.91%, 18.63%, 32.52%, and 82.61%, respectively. Similar to CIN2, WLWH exhibited higher sensitivity (93.55%) compared to HIV-negative women (84.62%), although with lower specificity. The NPV remained high across cohorts, particularly in WLWH (75.00% [95% CI 39.12 – 93.34]).

Table 5.5 Performance of DNA-methylation analysis for histologic outcome CIN2.

	Sensitivity, % [95% CI]	Specificity, % [95% CI]	PPV, % [95% CI]	NPV, % [95% CI]
Total n=146	83,33 [67,19 – 93,63]	16,19 [9,72 – 24,65]	25,42 [22,36 – 28,75]	73,91 [54,77 – 86,90]
WLWH n=92	92,00 [73,97 – 99,02]	8,96 [3,36 – 18,48]	27,38 [24,73 – 30,21]	75,00 [39,31 – 93,29]
HIV – n=54	63,64 [30,79 – 89,07]	25,58 [13,52 – 41,17]	17,95 [11,92 – 26,12]	73,33 [51,96 – 87,49]

Table 5.6 Performance of DNA-methylation analysis for histologic outcome CIN3.

	Sensitivity, % [95% CI]	Specificity, % [95% CI]	PPV, % [95% CI]	NPV, % [95% CI]
Total n=146	90,91 [78,33 – 97,47]	18,63 [11,60 – 27,55]	32,52 [29,70 – 35,47]	82,61 [63,17 – 92,94]
WLWH n=92	93,55 [78,58 – 99,21]	9,84 [3,70 – 20,19]	34,52 [31,77 – 37,38]	75,00 [39,12 – 93,34]
HIV – n=54	84,62 [54,55 – 98,08]	31,71 [18,08 – 48,09]	28,21 [22,34 – 34,92]	86,67 [62,72 – 96,17]

5.4 DISCUSSION

5.4.1 Triage capability of the QIAsure DNA methylation assay

The objective of this study was to evaluate the triage capability of the QIAsure assay for detecting biomarkers FAM19A4 and mir124-2 in other hrHPV positive women, excluding HPV 16 and HPV 18. Additionally, the study aimed to compare DNA methylation results with increasing severity of histopathology results, and to calculate the sensitivity, specificity, and predictive values of DNA methylation.

The findings of this study highlight notable differences in the performance of DNA methylation analysis between CIN2 and CIN3 lesions. While the sensitivity of FAM19A4/hsa-mir124-2 methylation was relatively high for both CIN2 (83.33%) and CIN3 (90.91%), there were significant variations in specificity and PPV. Specifically, the specificity was lower for CIN2 lesions compared to CIN3, indicating a higher rate of false positives in detecting less severe lesions.

The study also revealed distinct differences in the performance of DNA methylation analysis between WLWH and HIV-negative women. WLWH exhibited higher sensitivity for detecting both CIN2 (92.00%) and CIN3 (93.55%) compared to HIV-negative women. This observation suggests that DNA methylation markers may be particularly effective in detecting cervical neoplasia in WLWH, potentially serving as a valuable tool for early detection and management in this population. However, it is important to note that WLWH also showed lower specificity compared to HIV-negative women, indicating a higher rate of false positives.

The 95% confidence intervals, in all instances, have quite a broad range, which indicates uncertainty in the estimate. A narrower confidence interval would imply a more precise estimate of the test performance measure, while a wider interval implies less precision. For example, the sensitivity for detecting CIN3 using the FAM19A4/miR124-2 methylation test in WLWH is 93,55% [78,58 – 99,21]. This variability suggests that while the point estimate of 93,55% sensitivity is a useful benchmark, the actual sensitivity of the test could be as low as lower limit of the range or as high as the upper limit of the range. This is likely due to the small sample size under investigation in our analysis.

Nevertheless, consistent trends in the performance of DNA methylation markers for cervical neoplasia detection have been reported, showing adequate sensitivity and varying specificity across various populations and settings.

In a European multicentre study including Scotland, Denmark, Slovenia, and The Netherlands, Bonde et al. observed a sensitivity of 77% (n = 228; 95%CI: 71-82) for detecting CIN3 using the FAM19A4/miR124-2 methylation test. For CIN2, sensitivity varied across the four centres, ranging from 33.3% in Scotland to 61.1% in Denmark.

Regarding specificity, the FAM19A4/miR124-2 methylation test demonstrated an overall specificity of 78.3% (n = 2012; 95%CI: 76-80) among women positive for hrHPV, with variability observed across regions, from 71.1% in Scotland to 80.3% in The Netherlands. The PPV of hrHPV-positive and methylation-positive outcomes for detecting CIN3 or higher was 28.3% (N = 690; 95%CI: 25-32), while for detecting CIN2 or higher, it was 36.7% (95%CI: 33-40). The authors conclude by acknowledging that cytology will remain the triage method of choice in primary HPV screening but recommends the introduction of the FAM19A4/miR124-2 methylation test into pilot cervical cancer screening programmes. This will offer additional data and insights to help shape a comprehensive molecular cervical screening programme that benefits both women and healthcare services. [31]

5.4.2 A journey through DNA-methylation analysis

In research, the journey to uncovering new knowledge is frequently marked by obstacles, setbacks, and unexpected twists. Such was the case in our endeavour to perform the QIAsure DNA-methylation assay, a journey marked by resilience, collaboration, and ultimately, a eureka moment. Our journey into the intricate world of DNA-methylation began with a simple

directive: use the EpiTect Bisulfite Kit and apply bisulfite treatment directly on cervical specimens. The advice seemed straightforward, but it raised some red flags in our minds. Most sources suggested a different approach, one that recommended starting with DNA extraction before bisulfite treatment. Scepticism lingered, especially for me, as I recalled my own encounter with DNA degradation during the Linear Array HPV genotyping test on cervical scrapes, that I performed during my master's degree.

Despite reservations, we sought the wisdom of experienced professionals. Conversations with the research and development team at Qiagen reinforced the notion that direct bisulfite conversion was our path forward. Their rationale was that this method was optimal for individuals with limited time in the laboratory, especially those not accustomed to routine laboratory work, given the high volume of specimens requiring testing.

The protocol under investigation had never been performed in South Africa prior to this study. I had the remarkable opportunity to undergo training at the Self-screen laboratories, where the assay was originally developed. While my stay was brief, spanning only a week, we meticulously ran through the entire protocol at least four or five times. This thorough training instilled in me a keen sense of confidence to independently carry out the procedure at Stellenbosch University. The initial plan was to conduct the laboratory testing at our institution within the Department of Medical Virology. However, neither the Department of Medical Virology nor any other laboratory that we had contacted in Cape Town had a suitable centrifuge required for the "cleanup of bisulfite-converted DNA using a centrifuge" step in the protocol. Several weeks had passed, yet we remained without a solution. We maintained continuous communication with Qiagen throughout this entire process, but even they were unable to offer a resolution. After numerous email exchanges, several meetings, and visits to various laboratories, we eventually identified a private laboratory in Johannesburg, Lancet Laboratories, that could provide assistance. With this discovery, we anticipated that the remainder of the process would proceed smoothly. We were wrong.

Initially, our challenges were unrelated to the assay itself; we experienced sample spillage during transport in air cargo, resulting in an incorrect input volume (we had less than the required amount of sample). Additionally, the Qiagen Sales Application Specialist encountered difficulties installing the Rotor-Gene Assay Manager application, causing an

unexpected delay in our planned progression. Nevertheless, towards the end of the visit, once all technical hurdles were resolved, we decided to proceed with a trial run. Unfortunately, out of the 32 samples tested, 26 produced invalid results (using approach 1).

The internal sample quality control, β-actin (ACTB), was used as a reference to confirm successful bisulfite conversion and ensure sample quality. For results to be interpretable, a minimal amount of bisulfite-converted DNA must be present, indicated by a ΔΔ cycle threshold (Ct) value of the housekeeping gene ACTB of ≤ 26.4. Samples meeting this criterion are validated by the Rotor-Gene Assay Manager.

Methylation was considered positive if the QIAsure methylation test results surpassed the established Ct value for either FAM19A4 or miR124-2, as per the manufacturer's guidelines. If the internal sample quality control is negative (i.e., ACTB Ct values > 26.4), it indicates that the bisulfite-converted DNA preparation is of insufficient quantity and/or quality, resulting in an invalid score for the sample.

For the subsequent round of testing, we prepared new aliquots to ensure accurate specimen volumes (this was done at the laboratory where the samples were stored). We followed the same workflow procedure and conducted four runs, testing a total of 218 samples. However, disappointingly, 143 of these samples yielded invalid results (using approach 1).

We had met with both the Qiagen and Self-Screen (legal manufacturer of the FAM19A4/miR124-2 methylation assay) teams to discuss the unsuccessful laboratory attempts. Subsequently, both Qiagen and Self-Screen advised we follow the DNA extraction prior to bisulfite conversion, workflow. We thus prepared new aliquots and conducted DNA extraction on the previously invalid samples. Following this, we quantified the concentration of each sample and proceeded with DNA-methylation analysis, prioritizing those with the highest concentrations. Out of the 64 samples tested, 39 yielded invalid results (using approach 2).

The research and development scientist supervising me in the laboratory suggested we proceed with a different bisulfite kit, one that had a simpler workflow and that she was familiar with…this is where we had our first eureka moment. Of the 20 samples tested, only two were invalid. Had we opted for this kit from the start, we could have avoided the hassle of finding a centrifuge with a compatible deep well plate adapter. Moreover, this alternative

kit comes at a significantly lower price compared to the original kit supplied. We achieved consistent success with all subsequent runs (using approach 3).

Regarding the differences between the EZ-96 DNA Methylation™ Kit (Zymo Research, Irvine, California, USA) and the EpiTect Fast 96 Bisulfite Kit (Qiagen, Hilden, Germany), there are notable distinctions worth considering. Firstly, the Zymo kit operates on a column-based system, offering flexibility in the number of columns utilized without the need to wait for a full batch of 96 specimens or risking contamination by leaving certain wells uncovered.

Secondly, the diameter of the filter in the columns differs between the two kits. The Zymo-kit's filter has a diameter of 2mm, while the 96-well plate's filter is 6mm. Consequently, when adding 10 µl of elution buffer to both, the eluate from the Zymo kit yields approximately 5-7 µl of concentrated DNA, whereas the original supplied kit produces only 2-3 µl. This discrepancy indicates that the Qiagen filter may not fully elute all DNA, resulting in a lower concentration in the eluate. We added around 6.5 µl of elution buffer to the Zymo column, which still provided adequate eluate for PCR. However, with the 96-well plate, insufficient eluate for PCR is obtained unless at least 10 µl of elution buffer is added. This disparity in elution volume can significantly impact the final DNA concentration in the eluate and subsequently influence PCR results.

The observed invalid rate is much higher compared to other studies utilizing the same test and platforms.[31,32] This discrepancy may stem from numerous factors, such as sample age, sample storage or transport challenges, amongst others.

While molecular techniques are invaluable in cervical cancer screening and triage their intricate nature heightens the risk of errors. Numerous factors, ranging from specimen quality to laboratory conditions during analysis, can significantly influence outcomes. Package inserts and protocols accompanying assay kits often highlight procedural limitations. The protocol of the QIAsure DNA-methylation assay, for instance, states that reliable results are dependent on adequate specimen collection, transport, storage, and processing procedures. There is also a possibility of obtaining false positive and false negative results because of the aforementioned factors.

The current protocol could be considered laborious. In recognition of this, Self-screen BV, in collaboration with GC Biotech, have developed an automated workflow for the protocol

which promises an improved throughput, reproducibility, robustness and accuracy (presented at Eurogin Congress 2024). [40]

5.5 CONCLUSION

Based on the findings of this study, we cannot draw any strong conclusions about the utility of methylation for triage. The data suggests certain trends and patterns; however, these are not robust enough to make definitive statements. Further research with a larger sample size is needed to validate these preliminary observations and reach more concrete conclusions. This study highlights the complexity of DNA-methylation analysis and the importance of continued investigation.

Further randomized trials and longitudinal follow-up data across diverse populations and resource settings are essential for a comprehensive clinical and epidemiological validation of the assay. As with the stringent criteria governing the use of HPV DNA screening, a similarly rigorous approach should be adopted for DNA-methylation testing, particularly given its potential role in primary screening. The assay's inflated cost at this time presents a significant barrier, making its integration into national screening programmes in LMIC unfeasible at this stage.

Considering the observed discrepancy in the invalid rate, future research initiatives should prioritize the adoption of the automated workflow. This advanced approach not only promises enhanced throughput, reproducibility, robustness, and accuracy, as demonstrated by Self-screen BV at Eurogin Congress 2024, but also offers a potential solution to mitigate the factors contributing to variability in sample processing and analysis.

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CHAPTER 6

Clinical trial protocol: Cervical precancer thermal ablation versus LLETZ excision comparative efficacy study (TALL STUDY).

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6.1 ABSTRACT

Background: Cervical cancer remains a significant global health concern and is the fourth most prevalent cancer among women. In South Africa, it is the leading cause of cancer-related deaths in women aged 15 to 44 years. The disease is typically preceded by persistent high-risk HPV (hrHPV) infection, leading to cervical intraepithelial neoplasia (CIN) and eventually cancer. Current management involves excision, particularly through large loop excision of the transformation zone (LLETZ), which has associated risks and limitations. Thermal ablation (TA) is an alternative cost-effective treatment method, providing a straightforward approach to treatment, particularly advantageous in environments characterized by limited resources. The study aims to assess the efficacy, safety, and patient experience of TA, providing valuable data for potential integration into South Africa's cervical cancer prevention policies.

Methods: Four hundred twenty women aged 30 to 60 years will be recruited from the Colposcopy Clinic at Tygerberg Hospital and will be followed up for a period of two years. The primary study endpoint is test of cure that will be assessed by HPV testing and cervical cytology. Other endpoints include the occurrence of adverse events and cervical structural changes that will be evaluated by colposcopy and transvaginal ultrasound.

Discussion: This paper outlines a study protocol for a randomized trial comparing the cure rate and effects of thermal ablation to those of LLETZ in treating cervical precancer. In the context of South Africa's HIV burden and hrHPV prevalence, understanding the effectiveness of TA is crucial for developing cost-effective and accessible treatments. This research will contribute to improving cervical cancer management and guiding evidence-based treatment guidelines. The outcomes may influence the integration of TA into South Africa's cervical cancer treatment framework, impacting public health.

Trial registration: SANCTR unique number DOH-27-062023-4853;

https://sanctr.samrc.ac.za/TrialDisplay.aspx?TrialID=9313 Protocol Version 3

Keywords: cervical intraepithelial neoplasia, large loop excision of the transformation zone, thermal ablation

6.2 BACKGROUND

6.2.1 The burden of cervical cancer

Ranked as the fourth most frequently diagnosed cancer and fourth most common cause of death in women, cervical cancer resulted in approximately 604 127 diagnosed cases and 341 831 deaths in 2020 worldwide.[1] The Catalan Institute of Oncology/International Agency for Research on Cancer (ICO/IARC) HPV Information Centre estimates for 2023 indicate that in South Africa, 10 702 women are diagnosed with cervical cancer annually, while 5 870 women die from the disease. [2] Cervical cancer remains the number one cause of cancer death in women in South Africa, particularly among women of reproductive age, 15 to 44 years. [1,2]

6.2.2 HPV infection and pathological changes in the cervix

The cervix is a cylindrically shaped fibromuscular organ, approximately 4 cm in length and 3 cm in diameter, that links the uterine cavity to the vagina through the endocervical canal. Within the cervix, the anatomy is subdivided into the endocervix and the ectocervix. The endocervix contains columnar glandular epithelium, while the ectocervix comprises stratified squamous epithelium extending onto vaginal skin. The border between the endocervix and ectocervix is the squamocolumnar junction (SCJ), which is the most common site at which the human papillomavirus (HPV)-induced premalignant cervical intraepithelial neoplasia (CIN) occurs.[3] Other sites of occurrence include the vaginal wall and vulvar epithelium.[4,5]

Cervical cancer is almost always preceded by persistent oncogenic HPV infection resulting in CIN graded from CIN 1 to CIN 3 depending on the severity of dysplasia.[6] Most cases of CIN remain stable or regresses spontaneously without intervention, depending on the hosts immune response while only a few lesions persist or progress to invasive carcinoma of the cervix, typically squamous cell carcinoma and adenocarcinoma.[7–10]

It is assumed that it takes an average of 10 to 20 years before a persistent high-risk HPV (hrHPV) infection may ultimately lead to invasive cervical carcinoma.[8,9] Since malignant transformation has a long latency period, the detection and effective treatment of cervical precancer are crucial for preventing disease progression.[11]

6.2.3 Management of cervical intraepithelial lesions

The approach to managing CIN is based primarily on the patient's risk for progression to cancer. Risk factors include age and grade on CIN. Women younger than 25 years have a lower risk of developing cervical cancer than those 25 years and older. [1,12] CIN grade, CIN 1 has a low potential for progression to malignancy and a high likelihood for regression, while CIN 2/3 has a greater potential for progression and a lower potential for regression.[13,14]

The two general approaches to management are as follows: 1) observation, with HPV testing, cytology, and/or colposcopy; and 2) treatment, with excision or ablation of the cervical transformation zone, which is the dynamic area containing the transition from the squamous epithelium of the ectocervix to the glandular epithelium of the endocervix and specialized cells that are thought to be susceptible to HPV infection and transformation. [3]

6.2.4 Large Loop Excision of the Transformation Zone

Currently, the preferred treatment method of premalignant lesions in South Africa is excision using large loop excision of the transformation zone (LLETZ). The LLETZ procedure is advantageous in that it allows for histopathological diagnosis. The LLETZ procedure, however, requires electricity, sophisticated equipment, and trained healthcare personnel to provide the needed care in addition to access to histopathology laboratories.

Risks associated with the LLETZ procedure include treatment failure (TF) or disease recurrence, especially in women living with HIV (WLWH) and impaired pregnancy outcomes. In addition to treatment-specific concerns, procedural complications such as cervical bleeding requiring suturing, bowel and bladder injury, and infection have been documented in the literature. [15–18]

Reimers et al., reported TF in 55% of WLWH with CIN 2+ and concluded that “clinicians treating CIN in WLWH should avoid raising expectations of cure and instead focus on the achievable goal of cancer prevention until there are better therapies for this patient population”.[19] Similarly, Debeaudrap et al., reported a TF prevalence of 21.4% (95% confidence interval [CI] 15.8–27.0) in WLWH. Treatment failure was significantly greater in

WLWH than in immunocompetent women (OR 2.7, 95% CI 2.0–3.5). A predictor of TF was positive margin status (OR 3.4, 95% CI 1.5–7.7).[20]

There is a strong and consistent association between LLETZ and subsequent preterm birth, as summarized in meta-analyses and observed in several countries.[21,22] However, more recent research suggests that increased risk may be associated with the depth of excision (10–14 mm, and particularly >15 mm) and that the reason for the lack of association in some studies was that the majority of women treated had small excisions. [23]

6.2.5 Thermal Ablation

Despite compelling reasons for operationalizing the inclusion of thermal ablation (TA) (also referred to as cold coagulation or thermocoagulation) in treatment guidelines, TA has not yet been widely implemented. Other than clinician concerns that ablation may overlook early cancer and is proven to be inadequate for treating large lesions, study trends suggest that both the LLETZ and TA have comparable efficacy. In a meta-analysis, Dolman et. al., reported an 87%–95% rate of CIN 2+ cure by cold coagulation.[24] Similarly, in a meta-analysis including 23 studies and 6 371 patients, six of which were analysed in LMIC, TA showed a treatment efficacy of 93.8% for CIN2+ lesions, as determined by biopsy. The study concluded that TA appears to be an effective treatment for CIN2+ lesions across a variety of settings, including LMICs, where its other commonly cited advantages include ease of use and simplicity.[25] A 2017 retrospective cohort study revealed that the cure rate for cold coagulation was 91.6%, and that for LLETZ was 97.1% at the six month follow-up, but the difference in cure rate between the LLETZ and cold coagulation was negligible at the 12 month follow-up.[26]

Furthermore, TA has proven to have additional advantages for LMICs, including requiring minimal electricity to charge batteries for battery-driven devices and using solar power for some models. Notably, many health care providers may find TA easier to provide because it is less time consuming and easier to perform. Additionally, it has been found to have fewer side effects, to be relatively less expensive than other treatment methods and to be technically simpler to implement, as it can be portable and delivered by trained nurses rather than doctors.[27–29]

The World Health Organization (WHO) guidelines for the use of thermal ablation for cervical precancer lesions recommend "thermal ablation as the treatment of choice and LLETZ only when ineligible for ablative treatment for patients with histologically confirmed CIN2+", as well as for patients who screen positive for hrHPV or visual inspection with acetic acid (VIA) or hrHPV followed by VIA.[30] Consistent with the above-cited efficacy and feasibility considerations for TA, the WHO Technical Guidance and Specifications of Medical Devices for Screening and Treatment of Precancerous Lesions in the Prevention of Cervical Cancer reiterates that TA has documented efficacy and is recommended for low-resource settings.[31]

6.2.6 HPV DNA prevalence in South Africa

Cervical HPV infection rates exhibit variability across continents, countries, regions, and population subgroups, with the highest reported prevalence observed in studies conducted within populations in sub-Saharan Africa and among WLWH. [1,32,33]

As reported previously, cervical cancer remains the number one cause of cancer death in women in South Africa, particularly among women of reproductive age, 15 to 44 years, and approximately 25% of these women are living with HIV.[34] Furthermore, it has been estimated that approximately 3.2% of South African women in the general population harbour HPV16/18 infection at a given time. [34,35]

A retrospective descriptive study conducted by Van Aardt et. al., found that in 1 238 women without cervical cytological abnormalities, the prevalence of hrHPV infections was 44.92%. [36] Similarly, Mbulawa et al., demonstrated an HPV infection prevalence of 36.7 % (76/207; 95 % confidence intervals (CI): 30.4–43.4 %) in HIV-negative women and a high HPV prevalence of 74.0 % (205/277; 95 % CI: 68.5–78.8 %) among WLWH. [37] Denny et.al., reports HPV-positivity in 41.5% of WLWH and 17.4% of HIV negative women, in a demonstration study conducted in South Africa, which included 3 062 women aged 30–65 years. [38]

Considering the above, South Africa could improve current cervical precancer treatment standards by introducing TA to current treatment policies. However, there are no in-country efficacy data supporting South Africa's introduction of TA in addition to the LLETZ.

The aim of the current study is to assess the comparative efficacy of thermal ablation as a treatment option for eligible participants with confirmed precancerous lesions compared to excision treatment. Patient safety and experience will also be evaluated.

The evidence generated by this study may be useful for introducing TA to the South African cervical cancer treatment landscape.

6.3 MATERIALS AND METHODS

6.3.1 Study design and overview.

The randomized controlled trial will include at least 420 evaluable participants, aged 30 to 60 years. Participants will be invited to participate from the Colposcopy Clinic, as described in Figure 6.1., and will be followed for a period of two years, as described in Figure 6.2. The following selection criteria will be applied for the screening segment of the study (Table 6.1):

Table 6.1: Inclusion and exclusion criteria for the screening segment of the study.

Inclusion:	Exclusion:
Female gender	Previous hysterectomy/Current pregnancy
Age 30 years and above; younger than 60 years	Age younger than 30 years or older than 60 years
Negative pregnancy test	Hesitant or unable to undergo screening and treatment if indicated
Never treated for precancer/cancer	Current or previous treatment for gynaecological cancer
Two episodes of Low-grade squamous intraepithelial lesions (LSIL) at least six months apart on cytology or one High-grade squamous intraepithelial lesion (HSIL)	
Informed consent accepted and signed	

<u>Screening visit</u>

- Confirm eligibility criteria.
- Informed consent and discussion, including optional donation of leftover specimens.

- If the HIV status is negative or unknown, a rapid HIV test will be offered.
- Demographics and medical history.
- Self-sampling for HPV testing.
- Perform TVUS for cervical length.
- Speculum examination and collection of cervical specimens for HPV testing, storage, and cervical cytology.
- Perform colposcopy to determine whether lesion is eligible for TA.

  Inclusion:

  - Type 1 transformation zone with treatable lesion.
  - Type 2 transformation zone where the probe tip will achieve complete ablation of the squamocolumnar junction epithelium and lesion.

  Exclusion:

  - If there is any suspicion of invasive or glandular disease (i.e., adenocarcinoma or adenocarcinoma in situ).
  - The transformation zone is not fully visible because it is endocervical (Type 3 transformation zone).
  - It is a Type 2 transformation zone where the squamocolumnar junction is out of reach of the probe tip.
  - A lesion where the upper limit is not visible.

<u>Randomization (screening and randomization at same visit)</u>

- Randomize to TA or LLETZ.
- If randomized to the TA treatment arm, obtain cervical biopsies.
- Perform TA or LLETZ according to randomization.

<u>6 Week telephonic follow-up (-1 + 3)</u>

- Participants will be contacted 6 weeks after the procedure. A standardized questionnaire will be completed via telephone to assess the participants experience and possible side effects after the procedure.

26 Week follow-up (-1 + 3)

- Self-sampling for HPV testing conducted at research site.

52 Week follow-up (-1 + 3)

- If the HIV status is negative, a rapid HIV test will be offered.
- Speculum examination and collection of cervical specimens for HPV testing, storage, and cytology.
- Perform colposcopy.
- Transvaginal ultrasound (TVUS) for cervical length.

Interim treatment visit (within 10 weeks of receiving Week 52 follow-up cervical cytology and HPV results)

Treat with LLETZ procedure if:

- ASC-H or HSIL on cervical cytology.
- ASCUS or LSIL on cervical cytology and any positive result on HPV test.
- NILM on cervical cytology and positive HPV16 or HPV18 on HPV test.

78 Week follow-up (-1 + 3)

- Self-sampling for HPV testing conducted at research site.

104 Week follow-up (-1 + 3)

- If the HIV status is negative, a rapid HIV test will be offered.
- Speculum examination and collection of cervical specimens for HPV testing, storage, and cytology.
- Perform colposcopy.
- TVUS for cervical length.

Interim treatment visit (within 10 weeks of receiving Week 104 follow-up cervical cytology and HPV results)

Treat with LLETZ procedure if:

- ASC-H or HSIL on cervical cytology.
- Positive HPV16 or HPV18 on HPV test.

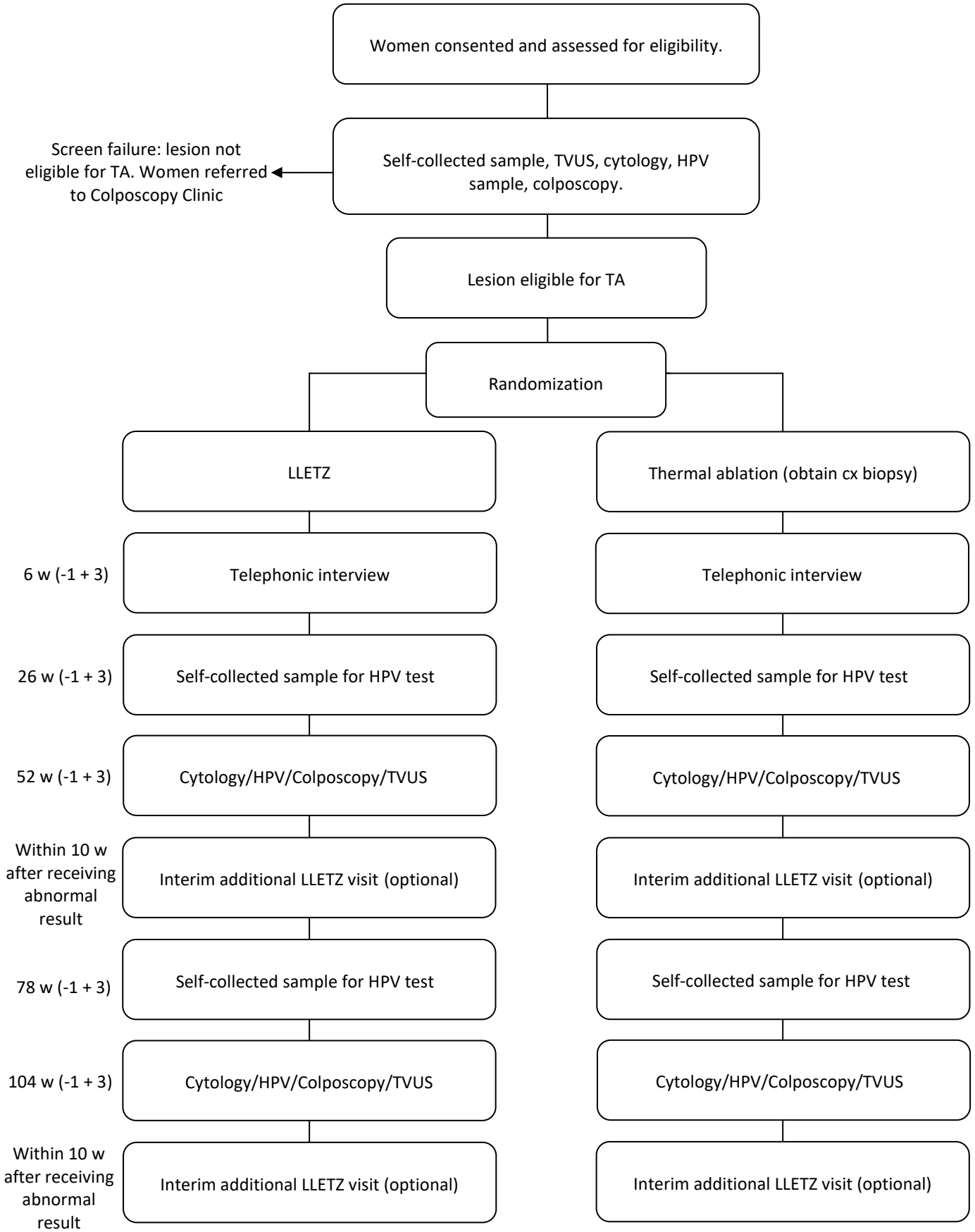

Figure 6.2 TALL study schematic.

6.3.2 Ethics

The protocol of this study was submitted and approved by the Health Research Ethics Committee (HREC) of Stellenbosch University (Ethics Reference No: M20/11/035). The protocol has also been submitted and approved by the Western Cape Department of Health, via the National Health Research Database (NHRD) to conduct research at Tygerberg Hospital (WC_202109_016).

All study procedures will comply with ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice, and the Medical Research Council (MRC) Ethical Guidelines for Research. Furthermore, all the participating researchers and investigators are familiarised with the ethical code of conduct and are certified in good clinical practice (GCP).

The following cover has been arranged for clinical trials (insurance for research-related adverse events) in terms of the following two policies:

1. No fault Compensation Insurance policy number 73112119A017 underwritten by Lloyds for a limit of USD5 000 000 any one claim and in the aggregate.
2. Professional Liability Insurance policy number 4000/24901 underwritten by Stalker Hutchison Admiral for a limit of ZAR150 000 000. This policy has been extended to include Medical Malpractice.

6.3.3 Participant recruitment

The research team, comprising the principal investigator (PI), sub investigator, medical doctor, field translator (for Afrikaans and isiXhosa or other local languages), and research assistant will recruit volunteers from the Colposcopy Clinic. Ultimately, the PI is responsible for ensuring that informed consent is appropriately obtained and documented. The potential participant will be briefly introduced to the study and asked whether she will be interested in participating in the study. In the case where the potential participant indicated that she is interested to be enrolled in the study, a formal informed consent process will be done in a private area to ensure confidentiality. The person conducting the informed consent process must discuss all the information in the consent form. The informed consent discussion

includes an explanation of all aspects of the informed consent form. The person obtaining informed consent will assess the potential participants' understanding of the contents and process of providing informed consent. If needed, the understanding of the study will be verified by the individuals mentioned above and proper notes will be made in the source notes.

If the potential participant indicates interest in being enrolled, she will be asked to sign the informed consent form. Initials only must not be allowed to serve as a signature. It should be explained to the potential participant that full names (as indicated on the birth certificate or South African Identity document), signatures and dates should be on the document as per ICH clinical guidelines and South African Good Clinical Practice Guidelines.

If the potential participant declines to participate in the study, she must be reassured that this will not affect her clinical treatment.

The person who provided informed consent will then provide his/her full name, sign, and date the informed consent form only after ensuring that the potential participant understands the informed consent process and has signed the form. The time that consent was obtained must be captured on the document to verify that consent was obtained prior to performing any study-related procedures. Inform the potential study participant that they can withdraw at any time during the study and that the withdrawal will not affect their clinical treatment. Copies of consent forms will be made for each volunteer.

The goal is to recruit all study participants within 16 months. To meet this goal, 25 participants must be randomized each month.

Investigators are expected to work with other departments within the institution, the Community Advisory Board (CAB), and outside referral centres to ensure that recruitment goals are met. A referral information form may be used to notify other clinics or departments within the institution of the study.

If recruitment is low at any time, the following strategies will be used:

1. Clinical investigator(s) reach out to other members of the department and other clinics to inform physicians and nurses about the study.

2. Clinical investigator(s) will engage the local Community Advisory Board to help participants know about the study.
3. Clinical investigators will contact local clinical sites, including affiliates and NGOs where pelvic exams and cervical cancer screenings are performed to request referrals.

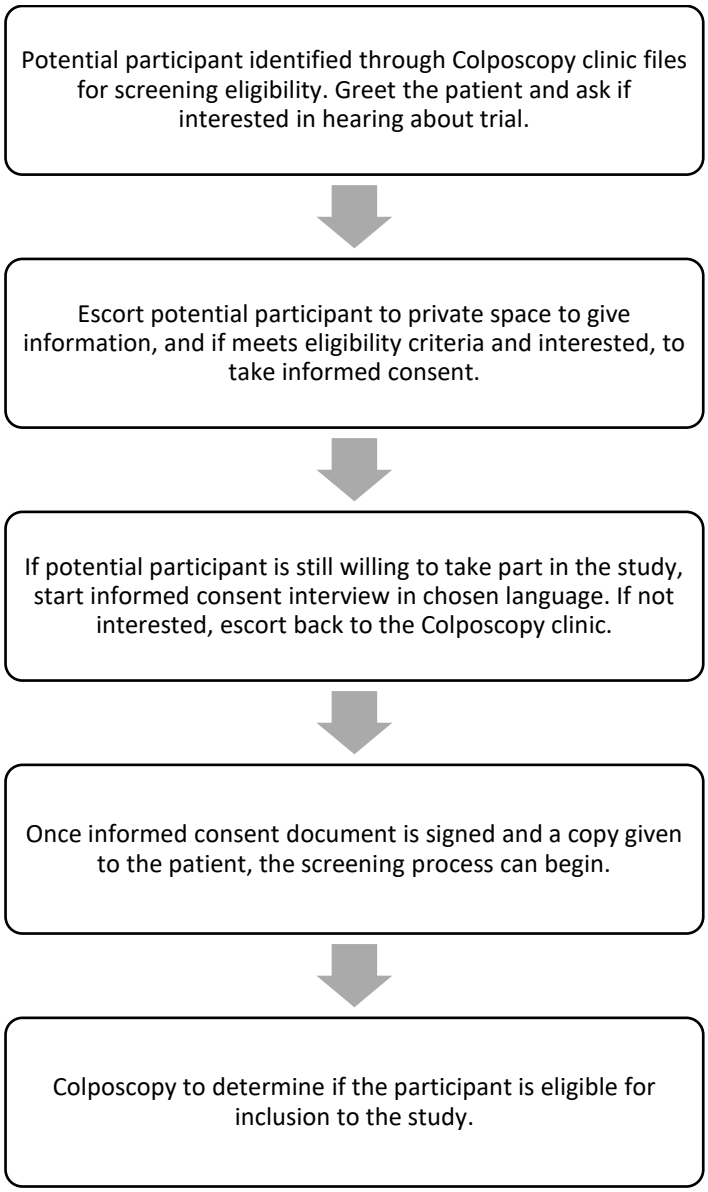

Figure 6.1. Flowchart of participant recruitment for the TALL study.

6.3.4 Procedures

6.3.4.1 Transvaginal ultrasound

As discussed previously, cervical length measurement is crucial during pregnancy to assess the risk of preterm birth. A shortened cervix is considered a risk factor for preterm labour and delivery. Cervical length will thus be measured by TVUS, before and after treatment.

6.3.4.2 Cervical cytology

Samples will be collected by trained health care workers during speculum examination with a cervical collecting brush. Cytology will be used as one of the measures of "test of cure" after treatment of precancerous lesions.

6.3.4.3 BD Onclarity™ HPV Assay

The BD Onclarity™ HPV Assay is a qualitative in vitro test for the detection of HPV in cervical specimens using an endocervical brush/spatula combination or broom and placed in a BD SurePath™ vial. The test utilizes amplification of target DNA by polymerase chain reaction (PCR) and nucleic acid hybridization for the detection of 14 hrHPV types in a single analysis. The test specifically identifies types 16, 18, 31, 45, 51, and 52 while reporting the other HR HPV types in groups (33/58, 35/39/68, and 56/59/66).

6.3.4.4 Colposcopy

Colposcopy aims to identify and diagnose potential cervical pathologies such as CIN and invasive cancer. Colposcopy examinations will take place at the screening visit and at weeks 52 and 104. Digital images of the cervix will be obtained. Each participant undergoing colposcopy will have a low magnification image before and after the application of 5% acetic acid. Each image will be annotated, noting biopsy sites (where applicable) and/ or lesions if present.

6.3.4.5 Cervical biopsy

During colposcopy examination a biopsy for histology will be taken at enrolment for participants who are randomized into the TA treatment arm. The biopsy will be taken using standard forceps (e.g., Berger, Tischler or Kevorkian). A biopsy will be taken from the most

abnormal area on colposcopy or from a normal area of the cervix if no abnormalities are identified.

6.3.4.6 LLETZ

A large loop excision of the transformation zone is an excisional procedure of the cervical transformation zone, that uses an electric current. The depth of the excision should be at least 5 mm. The procedure is performed with the patient in the lithotomy position using a wire loop electrode and an electrosurgical unit (ESU). Loops are available in a range of sizes and are insulated along the shaft to prevent injury to the patient and thermal damage to the vaginal mucosa.

An insulated speculum is inserted into the vagina to facilitate adequate visualization of the ectocervix and transformation zone. The speculum is connected to suction during the procedure to evacuate smoke generated as the cervical tissue is excised.

A colposcopy will be performed to assess the cervix and lesion size. Local anaesthetics such as 1% lidocaine with 1:100,000 adrenaline will be used for LLETZ procedures. Excised tissue will be submitted for histology.

6.3.4.7 Thermal ablation

Thermal ablation will be performed using the Liger thermal ablator as described in the IARC colposcopy manual. The device is powered by a small removable 12-volt battery that is incorporated into the handle, which can be recharged over 2–3 hours and holds enough charge to complete at least 20 treatment procedures. Local anaesthetics such as 1% lidocaine with 1:100,000 adrenaline will be used. The thermal ablator probe will be heated to 100°C and applied over the TZ of the cervix for a period of 40 seconds. If the probe is not large enough to treat the entire transformation zone with one application, overlapping applications are allowed.

6.3.4.8 Wong-Baker Pain Rating Scale

Immediately after the treatment procedure, women will be interviewed and asked to report the level of pain experienced during thermal ablation and LLETZ treatment using the Wong-

Baker FACES® pain rating scale. This validated scale consists of an illustrated series of six faces with a range of emotions from happy (0) to crying (10) to indicate the level of pain. Scores are numbered from 0 to 10 in increments of two.

6.3.4.9 Six-week post-treatment telephone interview

Participants will be contacted six weeks after the procedure. A standardized questionnaire will be completed via telephone to assess the participants' experience and possible side effects after the procedure.

6.3.4.10 Self-collected samples for HPV testing

Cervicovaginal self-sampling will be performed at screening and at weeks 26 and 78. The device that will be used for self-sampling is the Evalyn brush. The Evalyn brush is approximately 20 cm in length and consists of a transparent case with wings. Within the casing is a pink stick with a pink plunger at one end and a white brush at the other. The depth of insertion is controlled by the wings. The brush will be inserted into the vagina and rotated five times. At each rotation, there is an audible click indicating the number of rotations. The device comes with an information leaflet detailing how the device is to be used**.**

6.3.4.11 Optional specimen donation

The participants will be informed (included in informed consent form) that to perform the research as discussed, the collection and storage of cervical specimens and health information from people with cervical abnormalities is needed. Following the completion of the clinical/laboratory tests outlined in this protocol, we propose to store residual specimens and/or health information. Globally, other investigators can ask whether these specimens be used in future cervical cancer research. Participants will be assured that measures will be taken to protect their privacy, i.e., their names will be replaced with a unique study number. The specimens will be stored at -80°C at the biorepository.

6.3.5 Treatment-related adverse events or study related injuries

In the unlikely event of an adverse event as a result of any study procedure, the participant will be referred to the on-site emergency gynaecology service at Tygerberg Hospital.

6.3.6 Randomization

Randomization is a process in which participants are assigned by chance (rather than by choice) to specific groups, typically for clinical research and clinical trials. The randomization module in REDCap will be used to implement a defined randomization model within the project. The randomization list was generated by an independent statistician, and participants will be randomized in one of two arms at a 1:1 ratio. The randomization process will be initiated by the site investigator or delegate, who accesses the web-based system after confirming the participants' eligibility and informed consent. The participants' unique study identifier and open-label study treatment allocation will then be automatically and electronically delivered to the local site investigator or delegate. Following randomization, the research staff will inform the study doctor of the participants' allocation group. Following randomization, the participant will undergo treatment immediately.

6.3.7 Criteria for discontinuation or removal from the study

Premature study discontinuation

- Request by the participant to withdraw.
- Request of the primary care provider if s/he thinks the study is no longer in the best interest of the participant.
- At the discretion of the health research ethics committee, investigator, or other country-specific government agencies as part of their duties to ensure that research participants are protected.

6.3.8 Early discontinuation evaluations

Participants who withdraw consent should be taken off study without further evaluations. Otherwise, participants should undergo week 104 evaluations unless it has been less than 8 weeks since their last cervical biopsy. If withdrawing less than 8 weeks after the last cervical biopsy, then no further evaluations should be performed.

6.3.9 Missing data management

Multiple imputation (using the statistical programming language R; R Foundation for Statistical Computing, Austria) will be employed to adjust for bias. Multiple imputation techniques will be used to impute missing values for variables of interest. This involves creating multiple complete datasets, each with imputed values, and combining results to provide unbiased and reliable estimates.

6.3.10Measures

The baseline characteristics of the participants will be summarized by study arm and presented as proportions, means with standard deviation or medians with interquartile ranges. Fisher's exact test, unpaired t-test or rank sum test will be used to compare characteristics in the two study arms.

Primary study endpoint:

Treatment success as measured by HPV clearance and/or cytological normality. For the purposes of these analyses, clearance will be equivalent to treatment success.

Other endpoints:

Occurrence of adverse events at 6 weeks post treatment and cervical structural changes as measured by changes in length of the cervix.

Analyses:

Treatment efficacy: Survival analyses will be performed to assess study outcomes. These analyses will be applied as this study is a comparative, longitudinal study on the efficacy of surgical treatments. Following treatment, efficacy outcomes will be measured every 26 weeks over a two-year period. Measurements will be taken from time of treatment until time at which event (the lesion) has cleared. Specifically, we will measure hazard ratios and present data in Kaplan-Meier curves with the associated survival tables. We will use log rank tests to compare groups in a univariate analyses and Cox regression to test for multivariate analyses. Exposure variable will be the treatment arm and outcome variable will be clearance as measured by cytology and HPV test. Confounding variables will include age and HIV status.

Event time ratio with 95% confidence interval (CI) and *p*-value less than 0.05 will be used to measure the strength of association and to declare statistically significant predictors, respectively.

Safety: Adverse events/safety will be measured based on telephonic interview results at 6 weeks post treatment. The analyses will concentrate on local (genital) adverse events even though other adverse events will be recorded. We will describe the number of adverse events as average number of events per group. Since we have two independent groups, and we are reporting average number of events, we will conduct independent t-tests. Analyses will be more exploratory since the study was powered to assess the primary study endpoint i.e., treatment efficacy, and not safety per se. If we do, however, find differences that are significant (higher number of adverse events in one treatment group compared to the other), we will conduct further statistical investigation.

Cervical structural changes: Cervical length will be measured on transvaginal ultrasound before and after respective treatments at weeks 52 and 104. Change in cervical length will be presented as a continuous variable and linear regression analysis will be used to examine significant changes during the study. For the regression model where cervical length at week 52 is the outcome we will include the treatment variable to see the effect of treatment on length and will adjust it for the baseline cervical length measurement. For the regression model where cervical length at week 104 is the outcome we will include the treatment variable to see the effect of treatment on length and will adjust it for the baseline cervical length measurement.

6.3.11 Power and sample size

Sample size calculations were performed based on the proportions of participants treated successfully from the "Efficacy of point-of-care thermal ablation among high-risk human papillomavirus positive women in China".[39]

The sample size was computed using WinPepi [40]. The calculations are as follows:

Assumed proportion successfully treated in standard treatment (LLETZ) = 87%, assumed proportion successfully treated in new treatment (Thermal Ablation) = 85%, difference

between proportions of 7% is defined as negligible, 1-sided test, significance level set at 5% and power 80%. Sample size required for noninferiority of new treatment: n = 189 in the new treatment arm and n = 189 in the standard treatment arm (N = 378). For the study to be considered a superiority study, the minimum important difference one would want to detect to consider one treatment superior to the other in terms of treatment success would be 10%; hence, for noninferiority, a percentage lower than that would be needed for it to be defined as negligible. If we anticipate a percentage of 10% loss to follow-up using the following formula, the adjusted sample size is: N (new)= N/(1 - % loss to follow-up) = 378/(1 - 0.10) = 420 (210 in each treatment arm).

6.3.12 Data and safety monitoring plan

The PI will be responsible for all the data monitoring and for compliance with all the national and institutional IRB policies and procedures for monitoring progress, and safety, reporting unanticipated problems or adverse events, and assuring actions resulting in suspension of the study are reported. All modifications to the protocol will be submitted for IRB approval. Summaries of all relevant discussions will be promptly disseminated to study personnel via e-mail, and retraining procedures will be implemented as needed.

The treatment options that will be used during this trial have been approved by the South African Health Products Regulatory Authority (SAHPRA) and have been registered for the treatment of cervical intraepithelial neoplasia (cervical precancer), for which it will be used in this specific project. No investigational products are involved in this trial. The study will evaluate and compare established clinical practice options only. For this reason, no interim analyses will be performed; however, adverse events and serious adverse events will be closely monitored throughout the study.

Each participant will be assigned a unique study ID. Participant information will be stored on a password protected computer and only those directly involved in the study will have access to it. The participant files will be stored in a locked cabinet.

Data will be captured into the REDCap database. Confidentiality will always be maintained. The data collected will be held on a password protected computer and will only be accessible to those directly involved in the study.

All source documents will be checked for quality before the data are entered into the REDCap database. This involves ensuring data integrity and accuracy, handling errors/queries and missing data, checking all the information transcribed from another source, tracking data changes and corrections in accordance with the GCP.

Throughout this process, the flagging system will be utilized. The flagging system is a source of communication between the study nurses, study coordinator, study doctors and the data capturers. The system involved the use of three different colours of stick-on flags namely:

Orange indicates STOP: This flag is used to indicate that there is missing information on the source document for a specific participant.

Green indicates GO: This flag is used to indicate that the information is complete in the relevant source document and that the information is correct and ready to be checked and captured.

Yellow indicates PLEASE CORRECT: This flag is used to indicate that the file/source documents have gone through the QC process and that there were some errors requiring correcting or that clarification is needed concerning a certain form before the information can be captured.

6.3.13 Data dissemination plan

The trial results will be disseminated to the scientific community through presentations at relevant national and international conferences, dissemination through professional associations, and via peer-reviewed journals. The results of this trial will also be used to develop and distribute educational materials to healthcare professionals to aid in interpreting and implementing the trial findings in clinical practice. A layperson's summary of trial results will be made available to CAB, which will assist in developing strategies to disseminate research findings back to the community in an accessible and meaningful way.

6.4 DISCUSSION

In this paper, we present the research protocol “a randomized controlled trial assessing the comparative cure rate and treatment effects of thermal ablation to those of LLETZ”. The assessment will focus on the cure rate, examining cytological normality and hrHPV negativity at 26-week intervals over a two-year period, post-treatment. Additionally, the study aims to document and compare patient experiences, side effects, and safety between thermal ablation and excision as well as TVUS and colposcopy with mobile imaging to assess cervical anatomy for structural changes affecting the reproductive system before and after treatment.

Cervical cancer, a leading cause of female mortality, disproportionately affects South African women. Determining the effectiveness of TA, particularly in a population with a substantial HIV burden and hrHPV prevalence, provides valuable insights into its potential as a cost-effective and accessible treatment alternative. This research addresses the urgent need to enhance cervical cancer management, by informing evidence-based decisions for cervical cancer screening and treatment guideline implementation. The study's outcomes could significantly impact public health by introducing a more efficient and resource-friendly treatment option tailored to South Africa's healthcare landscape.

The findings of this study have the potential to inform the incorporation of TA into the cervical cancer treatment framework in South Africa.

6.5 DATA AVAILABILITY

All the study data shall be the sole and exclusive property of Stellenbosch University, and the PI shall maintain all the original patient records as stated in the protocol (as per Investigator-Sponsored Study Agreement CyberGrant 76172193).

The datasets generated and/or analysed during the current study will be available from the corresponding author upon reasonable request.

6.6 ADDITIONAL INFORMATION

The current protocol follows the SPIRIT guidelines and has been registered on the South African National Clinical Trials Register: SANCTR unique number DOH-27-062023-4853; https://sanctr.samrc.ac.za/TrialDisplay.aspx?TrialID=9313.

Protocol version history:

HREC New Application Form – Version 1, 06 April 2021

HREC Protocol Amendment Form - Version 2, 26 October 2021

HREC Protocol Amendment Form - Version 3, 09 January 2024

6.7 CANDIDATE CONTRIBUTION

As a Co-Investigator for this study, I contributed to various aspects of the project, including:

1. Protocol and documentation: Protocol writing, source documentation, and budget development. Additionally, I created and maintained our web-based REDCap data collection tool.
2. Ethics and compliance: Completed research ethics applications, including submissions to the Health Research Ethics Committee (HREC) and the National Health Research Ethics Committee (NHREC).
3. Trial registration: Successfully registered the trial on the South African National Clinical Trials Register (SANCTR).
4. Grant applications: Authored the BD Investigator Sponsored Studies (ISS) grant application, which resulted in an awarded grant.
5. Project management: Developed and monitored project workplans in consultation with the Principal Investigator and study sponsors.
6. Logistics and procurement: Oversaw procurement of study equipment, supply inventory, and storage.

6.8 CONFLICT OF INTEREST

The authors state that they are not aware of any financial or interpersonal conflicts that might appear to have an impact on the research conducted in this trial.

6.9 ADDENDUM B

B1: General information

Project name

Cervical Precancer Thermal Ablation versus LLETZ Excision Comparative Efficacy Study (TALL Study).

Funding sources:

BECTON DICKINSON ITALIA (BD) (CyberGrant 76172193)

Department of Obstetrics and Gynaecology (Departmental funding)

Name of clinical site

Stellenbosch University, Tygerberg Hospital

Capabilities

The research team is led by Dr Hennie Botha and Dr Haynes van der Merwe. Both are registered gynaecologic oncologists. Dr Botha served as the site PI for the internationally funded EVRI and COVENANT trials. The rest of the team includes amongst others another study doctor, a coordinator, and data manager. The team has experience in community interventions, clinical trials, and laboratory testing.

The research site is supported by an onsite emergency service, if necessary, a study pharmacy, a local health research ethics committee and a community advisory board.

The colposcopy clinic, which will be the main recruitment site, manages approximately 1 500 patients with abnormal cytology per year. The referral area for the clinic includes the metro-east area of Cape Town with an estimated population of approximately 1,8 million people.

Role of the Site Investigators

- Accept responsibility for the conduct of the study.
- Disclose all potential conflicts of interest.
- Identify need for subrecipient agreements.

- Prepare budget.
- Submission of an accurate and completed study proposal and obtains appropriate ethics approval.
- Ensure procedures are consistent with research proposal.
- Manage the study to his/ her highest professional conduct.
- Obtain, maintain, and safeguard equipment.
- Ensure that QA is ongoing.
- Submit accurate and timely documents on the status of the study.
- Prepare and maintain all documents related to the informed consent process.
- Promptly report any unanticipated problems to research participants and sponsors.
- Record, prepare, and maintain complete and accurate written records, files, accounts, notes, raw data, reports, data, and other information relating to the study in accordance with the protocol.
- Adhere to all South African regulations and sponsor policies including Good Clinical Practice (GCP) principles.
- Oversee investigational product accountability.
- Conduct regular site visits to ensure that all identified issues are addressed in an appropriate and timely fashion and are communicated to study staff.

Role of BD

- To provide funding for the implementation of the study.
- To provide DNA testing commodities for the HPV testing that the study entails.
- Future collaboration for testing of cervical specimens with a BD methylation assay on a BD instrument, in collaboration with PI and methylation team.

B2: TALL Study author details

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***Corresponding author**

****Trial PI, responsible for funding and conduct of the trial (investigator-initiated)**

B3: Informed consent form

INFORMED CONSENT

Study Title:

Cervical Precancer Thermal Ablation versus LLETZ Excision Comparative Efficacy Study (TALL Study)

We would like to invite you to take part in a research project.

The Principal Investigator of this study at this site is: Prof MH (Hennie) Botha

Local Contact Number: 021 938 9209

This is an informed consent form (ICF). It will tell you more about this study, who can take part in the study and what people taking part will agree to. Before taking part in the study, you will be asked to sign this form, to show that you understand the information. You will also get a copy of this form to keep. The form will give information about giving specimens and medical samples/data, how it may be used and how it will be stored. The study will also test you to find out your HIV status.

Please take some time to read this form, which will explain the details of this project. Please ask the study staff or doctor any questions about anything that you do not fully understand. It is very important that you are completely happy that you clearly understand what this research is and how you could be involved. Taking part is also **entirely by choice,** and you are free to decline to participate. In other words, you may choose to take part, or you may choose not to take part. Nothing bad will come of it if you say no: it will not affect you badly in any way whatsoever. Choosing not to take part will not lower the level of your care or cause any penalty or loss of benefits. Even if you do agree to take part, you are also free to withdraw from the study at any point.

The Health Research Ethics Committee at Stellenbosch University has approved this study (M20/11/035). The study will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, the South African Guidelines for Good Clinical Practice (2006), the Medical Research Council (MRC) Ethical Guidelines for Research (2002),

and the Department of Health Ethics in Health Research: Principles, Processes and Studies (2015).

What is this research study all about?

The aim of this study is to compare two methods of treatment for cervical lesions and see which is the most effective. We would like to test and compare how well a method of treatment called thermal ablation is to a method of treatment termed a Large Loop Excision of the Transformation Zone (LLETZ). Our aim is to get evidence to introduce thermal ablation in South Africa's cervical cancer treatment guidelines. We want to use it as a treatment alternative for precancerous cervical lesions in addition to the current use of LLETZ treatment. The study will be conducted at Tygerberg Hospital, Cape Town, Western Cape.

This study has two groups:

Group 1 will receive LLETZ.

What is a LLETZ?

LLETZ is a simple treatment to remove precancer changes from the cervix. A local anaesthetic is given to numb the cervix, then a fine wire loop is heated by electricity to scoop off the affected skin on the cervix. A diathermy ball is then applied to the cervix to cauterise it. The skin removed is sent to the laboratory to be checked. It is usually performed as an outpatient procedure in the Colposcopy Clinic. A small number of LLETZ treatments may be done under a general anaesthetic for certain reasons. Further details and explanation will be given if this applies to you.

Group 2 will receive a method of treatment called Thermal Ablation.

What is Thermal Ablation?

Thermal ablation is a type of procedure where a heated probe is placed directly on the cervix. This kills the abnormal cells by burning them. It is used to treat precancerous lesions of the cervix. This can be done in a doctor's office or clinic. After the procedure, you may have a watery brown discharge for a few weeks.

Randomization:

A computer will assign you to one of the study groups by chance. This is called randomization. This is done by chance because no one knows if one treatment is better than the other. You have an equal chance of being in any group.

What will your responsibilities be?

- You will have to agree to come to all the study visits as per schedule.
- Inform us of any health information that becomes available when you visit your clinic or doctor.
- Inform us if you wish to withdraw or have plans to move.

Can you stop taking part in the study?

Yes. You can decide to stop at any time. If you decide to stop for any reason, it is important to let the study doctor know as soon as possible so you can stop safely. If you stop, you can decide whether to let the study doctor keep giving your medical information to the organization running the study.

The study doctor will tell you about new information or changes in the study that may affect your health or your willingness to continue in the study.

The study doctor may take you out of the study:

- If your health changes and the study is no longer in your best interest.
- If new information becomes available.
- If you do not follow the study rules.
- If the study is stopped by the Ethics committee, or any other agency responsible for oversight of the study.

Are there any reasons that you should not be in the study?

You should not be in this study if you have:

- A sexually transmitted infection requiring treatment (you may choose to be in the study after you have completed the treatment).
- Other illnesses that would limit your ability to complete the study requirements.
- Prior hysterectomy (removal of the uterus or womb) with removal of the cervix.
- Prior treatment for cervical HSIL.
- Prior history of cervical cancer.

How long will I be in this study?

- You will be in the study for about 2 years.

How many women will be part of this study?

- 420

Will you benefit from taking part in this research?

This study may help researchers learn things that may help other people in the future. It is not possible to know at this time if the study method is better than the usual method, so this study may or may not help you.

Are there any risks involved in your taking part in this research?

The screening tests do not carry any known risks. The procedures in this study include the use of a type of swab, in the same way a Pap smear is done, to obtain material for testing. Pap smears and cervical biopsies will be done using the same methods as your doctor usually would and will be done by experienced medical personnel. Some bleeding may occur after a biopsy is taken. You may experience pain from injections of numbing medication into the cervix. After the medical procedure, you may experience some vaginal bleeding and discharge for one to two weeks. This bleeding and discharge may be like a period. You may experience a dull ache or cramping during and after the procedure.

You will be required to do self-sampling for the detection of the human papillomavirus (HPV) which is the virus that has been linked to warts and cervical cancer. We will provide you with a swab called an Evalyn Brush so that you can collect cervical specimens. This procedure is like the collection of cervical specimens during a pap smear. The swab comes with an instruction leaflet on how to use the device. Your doctor can also answer any questions you have about self-sampling and explain what they are doing during visits.

A transvaginal ultrasound (TVUS) is a type of pelvic ultrasound used to examine female reproductive organs. This includes the uterus, fallopian tubes, ovaries, cervix, and vagina. A transvaginal ultrasound is done through the vagina. The patient lies on her back on an exam table. The transducer is covered in gel and a plastic or latex covering. The transducer is then inserted into the patient's vagina, much like a tampon. The test itself does not have risks. Unlike X-rays, ultrasound does not use radiation.

Reproductive risks:

You should not get pregnant while in this study. The cervical treatment used in this study could hurt the unborn baby. Check with the study doctor about what types of birth control, or pregnancy prevention, to use while in this study. If you are a woman and become pregnant or suspect you are pregnant while participating in this study, please inform your treating doctor or nurse immediately.

Risks to privacy:

Your privacy is very important, and the researchers will make every effort to protect it. Your test results will be identified by a unique code and the list that links the code to your name will be kept separate from your sample and health information. This means that the researchers working with your information will not know your name, but the doctor treating you will.

Additional risks of study procedures:

Cervical colposcopy, speculum exam, Pap test, transvaginal ultrasound, self-sampling, and HPV testing:

These tests can be uncomfortable. Occasionally there can be some bleeding, a tingling feeling, or slight stinging. These tests may cause embarrassment. Some people may feel anxiety while waiting for the test results.

Cervical biopsy:

- Pain during biopsy.
- Mild bleeding like a menstrual period.

Rarely, the following problems can occur:

- Heavy bleeding can occur requiring treatment to stop the bleeding.
- Infection of the cervix requiring antibiotics (drugs used to stop or slow down the growth of bacteria and germs).
- Damage to the cervix making it more difficult to carry a pregnancy.

Cervical thermal ablation:

- Pain and cramping during the procedure.
- Heavy, watery discharge from the vagina for 1 to 2 weeks.
- Heavy, foul-smelling discharge from the vagina.
- Mild bleeding like a menstrual period.

- Infection of the cervix requiring antibiotics.

Rarely, the following problems can occur:

- Severe cramps and stomach pain requiring pain medications.
- Infection of your uterus and surrounding areas.
- Heavy bleeding requiring treatment to stop the bleeding.
- Narrowing of the cervix making it difficult for menstrual bleeding to occur.
- Accidental freezing of the vagina during the procedure.
- Dizziness or fainting during the procedure.
- Damage to the cervix making it more difficult to carry a pregnancy.
- Damage to the cervix making it difficult to become pregnant.

LLETZ:

- Pain from injections of numbing medication into the cervix.
- Blood tinged or dark brown mucus from the vagina for 1 or 2 weeks.
- Dull ache or cramping during the procedure.
- Pain or cramping after the procedure.
- Infection of the cervix requiring antibiotics.

Rarely, the following reactions can occur:

- Dizziness, fainting or allergic reaction to the numbing medications.
- Severe bleeding of the cervix requiring sewing of the cervix.
- Accidental damage of the vagina.
- Accidental damage of the uterus.
- Infection of the uterus and surrounding areas.
- Narrowing of the cervix making it difficult for menstrual bleeding to occur.
- Damage to the cervix making it more difficult to carry a pregnancy.
- Damage to the cervix making it difficult to become pregnant.

Spreading HIV:

For some time after you have thermal ablation or LLETZ, it is possible the condition of the cervix may make it more likely to spread HIV to a sexual partner. For this reason, we ask that you not have vaginal sex for six weeks after the procedure. If you choose to have vaginal sex, we ask that your sexual partner use a suitable barrier contraceptive.

If you do not agree to take part in the study, what alternatives do you have?

If you decide not to take part in this study, you will still receive standard treatment at the colposcopy clinic for the cervical lesion. You may withdraw from this study at any time.

Who will have access to your medical records?

Your medical information collected will be treated as confidential and will be protected. You

will be allocated a study ID. Your name will not be on any of the documentation. If your information is used in a publication or thesis, your identity will remain anonymous. Only members of the study team will have access to your information.

Even though it is unlikely, what will happen if you get injured somehow because you took part in this research study?

If you are injured as a result of taking part in this study, please tell your study doctor. Stellenbosch University – Obstetrics and Gynaecology Department site staff will give you the immediate treatment for the injuries. The doctor will refer you for further treatment if needed. There will be no cost to you in the event of a study related injury, emergency care or hospitalization.

By agreeing to participate in this study, you agree that there is a risk that the study medicine(s) or procedure(s) may cause you harm. If it does, the sponsor will reimburse you for medical expenses without you having to prove that the sponsor was at fault. You may, however, still claim for emotional pain and suffering if you so choose. In this event, you will have to prove that the sponsor was negligent and did not take all reasonable and foreseeable steps to prevent the injury or emotional trauma. This will be a separate legal matter.

Will you be paid to take part in this study and are there any costs involved?

- You will not have to pay for anything if you do take part. You will be compensated to take part in the study and your expenses will be reimbursed for each study visit.
- The amount and method of payment to research participants should reflect the following three components:
    - Compensation for time
    - Compensation for inconvenience
    - Reimbursement of expenses

Is there anything else that you should know or do?

- You should tell your family practitioner or usual doctor that you are taking part in a research study.
- You should also tell your medical insurance company that you are participating in a research study.
- You can phone Prof Hennie Botha at 021 938 9209 if you have any further queries or encounter any problems.
- You can phone the Health Research Ethics Committee at 021 938 9075 if there still is something that your study doctor has not explained to you, or if you have a

complaint.

- You will receive a copy of this information and consent form for you to keep safe.

Permission to have all anonymous data shared with journals:

Please carefully read the statements below (or have them read to you) and think about your choice. No matter what you decide, it will not affect whether you can be in the research study, or your routine health care.

When this study is finished, we would like to publish results of the study in journals. Most journals require us to share your anonymous data with them before they publish the results. Therefore, we would like to obtain your permission to have your anonymous data shared with journals.

□ Yes □ No Date……..

Permission for sharing samples and/or information with other investigators

Please carefully read the statements below (or have them read to you) and think about your choice. No matter what you decide, it will not affect whether you can be in the research study, or your routine health care.

To do the research we have discussed, we must collect and store cervical swabs and health information from people like you with cervical abnormalities. We will do some of the tests right away. Other tests may be done in the future. Once we have done the research that we are planning for this research project, we would like to store your sample and/or information. Other investigators from all over the world can ask to use these samples in future research. To protect your privacy, we will replace your name with a unique study number. We will only use this code for your sample and information about you. We will do our best to keep the code private. It is, however, always possible that someone could find out about your name, but this is very unlikely to happen. Therefore, we would like to ask for your permission to share your samples and information with other investigators.

□ Yes □ No Date……..

Declaration by participant

By signing below, I …….. agree to take part in a research study entitled Cervical Precancer Thermal Ablation versus LLETZ Excision Comparative Efficacy Study (TALL Study).

I declare that:

- I have read this information and consent form, or it was read to me, and it is written in a language in which I am fluent and with which I am comfortable.
- I have had a chance to ask questions, and I am satisfied that all my questions have been answered.
- I understand that taking part in this study is **voluntary,** and I have not been pressurised to take part.
- I may choose to leave the study at any time and nothing bad will come of it – I will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan that we have agreed on.

Signed at (*place*) ..... on (*date*) ……..20…….

.....	…….
Name and surname of participant	**Name of witness**
.....	……
Signature of participant	**Signature of Witness (if applicable)**
Thumbprint of participant (if illiterate)	**Signature of witness (if applicable)**

Declaration by investigator

I *(name)* …… declare that:

- I explained the information in this document in a simple and clear manner to ……..
- I encouraged her to ask questions and took enough time to answer them.
- I am satisfied that she completely understands all aspects of the research, as discussed above.
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.)*

Signed at (*place*) .....on (*date*) …….. 20……

..... ……

Signature of investigator **Signature of witness**

B4: Study calendar for participants

TALL Study: STUDY CALENDAR FOR VOLUNTEERS

Baseline visit (screening and randomization)

- We will confirm if you are eligible for the study based on age, pregnancy test result and previous treatment for precancerous lesion.
- We will discuss the informed consent form with you as well as the optional donation of left-over specimens.
- If your HIV status is unknown and you are willing, we will do a HIV rapid test.
- We will document your demographic information, medical history, and HIV status.
- We will ask you to collect a self-sample for HPV testing.
- We will do a TVUS to determine cervical length.
- We will do a speculum examination and collect cervical specimens for HPV testing and cervical cytology.
- We will perform a colposcopy to determine if the cervical lesion is eligible for thermal ablation.
- Once we have confirmed eligibility, you will be randomized into treatment arms.
- If you are randomized into the thermal ablation treatment arm, we will obtain a cervical biopsy of the lesion present on your cervix.
- You will then be treated with either thermal ablation or LLETZ according to randomization.

6 Week telephonic follow-up

- We will contact you 6 weeks after your treatment. A standardised questionnaire will be completed telephonically asking you about your experience and possible side effects post treatment.

26 Week follow-up

- It will be required of you to do self-sampling at the study site.

52 Week follow-up

- If you are HIV negative, we will do a HIV rapid test.
- We will ask you to do self-sampling for HPV testing.
- We will do a TVUS to determine cervical length.
- We will do a speculum examination and collect cervical specimens for HPV testing and

cervical cytology.

Interim treatment visit (six to 10 weeks after receiving Week 52 follow-up cervical cytology and HPV results)

- If we find an abnormal HPV or cytology result, one of the study staff members will contact you and ask you to come to the hospital so that you can be re-treated with the LLETZ procedure.

78 Week follow-up

- It will be required of you to do self-sampling at the study site.

104 Week follow-up

- If you are HIV negative, we will do a HIV rapid test.
- We will do a TVUS to determine cervical length.
- We will do a speculum examination and collect cervical specimens for HPV testing and cervical cytology.

Interim treatment visit (six to 10 weeks after receiving Week 104 follow-up cervical cytology and HPV results)

- If we find an abnormal HPV or cytology result, one of the study staff members will contact you and ask you to come to the hospital so that you can be re-treated with the LLETZ procedure.

B5: SPIRIT-Outcomes 2022 Checklist

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
Administrative information				
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	-	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	-	Page 2
	2b	All items from the World Health Organization Trial Registration Data Set	-	Pages 1 - 40
Protocol version	3	Date and version identifier	-	Page 1
Funding	4	Sources and types of financial, material, and other support	-	Page 24
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	-	Pages 24 - 26
	5b	Name and contact information for the trial sponsor	-	Pages 26
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	-	Pages 24 - 26
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	-	Pages 24 - 26
Introduction				
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	-	Pages 2 - 5
	6b	Explanation for choice of comparators	-	Pages 2 - 5
Objectives	7	Specific objectives or hypotheses	-	Page 5

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	-	Page 8
Methods: Participants, interventions, and outcomes				
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	-	Page 6 - 8
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	-	Pages 6 & 11
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered (for specific guidance see TIDieR checklist and guide)	-	Pages 14 - 17
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	-	Page 18
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	-	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	-	Page 17
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	-	Pages 18 - 20

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
	12.1		Provide a rationale for the selection of the domain for the trial's primary outcome	Pages 18 - 20
	12.2		If the analysis metric for the primary outcome represents within-participant change, define and justify the minimal important change in individuals	Pages 18 - 20
	12.3		If the outcome data collected are continuous but will be analyzed as categorical (method of aggregation), specify the cutoff values to be used	Pages 18 - 20
	12.4		If outcome assessments will be performed at several time points after randomization, state the time points that will be used for analysis	Pages 18 - 20
	12.5		If a composite outcome is used, define all individual components of the composite outcome	NA
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	-	Page 9
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	-	Page 20
	14.1		Define and justify the target difference between treatment groups (eg, the minimal important difference)	Page 20
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	-	Pages 13 & 14
Methods: Assignment of interventions (for controlled trials)				
Allocation:				
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	-	Pages 17 & 18

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	-	Pages 17 & 18
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	-	Pages 17 & 18
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	-	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	-	NA
Methods: Data collection, management, and analysis				
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	-	Pages 16 - 18
	18a.1		Describe what is known about the responsiveness of the study instruments in a population similar to the study sample	Pages 2 - 5
	18a.2		Describe who will assess the outcome (eg, nurse, parent)	
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	-	Page 18

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	-	Pages 21 - 22
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	-	Pages 18 - 20
	20a.1		Describe any planned methods to account for multiplicity in the analysis or interpretation of the primary and secondary outcomes (eg, coprimary outcomes, same outcome assessed at multiple time points, or subgroup analyses of an outcome)	NA
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	-	NA
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	-	Page 18
Methods: Monitoring				
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	-	Pages 21 - 22
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	-	Pages 21 - 22
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	-	Pages 21 - 22

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	-	Pages 21 - 26
Ethics and dissemination				
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	-	Page 20
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	-	Page 21
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	-	Pages 12 & 13
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	-	Page 17
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	-	Pages 21 - 23
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	-	Page 23
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	-	Page 23
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	-	Page 10
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	-	Page 23
	31b	Authorship eligibility guidelines and any intended use of professional writers	-	NA

Section	Item No.	SPIRIT 2013 Item	SPIRIT-Outcomes 2022 item	Location Reported[b]
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	-	Page 23
Appendices				
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	-	Pages 27 - 36
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	-	Pages 27 - 36

[a]It is strongly recommended that this checklist be read in conjunction with the SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) Statement paper for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license and is reproduced with permission.
[b]Indicates page numbers and/or manuscript location: to be completed by authors.

6.10 REFERENCES

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CHAPTER 7

Conclusion and future direction in cervical cancer secondary prevention in South Africa.

7.1 GENERAL DISCUSSION

7.1.1 Summary of findings

Cervical cancer is a significant global health concern, affecting millions of women each year. While primary prevention strategies, such as vaccination against HPV, have shown promise, the importance of secondary prevention cannot be overstated. The aim of this thesis was to assess components of, and to describe the multifaceted nature of secondary prevention, including screening programmes, early detection methods, and interventions aimed at reducing the burden of cervical cancer.

Chapter 2 highlights the crucial lessons learned from South Africa's cervical cytology programme, which should inform the implementation of HPV DNA screening. Addressing systemic challenges, educating, and motivating healthcare staff, and ensuring robust laboratory quality control are key to optimizing the new screening approach. Integrating cytology as a reflex triage test alongside HPV DNA testing can provide comprehensive coverage. By applying these insights, South Africa can create a more effective and equitable cervical cancer screening strategy.

In **chapter 3**, our findings reveal that visual inspection methods perform more effectively in WLWH compared to HIV-negative women and the utilization of visual inspection methods in cervical cancer screening for WLWH may be justified.

While our study underscores the potential utility of visual inspection methods, especially in WLWH, it is crucial to acknowledge that, in most studies, VIA and VILI exhibit satisfactory sensitivity only when rigorous quality assurance measures are implemented. However, implementing such intensive quality assurance may pose challenges in non-academic settings, potentially leading to over-treatment due to a relative lack of specificity.

Despite this, visual inspection is the most advocated screening method in many LMIC’s, as described in **chapter 4**, aimed at reviewing, and comparing cervical cancer control guidelines in the 16 member states of the SADC region, to the most recent WHO guideline for screening and treatment of cervical precancer lesions for cervical cancer prevention.

The second edition of the WHO guideline prioritizes HPV DNA testing as the preferred screening test, with or without triage for the general population of women, and with triage for WLWH. The implementation of HPV-based testing represents a major step forward in cervical cancer screening technology. HPV DNA testing is the most sensitive test for cervical cancer screening and has a NPV approaching 100%, allowing for an increase in the screening interval for negative tests. As a result, fewer follow-up visits and additional tests are required, making it more cost effective than cytological testing.

The use of HPV DNA testing as a primary screening method, however, requires the use of a triage test to identify women with clinically relevant infections, as many of the detected infections are transient, and only a minority are associated with cervical abnormalities. HPV DNA testing in isolation may therefore not be clinically meaningful.

A pragmatic benefit of HPV testing is that the same sample can be used for triage testing, including assessments like host-cell DNA-methylation. This two-step use of the same sample streamlines the diagnostic process, reducing the need for additional sample collection. Fewer pre-analytical steps not only contribute to the efficiency of the testing process but also help in minimizing the discomfort or inconvenience for the individual undergoing the screening.

In South Africa, HPV testing is now almost routinely offered in the private sector and may become more widely available in the public sector as part of the national screening programme. The South African HPV Board, in the latest screening guidelines, recommends reflex cytology as the preferred triage test. The use of molecular biomarkers, however, have the potential to further risk-stratify women at risk of progressive disease. As described in **chapter 5**, methylation of host-cell DNA has been proposed as a promising biomarker for triage of HPV positive women at screening. A study by De Strooper et al. evaluating the long-term triage capacity of FAM19A4 and hsa-mir124-2 in hrHPV positive women 30 years and older, found that DNA-methylation had a high sensitivity for cervical cancer which translated to a low 14-year cancer risk of hrHPV positive women who were DNA-methylation negative of 1.7% (95% CI: 0.66-3.0). With the findings of our study, however, we cannot draw any strong conclusions. DNA methylation shows promise for triage in cervical cancer screening, but more evidence-based data are needed to support its inclusion into screening

programmes, particularly in LMICs. The adaptation of methylation assays for use in LMICs, focusing on cost-effectiveness should be considered. Longitudinal studies using methylation assays in WLWH are essential, as most existing research has been limited to cross-sectional studies, as highlighted by Burdier et. al. [1]

As part of the continuous journey of secondary prevention, after identification of cervical lesions in need of treatment, removal of the lesion can prevent further progression into cervical cancer. **Chapter 6** thus outlines a study protocol for a randomized trial comparing the cure rate and effects of thermal ablation to those of LLETZ in treating cervical precancer. Thermal ablation requires minimal electricity to charge batteries for battery-driven devices, is inexpensive, easy to perform, and has been found to have fewer side effects in comparison to other treatment methods. In the context of South Africa's HIV burden and high-risk HPV prevalence, understanding the effectiveness of thermal ablation is crucial for developing cost-effective and accessible treatments.

7.1.2 Contribution to knowledge

Collectively, our research has contributed to the expanding body of knowledge on cervical cancer prevention, addressing critical gaps in understanding and practice. By assessing the performance characteristics of various screening modalities, reviewing regional screening guidelines, exploring novel molecular biomarkers, and initiating treatment comparative effectiveness research, we have taken steps towards facilitating informed decision-making and implementing evidence-based interventions in South Africa.

7.2 FUTURE DIRECTION

In line with the WHO ambitious targets for the elimination of cervical cancer, our study underscores the importance of sustained efforts and strategic interventions. By 2030, the WHO aims to achieve a 90% vaccination coverage against HPV among girls by the age of 15, along with ensuring that 70% of women are screened with high-performance tests at ages 35 and 45. Additionally, the WHO targets to have 90% of women identified with cervical disease receive appropriate treatment and care. As we move forward, it is imperative to align our actions with these targets, focusing on enhancing vaccination programmes, improving

screening accessibility and accuracy, and strengthening healthcare infrastructure to ensure timely diagnosis and treatment. Collaborative endeavours among policymakers, healthcare providers, researchers, and communities will be pivotal in realizing the shared goal of eliminating cervical cancer as a public health concern.

7.2.1 Comprehensive cervical cancer screening guidelines and programme in South Africa

To ensure comprehensive cervical cancer screening, guidelines must detail the recommended primary screening test, the age to begin screening, the age to conclude screening, the screening frequency, the appropriate triage test, and the treatment method for precancerous lesions. Additionally, these guidelines should distinguish between high-risk groups, such as WLWH, and the general female population.

It is essential to provide metrics on the implementation of screening programmes, such as whether the programme has been initiated, if a pilot is planned, and the current screening coverage. If such data is unavailable, governments should plan to gather it. While collaboration with NGOs is valuable for initiating screening programmes, their involvement might be temporary or limited in duration. Therefore, it is crucial for countries to plan and budget for the programme's long-term sustainability independently. Guidelines should be regularly updated within specified intervals to incorporate the latest scientific evidence.

7.2.2 The role of the prophylactic HPV vaccine in cervical cancer secondary prevention in South Africa

Growing evidence suggests that HPV vaccination decreases the likelihood of recurrent cervical lesions in women who have undergone surgical treatment for high-grade cervical lesions. Prospective longitudinal studies are required to evaluate the impact of the prophylactic vaccine on the recurrence of cervical lesions.

7.2.3 The role of the therapeutic HPV vaccine in cervical cancer secondary prevention in South Africa

Various types of therapeutic HPV vaccines are currently under investigation, including peptide-based vaccines, protein-based vaccines, viral-vectored vaccines, bacterial-vectored vaccines, DNA-based vaccines, and cell-based vaccines. While none of these therapeutic vaccines have been approved by regulatory agencies for clinical use, several have undergone evaluation in phase II or III trials. [2] The potential role of therapeutic HPV vaccines in cervical cancer secondary prevention, particularly in South Africa, has yet to be evaluated. This research holds promise for contributing to the secondary prevention paradigm, offering new avenues for early intervention and treatment.

7.2.4 The introduction of self-sampling into the South African cervical cancer screening programme

Self-sampling represents a significant advancement in overcoming barriers to screening, particularly in underserved communities. Future studies should focus on optimizing self-sampling methods, evaluating their acceptability and efficacy, and addressing potential challenges such as sample quality and follow-up mechanisms.

7.2.5 The implementation of extended HPV genotyping for cervical cancer screening and triage in South Africa

The integration of extended HPV genotyping into screening programmes, with a focus on prevalent genotypes in specific populations such as South African women, could improve screening risk stratification. Immediate treatment recommendations are warranted for both the general population of women and WLWH upon screening positive for HPV types 16, 18, 31, 35, 39, 51, 52, 56, and 58. These particular HPV types consistently correlate with high-grade lesions and cancer, as shown in figure 6.1, underlining the urgency of intervention. Conversely, the detection of HPV types 33, 45, 51, 56, and 59 could be considered medium risk and be referred for triage. By utilising extended genotyping data, screening programmes

can more precisely identify individuals at elevated risk, facilitating timely and targeted interventions to mitigate the burden of cervical disease.

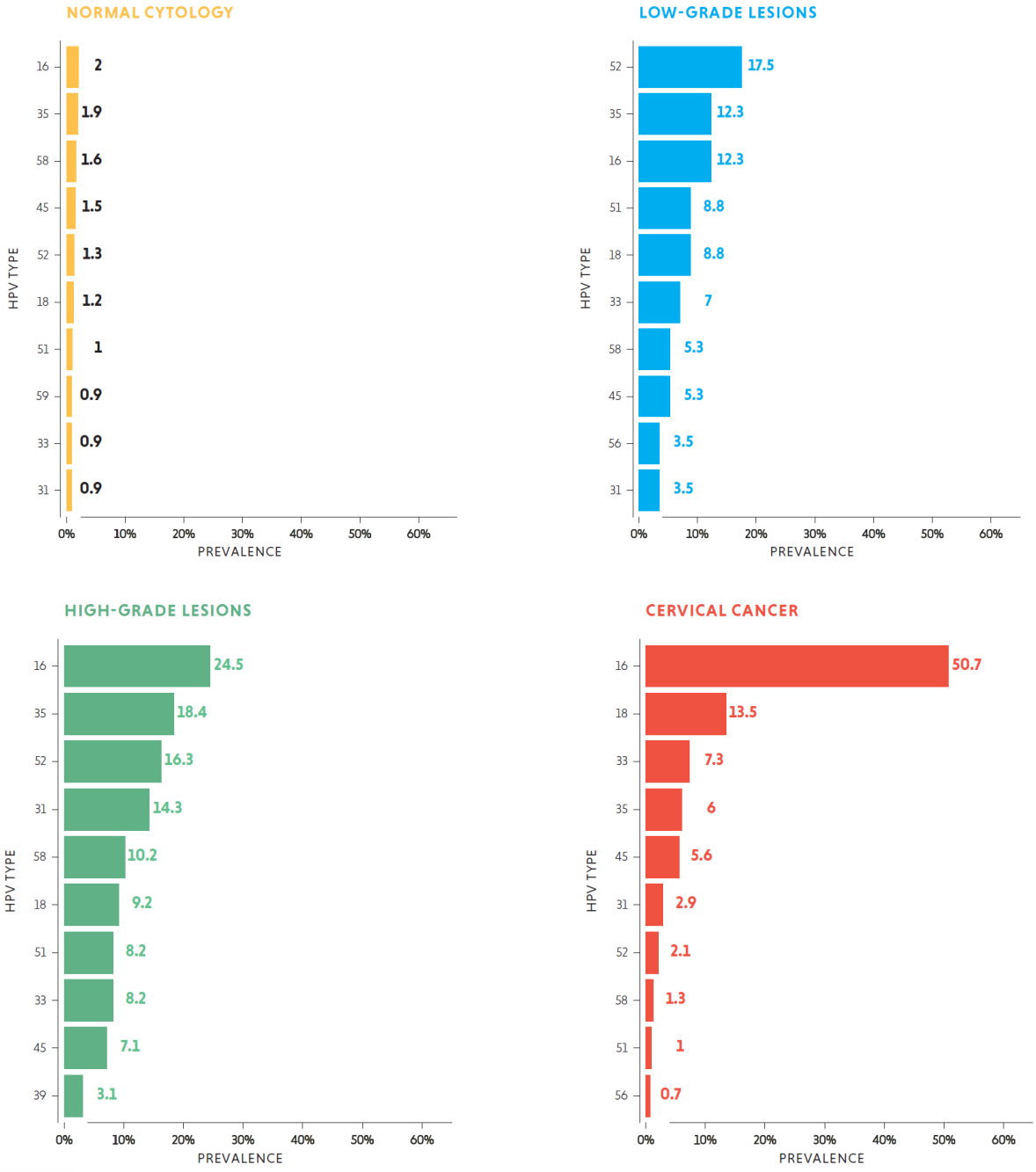

Figure 7.1 Comparison of the ten most frequent HPV oncogenic types in South Africa among women with and without cervical lesions, as reported by ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). [3]

7.2.6 Point-of-care testing

Point-of-care testing offers the promise of rapid diagnosis and immediate intervention, particularly in resource-limited settings. Future research should focus on developing affordable and user-friendly point-of-care tests, evaluating their accuracy and feasibility in diverse settings, and integrating them into clinics and hospitals.

7.2.7 Complete transition to molecular assays for cervical cancer screening and triage

Molecular assays offer a promising approach to improve the accuracy and efficiency of screening programmes, particularly in resource-constrained settings. Research efforts should concentrate on validating these assays in diverse populations, exploring their cost-effectiveness, and integrating them into existing screening algorithms. Adapting DNA methylation assays for LMICs holds great potential for enhancing early detection and risk stratification. However, challenges related to affordability, accessibility, laboratory complexity and scalability must be addressed through collaborative research and innovative implementation strategies tailored to LMIC settings.

7.2.8 Cervical precancer treatment options

Minimally invasive treatment modalities represent a significant shift in managing cervical precancer. Long-term studies are needed to evaluate their efficacy, safety, and impact on recurrence rates, especially in WLWH, where treatment failure is prevalent.

A systematic review and meta-analysis by Atemnkeng et al. assessed the role of ART in detecting CIN2+ post-treatment in WLWH. The study concluded that ART might reduce the risk of CIN2+ detection during follow-up. The authors suggest that this effect is likely enhanced by combining adequate HIV control with excisional CIN treatment. [4]

Given these findings, integrating HIV care with cervical precancer treatment could be highly beneficial.

7.3 CONCLUSION

Efforts to strengthen health systems and promote interdisciplinary collaboration are vital for the successful implementation and scale-up of cervical cancer prevention initiatives. Capacity building, community engagement, and advocacy efforts are essential to ensure equitable access to screening, diagnosis, and treatment services, particularly for marginalized populations who bear a disproportionate burden of disease. By prioritizing these research and implementation strategies, we can work towards the ultimate goal of eliminating cervical cancer as a global public health concern.

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