ASSOCIATION OF FOXP3 AND P16INK4A SERUM EXPRESSION WITH GRADES AND TREATMENT OUTCOMES OF CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS IN SOUTH WESTERN UGANDA

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AUGUST 2024

Declaration

I hereby declare that the work in this thesis is my own except for quotations which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for the award of any other degree.



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Dedication

I dedicate this work to the almighty God and my lovely family

Definition of terms

Reproducibility: The ability of a test to give similar results when repeated by the same individual or a different one (National Academies of Sciences et al., 2019).

Sensitivity: The ability of a diagnostic test to correctly identify people with a certain disease (true positive rate) (National Academies of Sciences et al., 2019).

Specificity: The ability of a diagnostic test to correctly identify people without a certain disease (true negative rate) (National Academies of Sciences et al., 2019).

Neoplasia: This means a new and uncontrolled growth of cells that is not under normal physiologic control (Cass, 2003).

Cytology: This is the study of the cells in relation to structure, function and biochemistry (Creason, 2010).

Accuracy: The degree to which a diagnostic test gives result of a measurement, calculation, or specification that conform to the correct value or a standard (National Academies of Sciences et al., 2019).

Treatment outcomes: In this study, we defined treatment outcome as either clearance, persistence or progression of a cervical lesion. Clearance was defined as absence of any lesion at 12 months post treatment. Persistence was defined as presence of the cervical lesion 12 months post treatment at the same grade as at diagnosis. Progression was defined as presence of a cervical lesion 12 months post treatment at a grade higher than the one at diagnosis.

List of abbreviations and acronyms

ASCO	American Society of Clinical Oncologists
ASCUS	Atypical Squamous Cells of Undetermined Significance
AUC	Area Under the Curve
BHLH	Basic Helix-Loop-Helix
CDC6	Cell Division Cycle protein 6
cGIN	Cervical Glandular Intraepithelial Neoplasia
CHFR	Checkpoint with Forkhead and ring finger
CHFR	Checkpoint with Forkhead and Ring Finger Domains
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma *in situ*
CKC	Cold Knife Conisation
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic Acid
E6AP	E6-associated protein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescent In Situ Hybridisation
FISH	Fluorescent Insitu Hybridisation
FOXP3	Forkhead Box P3
H and E	Haematoxylin and Eosin
HC	Hybrid Capture
hcGIN	High grade cervical Glandular Intraepithelial Neoplasia
HIF-1α	Hypoxia-Inducible Factor -1α subunit
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HR HPV	High Risk Human Papilloma Virus
HRP	Horseradish Peroxidase
HSIL	High-grade Squamous Intraepithelial Lesion
ICC	Invasive Cervical Cancer
IHC	Immunohistochemistry
IQR	Interquartile Range
LEEP	Loop Electrosurgical Excision Procedure
LSIL	Low-grade Squamous Intraepithelial lesion

MCM	Mini Chromosome Maintenance
MOH	Ministry of Health
mRNA	Messenger Ribonucleic Acid
MRRH	Mbarara Regional Referral Hospital
MUST	Mbarara University of Science and Technology
MUST	Mbarara University of Science and Technology
NACT	Neoadjuvant chemotherapy
OD	Optical Density
P53	Tumor protein **53**
Pap	Papanicolaou test
PCR	Polymerase Chain Reaction
Rb	Retinoblastoma protein
RNA	Ribonucleic Acid
ROC	Receiver Operating Curve
RT-QPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SCC	Squamous Cell Carcinoma.
SD	Standard Deviation
SOP	Standard Operating Procedure
T-reg	Regulatory T cells
UCI	Uganda cancer Institute
VEGF-C	Vascular Endothelial Growth Factor C
VIA	Visual Inspection with Acetic acid
WHO	World Health Organisation
WRN	Werner DNA Helicases

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Abstract

Background: Immunohistochemical expression of P16ink4A and FOXP3 is associated with increasing grades of cervical intraepithelial lesions. We determined the prevalence of cervical intraepithelial lesions and associated factors, the association between serum biomarkers P16ink4A and FOXP3 concentrations and cervical intraepithelial lesions; and the diagnostic accuracy of serum biomarkers P16ink4A and FOXP3 in the detection of cervical intraepithelial lesions; and treatment outcomes among women attending the cervical cancer clinic at Mbarara Regional Hospital (MRRH), South Western Uganda.

Methods: We conducted a cross sectional study (n=364), two case control studies (n=270 and n=279) and a 12 months prospective cohort study (n=180). Participants included women seeking cervical cancer screening and testing services at the cervical cancer clinic of MRRH, from April 1 2022 to March 30, 2024. We collected clinical and demographic data, collected blood samples and measured serum P16ink4A and FOXP3 concentrations using quantitative Elisa kits from Elabscience Biotechnology. All Pap smears were microscopically examined by a laboratory scientist together with a pathologist following the Bethesda grading system (2014). All cases were confirmed with cytology and/or histology and statistical analyses were done in STATA version 17.

Results: The prevalence of cervical intraepithelial lesions was 6.6%. We observed increased odds of cervical intraepithelial neoplasia (CIN) for P16ink4A and a significant reduction in odds of cervical cancer (CC) for serum P16ink4A. Also, increased FOXP3 concentrations increased the chances of CC by 2 times. FOXP3 concentration of >0.0545ng/ml showed moderate sensitivity (32.22% and 57.78%) and specificity (68.89% and 68.89%) for detection of CIN and CC from healthy controls respectively. P16ink4A concentration of >0.946ng/ml showed moderate sensitivities (50.00% and 60.00%) and specificities (56.67% and 55.56%) for the detection of CIN and CC from healthy controls respectively. A combination of elevated FOXP3 and P16ink4A showed high specificity of 83.33%. The mean relative risk for follow-up serum FOXP3 among participants with persistent and progressed lesions was 1.34 and 27.82 respectively while that of P16ink4A among persistent and progressed lesions was 5.16 and 0.44 respectively.

Conclusions: The prevalence of cervical intraepithelial lesions was lower than global estimates. Serum P16ink4A and FOXP3 concentrations were observed to not only be associated with, but also highly specific for detection of cervical lesions and their persistence and progression. Quantitative measurement of circulating P16ink4A and FOXP3 may aid in diagnosis as well as prediction of persistence and progression of cervical lesions; and their combination has a high potential to be applied for diagnosis rather than screening for cervical lesions.

CHAPTER ONE: INTRODUCTION

1.1 Background

Cervical cancer is one of the world's deadliest although most easily preventable forms of cancer in women accounting for over 270,000 deaths annually with about 85% of these occurring in developing countries (WHO, 2016). Overall cervical cancer is ranked as the second commonest cancer among women after breast cancer in women worldwide (Bray et al., 2018).

Clinical and laboratory screening or diagnosis are key to management and improving prognosis of cervical cancer (Campos et al., 2017). With adequate, accurate and affordable laboratory tests, cervical cancer can be detected earlier and managed adequately (Campos et al., 2017, Sherris et al., 2009). Since its introduction, the "Pap test" is the most widely used screening method of cervical lesions world-wide (Yabroff et al., 2009).

Over the years, vigorous screening programs have been implemented and have subsequently contributed to the reduction of mortality and morbidity, especially in the developed world (Sankaranarayanan, 2014, Bansil et al., 2015). In Uganda, screening for cervical cancer has been dominated with techniques such as visual inspection with acetic acid (VIA) and the Pap test (Bansil et al., 2015, Nakisige et al., 2017). These techniques have improved awareness of the disease, and recently mass immunisation against high risk human papillomavirus (HR HPV) subtypes is now recommended especially for young females (Mutyaba et al., 2006, Bansil et al., 2015).

Limitations with respect to Pap smear modest sensitivity and specificity (Wright et al., 2004) have prompted the search for new biomarkers specific for not only the different histologic subtypes of cervical cancer (Bast et al., 2005) but also for predicting diagnosis and prognosis (Qiu et al., 2020). Such biomarkers may be used as stand-alone tests or in conjunction with traditional screening tests to enhance diagnostic consistency and accuracy in detection of cervical cancer at an earlier stage (Onyango et al., 2020). A combination of different blood-based biomarkers, say proteins and micro RNAs can greatly improve accuracy in detecting cervical cancerous lesions (Cohen et al., 2018).

FOXP3 and P16ink4A have been widely and successfully applied in grading of cervical lesions using immunohistochemical methods (Cuschieri and Wentzensen, 2008, Beauséjour et al., 2003, Keating et al., 2001, Klaes et al., 2001, Klaes et al., 2002) though with several challenges including longer turn-around-time and poor reproducibility (Tringler et al., 2004). It is against such a background that serum concentrations of proteins like FOXP3 and P16ink4A should be

studied for their diagnostic utility for detecting and monitoring prognosis of cervical cancer and cervical intraepithelial neoplasia.

1.2 Problem statement

Diagnosis of cervical lesions remains a challenge, especially in resource limited countries. The traditional diagnostic techniques such as VIA and the Pap test are discomforting for patients during specimen collection and carry along many barriers for their uptake (Yeo et al., 2018a, Nelson et al., 2010, Nagendiram et al., 2020, Islam et al., 2017, Devarapalli et al., 2018, Chua et al., 2021). Both tests are based on subjective results and thus bear challenges with respect to uniformity (Dasari et al., 2015).

The Pap test has been reported to have low to moderate sensitivity (Wright et al., 2004) in screening for cervical neoplasms. The Pap test is said to lead to many repeat tests and many unnecessary coloposcopies (Ronco et al., 2016).

Usually an abnormal Pap test is relied on, and more invasive and expensive investigations are done to confirm presence or absence of cancerous or pre-cancerous lesions (Jia et al., 2015). These investigations include identification of HPV DNA, colposcopic examination, histology and/or immunohistochemistry to diagnose cervical cancer or pre-cancerous lesions.

However, it should be noted that histological investigations on cervical preparations are also not only discomforting during specimen collection, but also yield subjective results (Dasari et al., 2015, Siebers et al., 2009). There is also inter and intra-observer variability in terms of reporting as well as poor adherence to standards (Dasari et al., 2015, Stoler and Schiffman, 2001). Colposcopy and biopsy are invasive and are reported to delay treatment options and also lead to extra costs and risks to patients (Jia et al., 2015). Screening for cancer of the cervix by using HPV DNA tests has been shown to be more effective compared to cytology but its major shortfall is the low positive predictive value, especially for high grade lesions (Ronco et al., 2014).

A number of biomarkers have been reported to be associated with cervical lesions through studies that have suggested that they are simpler, non-invasive and with high sensitivity and specificity (Pereira et al., 2010, Hu et al., 2010). Some of these biomarkers have been used in immunohistochemcal studies on tissue sections. However, immunohistochemistry also has its general shortcomings of false positives and poor accuracy for low grades tumors (Chou et al., 2015). Biomarkers including HPV protein E7, HPV Protein E6, CEA, SCC Ag, CA19-9, **Cyclin-dependent kinase inhibitor 2A** (P16ink4A), **Forkhead box p3 (FOXP3) and** c MYC have been proposed as immunohistochemical techniques, rather than being objectively quantified and measured in blood using methods that can be standardised (Wielders et al., 1990, Henry and

Hayes, 2012). Plasma/serum levels of these proteins and microRNAs have been correlated with the presence of cervical lesions and are elevated or reduced compared to the normal state. However, the analytical performance and diagnostic utility of these biomarkers in detection of cervical neoplasms and treatment outcomes has not been rigorously assessed in low resource settings such as Uganda.

1.3 Study justification

This study provides scientific evidence on the current prevalence of cervical lesions and the potential usage of serum concentrations of FOXP3 and P16ink4A in detection and prognostic monitoring of cervical intraepithelial lesions. With more evidence, the results of this study are hoped to guide decision making on whether these biomarkers should be used in cervical cancer diagnosis and monitoring prognosis either as stand-alone tests or in combination with the traditional tests.

1.4 Research questions

1. What is the prevalence and factors associated with cervical intraepithelial neoplasia among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital?
2. What is the association between serum levels of FOXP3 and P16ink4A and grades of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital?
3. What is the individual and combined accuracy of FOXP3 and P16ink4A in detection of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital?
4. What is the relationship between follow-up serum levels of FOXP3 and P16ink4A and treatment outcomes (clearance, persistence or progression) of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital?

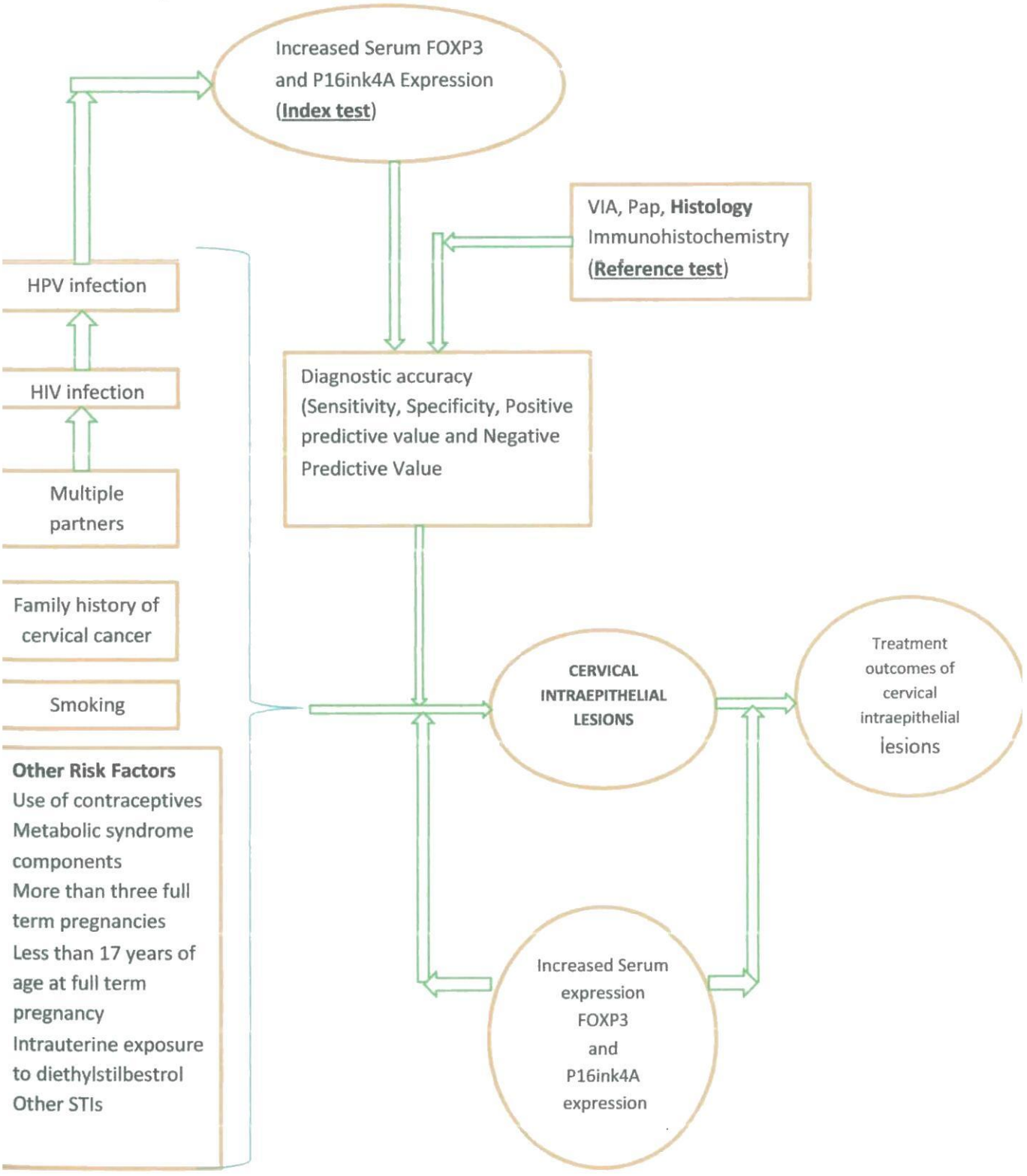
1.5 General objective

To assess prevalence and factors associated with cervical squamous intraepithelial lesions; association between serum levels of FOXP3 and P16ink4A and cervical lesions; the diagnostic utility of serum levels of FOXP3 and P16ink4A in diagnosis; and the association between serum levels of FOXP3 and P16ink4A and treatment outcomes of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital.

1.6 Specific objectives

1. To determine the prevalence and factors associated with cervical intraepithelial neoplasia among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital.
2. To determine the association between serum levels of FOXP3 and P16ink4A with grades of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital.
3. To establish the individual and combined accuracy of FOXP3 and P16ink4A in detection of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital.
4. To determine the relationship between follow-up serum levels of FOXP3 and P16ink4A and treatment outcomes (clearance, persistence or progression) of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital.

1.7 Conceptual framework



This study aimed at establishing the prevalence of cervical lesions and exploring factors associated. Some of these factors include smoking, HIV, multiple sexual partners, history of cervical cancer, contraceptive use among others. The study also determined the association between serum expression of p16ink4a and FOXP3 and cervical intraepithelial lesions. These biomarkers are thought to be highly expressed during development of cervical lesion and hence their serum expression can be used as a diagnostic test for cervical intraepithelial lesions. Also, these biomarkers are thought to reduce after treatment for cervical intraepithelial lesion. Basing on this, it is thought that their serum expression after treatment has an association with treatment outcomes (clearance, persistence or progression).

CHAPTER TWO: LITERATURE REVIEW

2.1 Burden of cervical intraepithelial neoplasia and cervical cancer

Cervical cancer has been reported to be the second most common cancer among women aged 15 to 44 years of age worldwide (Bruni et al., 2019b). Approximately 570 000 cases of cervical cancer have been reported worldwide, which makes it generally the fourth most common cancer among women (Arbyn et al., 2020). Cervical cancer has been reported to have a decreasing incidence in the developed world from 18.1 per 100,000 people to 11.1 per 100,000 from 1999 to 2013 (Jung et al., 2018). On a similar note, there has been a decreasing trend in the incidence of cervical cancer from 30.3 per 100,000 people in 2011 to 28.4 per 100,000 people in 2014 (Chang et al., 2018). Related findings were earlier reported by Schiffman and Solomon (2003) whereby the United States of America had a decreasing incidence of cervical cancer. However, there was an increasing incidence of cervical premalignant lesions including low grade lesions with over 1.4 million new cases) as well as high grade lesions with over 330,000 new cases (Schiffman and Solomon, 2003).

Cervical cancer is however ranked as the commonest cancer among women, responsible for deaths of more women than any other cancer in sub-Saharan Africa, Asia (including India), as well as some countries in Central and South America (WHO, 2012). On a similar note, 569,847 incident cases of cervical cancer were reported worldwide in 2018 by Bray et al. (2018), with an age standardised incident rate in East Africa being 40.1 per 100,000. South Africa had the highest age standardised incidence rate of 43.1 per 100,000. Majority of African countries, including Uganda, have been reported to have an age standardised incidence rate of at least 40 per 100,000 (Arbyn et al., 2020). In 2019, it was reported that 2784.9 million women worldwide aged 15 years and older were at risk of developing cancer of the cervix (Bruni et al., 2019b).

Over the years, the prevalence of Cervical intraepithelial neoplasia around different regions of Africa ranges between 3.7 and 22.6% (Donkoh et al., 2019, Thomas et al., 2012, Handlogten et al., 2014, Ajah et al., 2015b, Duru et al., 2015, Durowade et al., 2012, Allan et al., 2008, Wanyoike-Gichuhi et al., 2014, Kayumba, 2013, Xi et al., 2003, Keita et al., 2009, Assoumou et al., 2016). From the Ugandan perspective, Eilu et al. (2020) showed that the prevalence of cervical intraepithelial neoplasia stands at 38.6%.

Similarly, the incidence rate of cervical intraepithelial neoplasia has been shown to be 6.6 per 1000 persons in Uganda's neighbour, Kenya (Njagi et al., 2013). Chang et al. (2018) reported increasing incidence rates of CIN 1/2 from 2011 all through 2014, 317.9, 324.4, 317.7, 373.3, and 425.4 per 100,000 people. These figures illustrate an increasing trend in low grade cervical

intraepithelial lesions. In contrast, there has been a reported reduction in incidence rates of CIN 3, from 40.5 per 100,000 people in 2011 to 39.8 per 100,000 people (Chang et al., 2018).

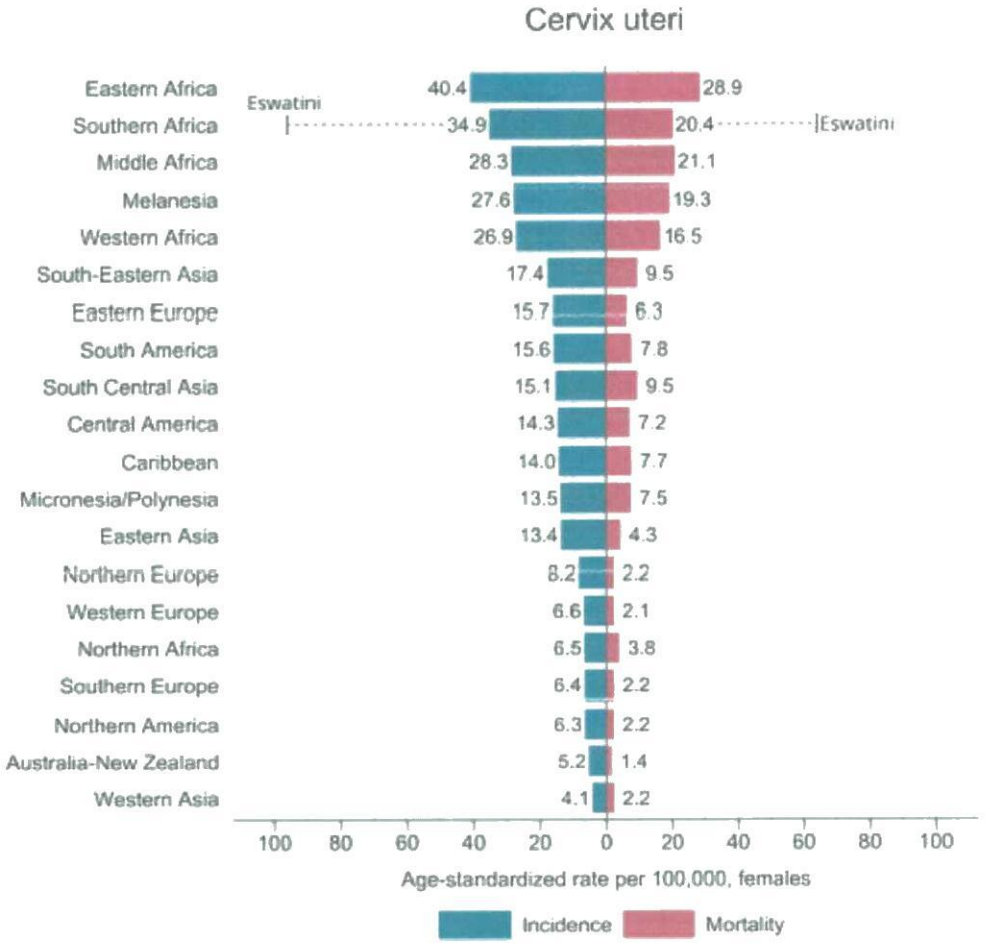


Figure 1: Bar chart of the region-specific incidence and mortality age-standardized rates for cervical cancer in 2022 (c.f (Bray et al., 2024)).

Rates are shown in descending order of the world age-standardized rate, and the highest national age-standardized rate for incidence and mortality is superimposed. Source: GLOBOCAN 2022 (Bray et al., 2024).

2.2 Pathogenesis of cervical cancer

Cancer means malignant, autonomous and uncontrollable growth of cells in tissues into tumours, which may invade surrounding tissues where they can cause new growths in distant and different parts of the body (metastases) (Tiltman, 2005, Sakuragi et al., 1999, Metcalf et al., 2000, Eifel et al., 1990, Thiery, 2002). Persistent infection with specific cancer-causing HPV genotypes, HR HPV, is the cause of most, if not all, cervical cancers (Tjalma et al., 2005), 90% of which are squamous cell cancers which are known to start in the squamocolumnar junction of the ectocervix; while the other 10% are adenocarcinomas, which arise in the glandular epithelial

lining of the endocervix (Schiffman et al., 2007, Tiltman, 2005). Cervical cancer develops from progressive epithelial cellular changes through multiple stages starting with infection with oncogenic human papillomavirus (HPV) infection (Giuliano, 2003, Tortolero-Luna and Franco, 2004, Fonseca-Moutinho et al., 2004). Cervical intraepithelial neoplasia are distinct changes, which occur in the epithelial lining cells of the transformation zone of the cervix (Tiltman, 2005)(Tiltman, 2005). They manifest when the cells develop in an abnormal fashion in the presence or persistent infection with HR HPV (Schiffman et al., 2007).

Cervical intra-epithelial neoplasia (CIN) is a detectable and curable pre-cancerous state of the cervical epithelium, and when left untreated can progress to a malignant state, cervical cancer (Njagi et al., 2013, Tiltman, 2005). Infection with any of the high-risk HPV (HR-HPV) genotypes is the major aetiological factor for cervical cancer and its pre-cancerous lesions (Walboomers et al., 1999). There are many genotypes of HPV, and are classified as either high risk or low risk (Tjalma et al., 2005). Fifteen HPV genotypes are classified as HR-HPV and they include HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (Shukla et al., 2009, Muñoz et al., 2003, Muñoz et al., 2006, Bouvard et al., 2009, Schiffman and Solomon, 2003, Schiffman et al., 2005), one more than the earlier 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) reported by Nindl et al. (1999) and Moody and Laimins (2010). HPV16 and HPV18 have been implicated to be high risk HPV subtypes highly associated with around 70% of all cervical cancers (Sankaranarayanan et al., 2015).

After infection with HPV, most lesions regress and clear on their own (Bosch et al., 2008, Baseman and Koutsky, 2005, Schiffman et al., 2007, Ostör, 1993). A few HR HPV infections tend to persist mostly in the basal and parabasal layers (Barrow-Laing et al., 2010, Zhang et al., 2006, Schiffman et al., 2007, Schlecht et al., 2001). Persistent infection with HR HPV does not always cause cancer in many individuals that get infected (Doorbar, 2006, zur Hausen, 2009). After a persistent infection with HR HPV genotypes, epithelial cells undergo a series of changes, typically epithelial-mesenchymal transition (Geiger et al., 2008), turning epithelial cells into mesenchymal cells which can metastasise; a property that is thought to contribute to the metastatic nature of human cancer cells (Thiery, 2002).

The human papillomavirus is a non-enveloped DNA virus with an icosahedral capsid consisting of a circular double stranded DNA, (7900 base pairs long). It has two functional genome regions: a coding region of early genes (E1, E2, E4, E5, E6, and E7) and another region of two late genes, the major and minor capsid proteins i.e. L1 and L2 respectively (Baker et al., 1991).

During infection and progression, different proteins are produced sequentially one after the other (Baker et al., 1991). During a productive stage of infection, the viral genome is relatively maintained at a low number of copies in the basal cells (Gupta and Mania-Pramanik, 2019, Pyeon et al., 2009, Parish et al., 2006, McBride, 2008, Maglennon et al., 2011). HPV undergoes several rounds of replication, making up 50–100 copies of viral genomes per cell (Gupta and Mania-Pramanik, 2019). The viral episomes remain maintained in basal cells by being replicated alongside with the host cell chromosomes (Baker et al., 1991, Doorbar et al., 2012). Thereafter, the viral life cycle goes on along with the differentiation program in keratinocytes and it is dependent on several cellular and viral factors and proteins (Baker et al., 1991).

For replication of the viral genome, the E1 viral initiator protein is a requirement and is recruited by E2 to the viral DNA, assembling a double hexamer at the viral replication origin (Hebner and Laimins, 2006). This makes E1 and E2 very important proteins for episomal maintenance (Egawa et al., 2012, Kim and Lambert, 2002). Differentiation of infected cells induces expression of late genes for further genome replication (Doorbar et al., 2012). Expression of viral proteins E6 and E7 detaches cell growth arrest from differentiation by inactivating P53 and pRb respectively (Doorbar et al., 2012). The result is that infected cells remain in proliferative state and continue with uncontrolled growth (Longworth and Laimins, 2004). The amplified viral genomes get packed into infectious viral particles using the L1 and L2 viral proteins (Doorbar et al., 2012).

HPV cervical carcinogenesis is mediated through viral E6 and E7 oncoproteins. These viral proteins E6 and E7 are of key importance in cervical cancer development (Doorbar et al., 2012). They compromise the usual effectiveness of cellular DNA damage responses and allow accumulation of secondary genetic mutations (Jeon et al., 1995, Pett et al., 2004). For instance, E6 degrades P53, a transcription factor which is responsible for regulating the cell cycle, DNA repair, cellular metabolism as well as apoptosis (Doorbar, 2006). So E6 induces breakdown of p53 by binding to the ubiquitin ligase E6AP and inhibiting p53-dependent cascade and eventually contributing to carcinogenesis (Gupta and Mania-Pramanik, 2019). On the other hand, E7 degrades pRb protein and as a result enhances uncontrolled cell proliferation (Longworth and Laimins, 2004). Thus, it is stated by Gupta and Mania-Pramanik (2019) that co-ordinated interactions of various viral and cellular pathways involving mainly proteins and other biomolecules like micro RNAs, E6 and E7, contribute towards progression of cervical epithelial cells to a cancerous state. Outgrowth of dysplastic lesions is said to be initiated by increasing expression of the two viral oncogenes, E6 and E7, which themselves interact with various proteins that regulate the cell cycle (Doorbar et al., 2012). Notable among these is the

retinoblastoma gene product (pRb). This is the one that gets inactivated after being bound by E7. pRb then inhibits transcription of the cyclin-dependent kinase inhibitor gene (P16ink4A). Increasing expression of these viral oncogenes in any dysplastic cervical cells might thus be reflected in increased expression of P16ink4A (Klaes et al., 2002).

2.3 Screening for cervical intraepithelial lesions

Screening is known as a public health intervention that is provided to any asymptomatic population, not for purposes of diagnosing disease, but to identify members within that population with a high risk of developing that disease or its precursors (Wilson et al., 1968).

A well-proven way of preventing cervical cancer is to screen and find pre-cancers before they can progress into invasive cancer (WHO, 2020). Cervical cancer is a very treatable disease when diagnosed early before progression (Sankaranarayanan, 2014). It is recommended that screening should be routinely done to have early diagnosis as well as treatment of cervical cancer (Sankaranarayanan, 2014). However, only 5% of women in low and middle income countries undergo cervical cancer screening (Sibiya, 2012). It has been reported that inadequate screening and treatment of cervical intraepithelial neoplasiais a contributing factor to existing high mortality due to cervical cancer (Forman et al., 2012, Hoppenot et al., 2012, Ditzian et al., 2011, Handlogten et al., 2014).

Screening should be done at least once for every woman; and the most benefit is achieved when women aged 30 to 49 years get screened at least once (WHO, 2013a). However, this is not the case for women positive with HIV. Such women should be screened for cervical cancer as soon as possible (WHO, 2006).

Recommended tests for cervical cancer screening include HPV testing, Pap smear cytology and VIA (WHO, 2013a). When Pap or VIA turns out negative, a woman is asked to retest after 3 to 5 years (WHO, 2006, WHO, 2013a, WHO, 2014c, WHO, 2014b, WHO, 2014a). For the case of HPV, a negative result leads to retesting after 5 years or three years among HIV positive women (WHO, 2013b). This screening interval can be made longer than five years for older women and even for those with a series of negative screening tests (WHO, 2014c).

International guidelines emphasise that the choice of screening and treatment approach, whether a "screen-and-treat" or a "screen, diagnose and treat" should depend on potential benefits and risks, the likelihood of women getting lost to follow-up, the cost, and availability of the required equipment and human resource in any given setting (WHO, 2014c).

Uganda has a cervical cancer screening program, employing a "screen, diagnose and treat" approach, in which the main screening tests include Pap smear cytology and VIA (Nakisige et al.,

2017). Women who undergo such screening are aged 25 to 65 years for cytology and 25 to 49 for VIA at a screening interval of 3 years (Nakisige et al., 2017). However, this screening program does not have any known quality assurance structure to supervise and monitor screening processes (Bruni et al., 2019a).

For women who test negative with VIA or Pap smear cytology, WHO (2014c) recommends that the interval for re-screening should be 3 to 5 years. It further specifies that for women whose test results turn out negative for HPV, re-screening should be done after 5 years. After repeated screening with negative test results, and also for older women, the screening interval can be prolonged to more than 5 years. Still according to (WHO, 2014b), women who have undergone treatment for cervical pre-cancerous lesions should receive post-treatment review and testing after 12 months.

The HPV DNA test is a molecular test that is based on detection of DNA from any of the high risk HPV types from either vaginal or cervical specimens (Hwang and Shroyer, 2012). This method is recommended for women aged 30 years and above while for those below 30 years, they are known to have transient HPV infections hence it is unnecessary to subject them to the test (WHO, 2006, WHO, 2013a). For older women, detection of high risk HPV is an indication of a persistent HPV infection and such a woman is categorised as either having an existing lesion or at risk of developing a pre cancer of a cancer (WHO, 2006). In Uganda, the ministry of health has started rolling out HPV test as a cervical cancer screening test for HIV positive women. Many new HPV DNA tests have been introduced in some countries as an adjunct to cytology or as primary screening test to be followed by a secondary, but more specific test, for instance Pap smear cytology (Bruni et al., 2019b).

Visual Inspection with Acetic Acid (VIA) is the method that enables the health care provider to visually detect cellular changes on the cervix after applying 3-5% acetic acid and is the most widely used method because of it being of low cost (Denny et al., 2006, Denny, 2012).

VIA has been said to be an effective screening tool for cervical cancer (Fokom-Domgue et al., 2015, Firnhaber et al., 2013, Huchko et al., 2015b, Huchko et al., 2015a, Basu et al., 2015, Qiao et al., 2015, Mustafa et al., 2016). In fact, Shastri et al. (2014) reported a significant reduction in mortality due to cervical cancer as a result of implementing screening with VIA. In Uganda, VIA is the main screening test, although its coverage is as low as 8% among all women of reproductive age (Nakisige et al., 2017).

The Papanicolaou (Pap) is the most widely used cytological cervical cancer screening method world over, and it has greatly reduced morbidity and mortality rates of cervical cancer (Papanicolaou and Traut, 1941, Lăără et al., 1987). This test involves microscopic examination of

stained cells from the cervix, especially the transformation zone. Cells are fixed onto a glass slide and for liquid based cytology, they are placed into a fixative medium (Papanicolaou and Traut, 1941). Several studies have validated cervical cytology against histology and have reported significant findings of accuracy (Dames et al., 2009, De Vuyst et al., 2013, Kitchener et al., 2007, Branca et al., 2001, Maiman et al., 1998, Boardman et al., 2008).

Diagnostic tests include colposcopy and histology or biopsy. Colposcopy involves examination of the cervix and nearby related organs with a colposcope, an instrument that provides a strong and bright light while magnifying the image (WHO, 2013b). Colposcopic examination is done following a positive screening test and it helps to verify presence of lesions, determine the degree and type of lesions, either pre cancer or cancer (WHO, 2013a, WHO, 2014a). During colposcopy, biopsies can be collected directly from any area that appears abnormal (WHO, 2013a). Also from colposcopy, it can be determined whether to use cryotherapy or LEEP as treatment strategies (WHO, 2006).

A biopsy is a piece of tissue that is removed from a living person (Whitehead, 1954). In this case, the procedure requires removal of a piece of tissue from areas that are either positive on VIA or appear suspicious for cancer (WHO, 2013a). Histology is considered to be the gold standard for diagnosis of cervical lesions (Hwang and Shroyer, 2012). However, it is worth noting that histologic assessment of cervical lesions is limited only to interpretation of the cellular morphology and arrangement, and does not cater for risk of persistence, progression, or regression (Hwang and Shroyer, 2012). It is on record that histologic assessment of cervical specimens is underscored by interobserver variability (Hwang and Shroyer, 2012, Stoler and Schiffman, 2001). In fact errors in histologic diagnosis can lead to overtreatment of suspected patients as well as under treatment for patients with high-grade lesions if they receive false negative diagnoses (Hwang and Shroyer, 2012). There has been overwhelming work to try and improve sensitivity of traditional screening methods (Pap test and VIA). Brown et al. (2012) reported that such innovations include use of liquid based preparations to address issues of specimen collection, handling and preparation as well as computer aided microscopy screening errors.

2.4 Treatment methods for cervical cancer and cervical intraepithelial neoplasia

Following a positive screening test, as a case in a screen and treat approaches, women found to have cervical intraepithelial neoplasia must receive treatment and this does not have to wait for diagnostic confirmation (WHO, 2014b). Cervical intraepithelial neoplasia of CIN2+ are

diagnosed histopathologically (Arbyn et al., 2012), and if left untreated, they may progress to cervical cancer.

The three treatment modalities that are recommended for low and middle income countries are cryotherapy, LEEP and CKC (WHO, 2006, Santesso et al., 2012, WHO, 2011) with or without histological confirmation (WHO, 2013b). Cryotherapy, one of the ablative methods (Castle et al., 2017), it is regarded as the first option for treatment of cervical intraepithelial neoplasia (WHO, 2013a). This is an ablative method that eliminated precancerous cells by freezing. It involves application of a cryoprobe to the affected area, thereby freezing and destroying the abnormal areas along with the nearby normal areas (Castle et al., 2017, WHO, 2014b). Through a cohort study, cryotherapy has been known to have success rate of 70% after one year post treatment for CIN3 lesions. Similarly, it has been reported that cryotherapy succeeds among 85 to 92% of CIN2 and CIN3 lesions (Sauvaget et al., 2013, Dolman et al., 2014).

Loop Electrosurgical Excision Procedure (LEEP) is a procedure that involves removal of a lesion from the cervix using a looped thin wire that is connected and powered by an electrical unit which cuts and coagulates at the same time (WHO, 2014c). Therefore, LEEP removes the lesion from the entire transformation zone, it can be performed using local anaesthesia and also provides a specimen for histopathological analysis (Castle et al., 2017). LEEP has been reported to have a success rate of 84% with is lower than the 76% of cryotherapy (Mitchell et al., 1998, Mitchell et al., 1999). It was reported that LEEP is as effective as some ablative methods more so thermocoagulation after one year post treatment (Papoutsis et al., 2017). Nessa et al. (2014) reported that 95% of cases treated with either LEEP or thermal coagulation clear lesions and show no evidence of lesions during follow up visits.

Cold knife conisation (CKC) is a surgical procedure that is used for treatment, by removing tissue and use the removed tissue to diagnose cervical dysplasia (Castle et al., 2017). Therefore, conization of the cervix is indicated for diagnostic purposes as well as treatment (Cooper and Menefee, 2017). This diagnostic excisional procedure is recommended to be done if there is a lesion that is deemed suspicious for invasive cancer or an adenocarcinoma in situ of the cervix (WHO, 2014b). CKC is also recommended if the entire lesion can hardly be completely visualized on colposcopy (Perrone et al., 2019, Schmidt, 2019, Chen et al., 2018). Cooper and Menefee (2017) indicated that CKC should not be performed when there is little cervical tissue, probably due to previous surgeries. It should be noted that CKC carries an advantage of having a

low recurrence rate (Melnikow et al., 2009) and the fact that it provides a histological specimen with good specimen margins (Castle et al., 2017).

The international Federation of Gynaecology and Obstetrics (FIGO) provides clear guidelines on staging and treatment of cervical cancer (Koh et al., 2015). Staging is done basing on size of the tumor and extent of spread of the disease within nearby tissues and organs of the pelvis, and also to distant organs through metastasis and it is such staging that determines treatment (WHO, 2020). It is in the global strategy of elimination of cervical cancer by WHO (2020) that 90% of women diagnosed with cervical cancer get treated and managed appropriately. Cervical cancer treatment options include surgery as the primary therapy (WHO, 2020, Small Jr et al., 2017), radiotherapy (Koh et al., 2019) and chemotherapy, either singly or in combination (WHO, 2020). Treatment options for cervical cancer depend on histopathology results from biopsies and also the need or possibility of preserving fertility (Koh et al., 2019).

The standard treatment for patients with early stage cervical cancer (FIGO stages IA to IB1) includes radical hysterectomy and lymph node dissection and/or radiation which can either be combined with chemotherapy or not (Landoni et al., 1997, Gadducci et al., 2003, Sedlis et al., 1999). Surgery is the treatment of choice for early stage cervical cancer which include IA, IB1 and some few IIA1 cases (Koh et al., 2019, Lei et al., 2020). However, for stage IA1, whenever a patient needs to preserve fertility, a cone biopsy with or without pelvic lymph node dissection is recommended (Andikyan et al., 2014) and in case fertility preservation is not a desire or impossible, a simple hysterectomy is recommended (Koh et al., 2019). Among patients with FIGO stage IA2, and there is a wish to preserve their fertility, radical hysterectomy and pelvic dissection is recommended. But if for any reason fertility preservation is not desired, surgery (radical hysterectomy and bilateral lymph node dissection) (Yoneda et al., 2015, Kokka et al., 2014) or radiotherapy (Small Jr et al., 2012) is recommended. The category of advanced cancer includes FIGO stages IIB up to IVA, but Koh et al. (2019) indicated that IB2 and IIA2 can also fall under advanced cancer. For women with advanced cancer, the cisplatin-containing concurrent chemoradiotherapy is superior and hence recommended (Gaffney et al., 2007). Locally advanced cervical cancer is treated with external beam radiotherapy with concurrent chemotherapy (cisplatin based) with brachytherapy (Pötter et al., 2011, Sturdza et al., 2016).

In Uganda, the Ministry of Health, (MoH, 2016) categorises cervical cancer into three categories, i.e., early stage, late stage and very late stage. Through the division of non-communicable diseases (NCDs), the ministry of health has played a significant role in developing strategic plans

for cervical cancer control and prevention. Uganda implements a test and treat approach towards cervical cancer (MoH, 2016). With increased government intervention, surgery, radiotherapy and chemotherapy are all offered at UCI (Nakisige et al., 2017). However, with the inconsistency of radiotherapy, Uganda opted for the neoadjuvant chemotherapy (NACT) based on the resource stratified clinical practice guideline from the American Society of Clinical Oncology (ASCO) (Chuang et al., 2016). Early cervical cancer stages of IB2 to IIIA are being managed following the modified NACT protocol, after a multidisciplinary discussion on a case by case basis, while stages IIIB to IV are managed with high doses of vaginal brachytherapy with or without chemotherapy (Nakisige et al., 2017).

2.5 Biomarkers for cervical cancer and cervical intraepithelial neoplasia

The current approaches in cervical cancer screening and diagnosis have challenges like delays in releasing results, demand for highly trained medical personnel, infrastructure (equipped laboratories) and follow-ups (Sibiya, 2012). It is however possible to have other methods of cervical cancer screening that are less costly and user-friendly as recommended for developing countries (Sibiya, 2012). Cancer biomarkers include genes, nucleic acids (DNA and RNA), proteins (enzymes and antigens) and all other molecules involved in carcinogenesis; and can be used to detect either carcinogenic processes, premalignant lesions or malignant lesions. Biomarkers in cervical cancer can be grouped as those of HPV infection and carcinogenesis (HPV DNA, E6/E7 mRNA p53, Rb, p16ink4a, telomerase RNA gene, serum SCC-Ag, OCVA1), cell cycle and proliferation biomarkers (Ki-67, cyclin D1, p53, p63), apoptotic biomarkers (P53, BCL-2, BCL-XL, BAX), biomarkers of differentiation (CK7, CK8, CK17, CK19), cell adhesion, invasion and metastasis biomarkers (E-cadherin, P-cadherin, CD44, ADAM9, MT1-MMP, TIMP-1, TIMP-2, MT1-MMP, MMP-2, MMP-1, MMP-9, MMP-14, proMMP-14 furin, gelatinase, TIMP-1 and TIMP-2), cancer stem cell biomarkers (Nanog, nucleostemin (NS), musashi1 (Msi1), SOX2, KLF4, CD133, Cd44, ALDH1, CD49f, ABCG2, BMI1, PIWIL2, LGR5, OCT4, CD117), biomarkers of angiogenesis (VEGF, podoplanin (PDPN), thrombospondin-1, CD31, CD34, CD105) and biomarkers of vaginal microbiome, inflammation and immune homeostasis (Volkova et al., 2021, Gadducci et al., 2015, Hwang and Shroyer, 2012, Noordhuis et al., 2011). More than 98% of cervical cancer is due to Human Papilloma Virus (HPV) infection. In a need to improve screening and diagnosis of cervical lesions, identifying main components in HPV-mediated carcinogenesis provides potential targets for clinically and diagnostically useful biomarkers.

2.6 HPV based biomarkers

2.6.1 HPV DNA

HPV DNA is the most researched and hence most used biomarker in many cervical cancer screening programs (Hwang and Shroyer, 2012). Currently there are numerous assays that have been developed to detect nucleic acids of HPVs in cervical specimens. It is documented that HPV nucleic acid tests have a very high sensitivity as well as a negative predictive value for CIN3 and above (Carozzi et al., 2013, Arbyn et al., 2012). There is also a reported high sensitivity of HPV DNA among women aged above 30 years (Ronco et al., 2010). It is also important to note that most of the precursor lesions of cervical adenocarcinoma are usually missed on cytology, but HPV DNA can be used to diagnose them with high sensitivity (Castle et al., 2010).

HPV DNA can be detected using various methods such as, polymerase chain reaction (PCR), immunohistochemistry (IHC), immunoytochemistry (ICC), Hybrid Capture (HC) 1 and 2 or fluorescent in situ hybridisation (FISH) (Hwang and Shroyer, 2012). In a meta-analysis conducted by Arbyn et al. (2012), it was discovered that different HPV DNA testing methods had a pooled sensitivity of 90.4% and a specificity of only 52.3% specifically for CIN2 and worse. However, it has been emphasised in several studies that the different methods of testing for HPV DNA have widely varying measures of accuracy (Belinson et al., 2011, Heideman et al., 2011).

Koliopoulos et al. (2017) conducted a systematic review and concluded that HPV DNA can hardly miss out any CIN2 or CIN3 or worse, but also emphasised that fact that such a benefit can lead to increased and unnecessary referrals for colposcopy. It was later presented by Zeferino et al. (2018) that cervical cytology can be used as a triage test for women with positive HPV DNA results, something Muwonge et al. (2014) had earlier proposed. More positives about the use of HPV DNA in cervical cancer screening had earlier been presented from a meta-analysis conducted by Arbyn et al. (2014) and these included the ease of training laboratory staff, acceptability of self-collected specimens and the reproducibility nature of the results.

HPV DNA is known to have several limitations with respect to cervical cancer screening and diagnosis (Hwang and Shroyer, 2012). Several assays of HPV DNA are as sensitive as 98-100% for CIN3+ and ASCUS (Einstein et al., 2010, Bartholomew et al., 2011). Most HPV DNA testing assays cannot differentiate between transient and persistent or transforming infections (Tornesello et al., 2013, Hwang and Shroyer, 2012). In fact, the four HPV DNA testing methods including the Hybrid capture 2, the Cervista HPV HR, the Cervista HPV 16/18 and the Cobas 4800 HPV only test for 13, 14, 2 and 14 HR HPV types respectively, out of the 15 known high risk HPV types (Tornesello et al., 2013). In many countries, HPV DNA is being used together with Pap smear

cytology to screen for cancer of the cervix. However, Aitken et al. (2019) reported that since 2017, the Dutch cervical cancer screening program has totally transitioned from older traditional methods to HR HPV DNA.

2.6.2 HPV viral load

The measure of viral DNA load per cell was earlier thought to be a very useful marker of determining risk of progression of cervical carcinogenesis (Carcopino et al., 2006). Hwang and Shroyer (2012) observed that elevated viral load is considered to be informative of a persistent infection and therefore progression of lesion, and a low viral load to mean HPV viral clearance and hence no progression. This concept is not true, in that CIN 1 is a productive lesion and has thousands of viral copies per cell yet CIN2 and worse have very low, if not a single DNA copy per cell (Snijders et al., 2003). To the contrary, Dong et al. (2018) reported that viral load measurements of certain HPV types i.e. 16, 31, 33, 52 and 58 were elevated women with a cytology result of at least a HSIL.

Serial HPV viral load measurements have been reported by Depuydt et al. (2015), Verhelst et al. (2017) and Depuydt et al. (2016) to be predictive of outcomes and that two measurements would be enough. This approach was emphasised by Berggrund et al. (2019) who proved that serial testing of HPV viral load distinguishes between women with CIN2 or worse from those without. However, basing on the relevance of serial testing, Luo et al. (2017) had earlier emphasised the need to establish optimal titre thresholds to help stratify women better. Constandinou-Williams et al. (2010) also had earlier on stated it clearly that a single random estimate of viral load cannot be a useful biomarker of cervical lesions in clinical settings.

Schmitt et al. (2013) reported that HPV viral load, other than just HPV DNA, can reduce the number of referrals, for treatment, by at least 52%. Moreover, with all limitations, more so, the presence of cervical cellular material that is derived from low-grade squamous lesions. Carcopino et al. (2006) had earlier observed that viral loads of HPV 16 and 18 were related to severity of cervical lesions.

Of late, the relationship between viral load of HR HPV is known to differ across HPV types (Gustavsson et al., 2019), and this calls for individual testing and measurement for each HPV type. This is something that has not yet been realised to date since the Hybrid Capture 2 and the Cobas HPV test only for HPV 16 and 18 types; have cross reactivity with several other HPV types and they don't even measure viral load as reported by Preisler et al. (2016) and de Thurah et al. (2018).

2.6.3 HPV mRNA

The proposed usability of HPV mRNA is based on an old concept that E6/E7 expression by HR HPV always results in transformation events, more so with unregulated cell-cycle progression as a result of degradation of p53 and Rb (Hudson et al., 1990).

Many studies have been conducted to establish the usability of HPV mRNA for oncoproteins E6 and E7 (Hwang and Shroyer, 2012) and this has been done mostly in comparison with conventional cytology as well as HPV DNA. For instance, Wang et al. (2019) evaluated two HPV tests, one for HPV DNA (Aptima HPV-GT) and another for E6 and E7 mRNA (Aptima HPV) for HPV type 16 and 18. In comparison with cytology, positive HPV mRNA (Aptima HPV) was found to be significantly higher in HSIL, with a reported sensitivity and specificity of 98.11% and 87% respectively (Wang et al., 2019). This meant that women with a positive Aptima HPV were more likely to have a cytological result of HSIL and worse (Hwang and Shroyer, 2012).

In a relatively similar comparison of HPV DNA, cytology and HPV E6/E7 mRNA, Han et al (2018) explored the clinical value of HPV E6/E7 mRNA in cervical cancer screening among HPV DNA positive women or with abnormal cytology in the Xinjiang region of China. They reported that HPV E6/E7 mRNA had a sensitivity of 85.15% and a specificity of 66.67%, and concluded that HPV E6/E7 mRNA was a better option for diagnosing high-grade cervical lesions compared with HPV DNA (Han et al., 2018). It had earlier been suggested by Liu et al. (2014) after studying the QuantiVirus(®) HPV E6/E7 mRNA test and concluded that mRNA testing may be such a valuable tool in triaging lesions of CIN2 and worse. The same test was tested by Yao et al. (2017) using cervical exfoliated cells and observed a sensitivity of 89.52% but reported a lower specificity of 48.96%. They further concluded that HPV E6/E7 mRNA can be used as a triage test for HPV positive women (Yao et al., 2017).

A report from a meta-analysis, where HPV E6/E7 mRNA test was studied among women with LSIL or ASCUS, (Yang et al., 2017) observed that those women who test positive with HPV E6/E7 mRNA are truly positive and should be rigorously followed. They could not conclude that HPV E6/E7 mRNA can be used to triage women with LSIL and ASCUS.

2.7 Host cell biomarkers

2.7.1 P16ink4A

This is cell cycle regulator protein whose amount and role are tightly regulated in normal cells (Gu et al., 1993, Serrano et al., 1993). It's a tumor suppressor protein which inhibits cyclin dependent kinases 4 and 6, by phopsphorylating the retinoblastoma (Rb) protein (Serrano et al.,

1993). During a transforming HPV infection, the viral oncogene E7 binds the Rb protein, which results in increased levels of P16ink4A (Sano et al., 1998). It is from such a background that P16ink4A is believed to serve as a surrogate marker for persistent HR HPV infection (Keating et al., 2001, Klaes et al., 2002). In fact, P16ink4A has been described as a sensitive and specific marker for dysplastic cervical cells and that it can be useful in cervical cancer screening and diagnosis (Guo et al., 2004, Nieh et al., 2003, Nieh et al., 2005, Tsoumpou et al., 2009, Yoshida et al., 2004). This is because IHC expression of P16ink4A is known to be related to degree of histological dysplasia and malignancy (Kishore and Patil, 2017). Relatedly, over expression of P16ink4A is associated not only associated to disease free survival but also to increased overall survival and hence a better prognostic marker (Lin et al., 2014).

In most of the times, the application of P16ink4A has been in Immunohistochemical studies and majority of such studies have used the E6H4 antibody clone (Keating et al., 2001, Klaes et al., 2002, Tringler et al., 2004, Horn et al., 2008, Zhang et al., 2007, Bergeron et al., 2010, Murphy et al., 2003). In other instances, P16ink4A immunohistochemical studies have been carried in combination with KI-67 (Kanthiya et al., 2016, Walts et al., 2006, Zhong et al., 2015). This duo staining is now used as an adjunctive test in screening for cervical cancer (Brown et al., 2012).

It had earlier been stated by Zhang et al. (2007) that the sensitivity of cervical cancer screening tests like HPV DNA and Pap smear cytology may be underestimated when compared with an imperfect gold standard, histology. It is from their study that they revealed that P16ink4A IHC has an impact on determining the performance of cervical cancer screening tests (Zhang et al., 2007). Moreover, there is an improved interobservor agreement of CIN2+ with the conjunctive use of histology with P16ink4A IHC compared with histology alone (Reuschenbach et al., 2014).

There have been developments in the usage of P16ink4A other than IHC on surgically obtained biopsies or cytology specimens. Wu et al. (2016) evaluated the performance of P16ink4A using an ELISA kit to measure serum concentrations of P16ink4A in serum of HIV positive participants. The P16ink4A ELISA kit did not perform well as a screening test for CIN2+ detection among the study population due to low specificity (Wu et al., 2016).
With the discovery of HPV vaccines and subsequent implementation of mass immunisation, Badiga et al. (2016) argued that expression of P16ink4A in cervical intraepithelial neoplasiamay not be affected by HPV vaccines.

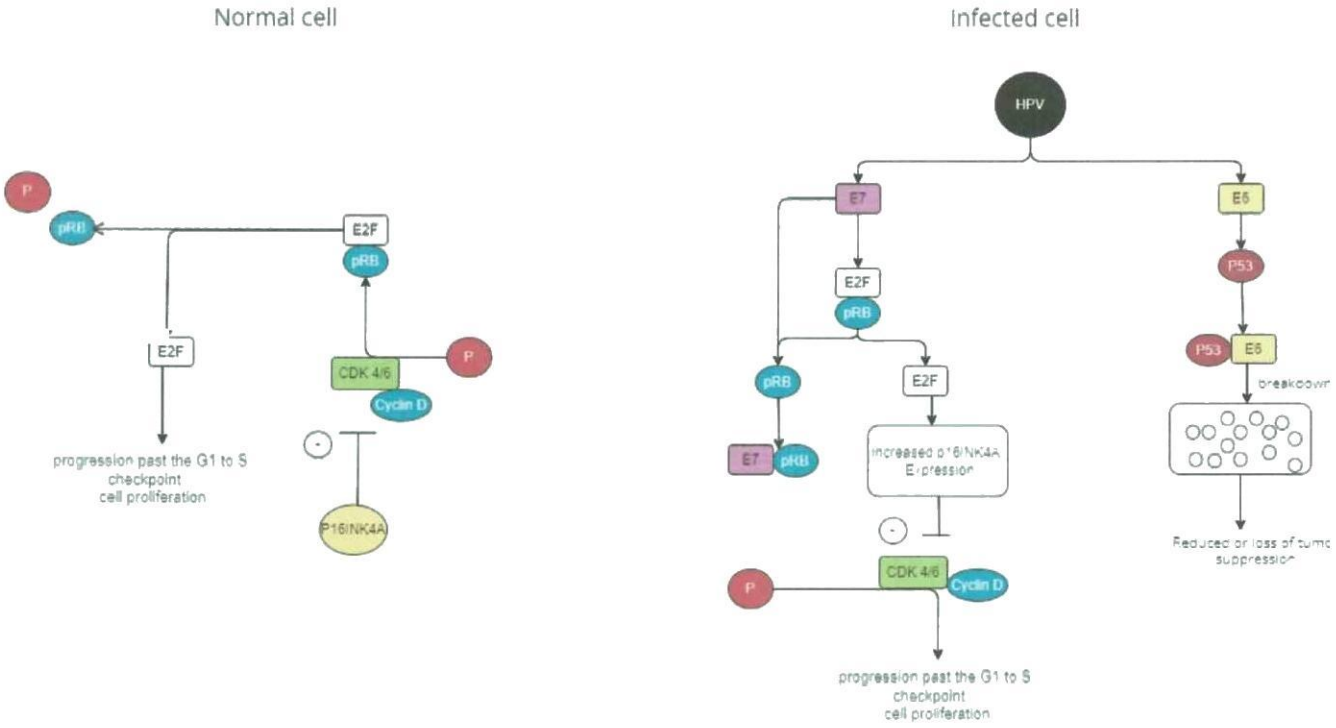


Figure 2. HPV related Cancer increases expression of P16ink4A.

The Left panel: Normal squamous cell. The complex of Cyclin D–cyclin dependent kinase (CDK) 4/6 phosphorylates the tumor suppressor protein, pRb. The hyperphosphorylation leads to cleavage and release of activated transcription factor E2F, which leads to expression of downstream gene expression products allowing cell cycle continuation from the G1 to S phase. As a cyclin kinase inhibitor, p16 serves as a tumor suppressor molecule and hence a negative regulator of the cyclin D–CDK 4/6 complex. Right panel: HPV infected cell. When E2F binds to pRb, it remains inactive until the overexpression of the E7 oncoprotein by high-risk HPV subtypes brings in its disruptive effects to the E2F–pRb complex by displacing E2F and itself binding to pRb. The release of activated E2F drives increased expression of downstream gene products, which allows increased cells cycle activation from the G1 to S phase. In an attempted regulatory feedback mechanism, to block further cell proliferation, p16 is itself upregulated. The overexpression of another oncoprotein, E6, leads to another mechanism. E6 binds to another tumor suppressor protein, p53, and this binding results into breakdown of p53. The resultant loss of the regulatory function of p53 causes propagation of the cell cycle hence preventing apoptosis.

2.7.2 FOXP3

Forkhead/winged-helix transcription factor box P3 (FOXP3) is a regulator for regulatory T cells (T reg) development and function; and belongs to the Fork head protein family of transcription regulators. In normal state, it is expressed on regulatory T cells (Luo et al., 2015).

Human FOXP3 gene is located on Xp1 1.23 and exists with ten introns and 11 exons (Luo et al., 2015). FOXP3 is reported to play very important roles in development and functionality of regulatory T cells, in that those regulatory T cells with high FOXP3 expression may have the potential to block an immune response (Hori and Sakaguchi, 2004, Sakaguchi, 2005, Sakaguchi et al., 2008, Sakaguchi et al., 2010, Sakaguchi et al., 2013, Fontenot et al., 2003). Earlier studies revealed that FOXP3 is expressed both in the cytoplasm and nucleus of cancer cells (Karanikas et al., 2008, Ma et al., 2013, Tan et al., 2014, Fu et al., 2007, Ladoire et al., 2011, Luo et al., 2015).

Immunohistochemical studies by Luo et al. (2015) showed that there is no demonstratable FOXP3 expression in normal cervical tissue yet there is a significant increase in FOXP3 expression from CIN1 all through to cervical cancer. This confirms earlier observations that FOXP3 expression is associated with development, progression and prognosis of cancers. (Karanikas et al., 2008, Ma et al., 2013, Tan et al., 2014, Fu et al., 2007, Ladoire et al., 2011, Luo et al., 2015, Tang et al., 2017).

It is also worth noting that FOXP3 expression had been shown to be significantly and positively related to P16ink4A expression. (Luo et al., 2015b). There is also a positive correlation that is reported to exist between expression of FOXP3 and VEGF-C as well as cervical cancer lyonhangiogenesis (Tang et al., 2017).

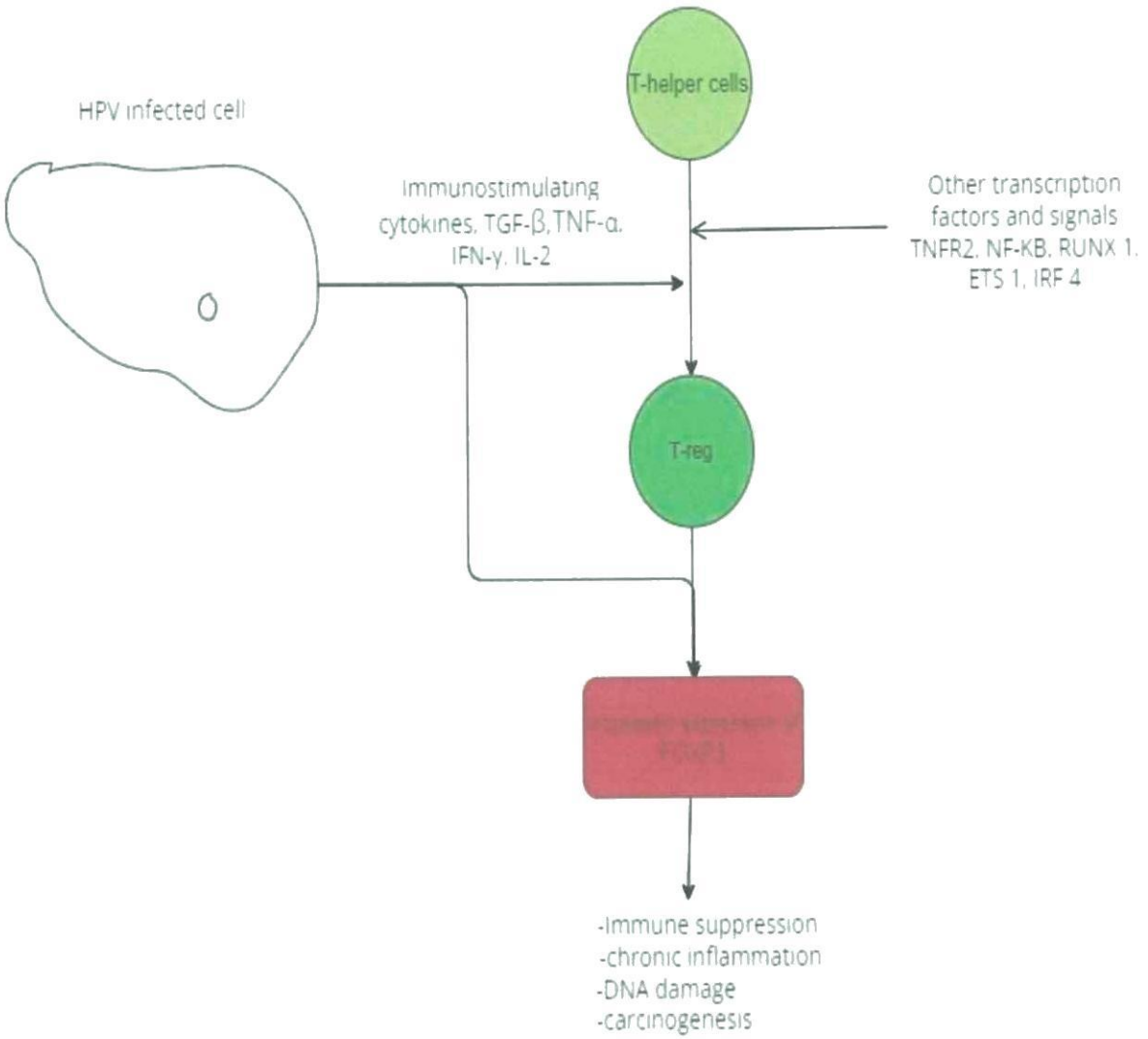


Figure 3. HPV related Cancer increases expression of FOXP3.

HPV infected cells secrete immunestimulating cytokines including the Transforming Growth Factor (TGF-β), Tumor Necrosis Factor-α (TNF-α), interferon-γ (IFN-γ), interleukin 2 (IL-2) etc. These cytokines result in development and maintenance of effector functions of regulatory T cells, including increased expression of FOXP3. This happens with the help of other transcription factors and cell signals. This leads to imunesuppression, chronic inflammation, DNA damage and increased susceptibility to neoplasia.

CHAPTER THREE: METHODS

3.1 Research paradigm

This research study was nested in the positivist research paradigm. In this philosophical orientation, we believe that there is one reality. There must be a population-specific prevalence of cervical lesions and associated factors. There also must be a blood-based test for detecting cervical lesions in our study population. This paradigm's conclusions are founded on deductive reasoning, hypothesis formulation and testing, operational definitions and mathematical equations, computations, extrapolations, and expressions, all with the goal of providing explanations and generating predictions based on observable outcomes. According to the four basic aspects or assumptions of a paradigm, the positivist paradigm's epistemology is objectivist, its ontology is naive realism, its methodology is experimental, and its axiology is beneficence. The results of this study are generalizable to other African low resource settings.

3.2 Ontology

This study was designed to know our population-specific prevalence of cervical lesions, factors associated and whether FOXP3 and P16ink4A are associated with cervical lesions, treatment outcomes and how accurate they are in detecting cervical lesions in our study population.

3.3 Epistemology

In this study, we described quantitative methods to enable us to know the nature and extent of the association between FOXP3 and P16ink4A and cervical lesions as well as their accuracy in detecting cervical lesions in our study population.

3.4 Study design

This study employed cross-sectional, case control and cohort study designs. First, we had a cross-sectional study for objective one, followed by two case-controls studies for objectives two and three, with one control group and two case groups each, after which we conducted prospective cohort study for objective four. Women confirmed to have any lesion of any grade were cases while those that were confirmed negative of any lesion were the controls.

The cases were followed up in a prospective cohort study for objective four. Those women who had a positive VIA and confirmed to have any cervical lesion of any grade (cases) were followed up as guided by the routine standard of care to compare serum concentrations of biomarkers before and after treatment. The prospective cohort took twelve months.

3.5 Study setting

The study was carried out at the cervical cancer clinic and the histopathology laboratory at Mbarara Regional Referral Hospital (MRRH) and the pathology department of Mbarara University of Science and Technology (MUST) respectively. MUST and MRRH are located in Mbarara city, south western Uganda. They are approximately 260km from Kampala, the capital city and 2km from the city centre on the Mbarara-Kabale highway. MUST and MRRH are government institutions and the university has over 3000 students, according to EduRank EduRank.org - Rankings of 14131 universities in 183 countries, while the hospital is a regional referral with a capacity of 350 beds. MRRH offers specialised services and runs specialised clinics for all medical conditions, including cancer of the cervix. Among these specialised clinics is the cervical cancer clinic which screens an average of 15 mothers per day (clinic records) and operates five days a week. The cervical cancer clinic serves all districts of western Uganda (Buhweju, Bulisa, Bundibugyo, Bunyangabu, Bushenyi, Hoima, Ibanda, Isingiro, Kabale, Kabarole, Kagadi, Kakumiro, Kamwenge, Kanungu, Kasese, Kazo, Kibaale, Kikuube, Kiruhura, Kiryandongo, Kisoro, Kitagwenda, Kyegegwa, Kyenjojo, Masindi, Mbarara, Mitooma, Ntoroko, Ntungamo, Rubanda, Rubirizi, Rukiga, Rukungiri, Rwampara and Sheema) as well as neighbouring countries including Tanzania, Rwanda and Democratic Republic of Congo. The population served by MRRH has originated from different parts of Africa as a result of conflict-driven displacement and is likely to have variable genetic make-up. The clinic is run by a gyneoncologist, medical residents, nurses and midwives.

At MRRH, the screening program employs a "screen and treat" approach, in which the main screening tests include Pap smear cytology and VIA (Nakisige et al., 2017). Women who undergo such screening are aged 25 to 65 years for cytology and 25 to 49 for VIA at a screening interval of 3 years (Nakisige et al., 2017). In Uganda, the ministry of health has started rolling out HPV test as a cervical cancer screening test for HIV positive women.

For women who test negative with visual inspection with acetic acid or Pap smear cytology, the WHO recommends that the interval for re-screening should be three to five years. It further specifies that for women who test negative with HPV DNA, re-screening should be done after five years. After subsequent screening with negative test results, and as well for older women, the screening interval can be prolonged to more than five years. Still according to WHO, women who have undergone treatment for cervical pre-cancerous lesions should receive post-treatment follow-up testing and review within 12 months.

The confirmatory tests for cervical intraepithelial lesions, include PAP smears or histology and are performed in the pathology department of the hospital/university. Diagnostic tests for cancer of the cervix include; colposcopy and histology or biopsy. Colposcopy is the examination of the cervix and nearby related organs with a colposcope, an instrument that provides strong and bright light while magnifying the image. Colposcopic examination is done following a positive screening test and it helps to verify presence of lesions, determine the degree and type of lesions, either CIN or cancer (WHO, 2013, WHO, 2014). During colposcopy, biopsies can be collected directly from any area that appears abnormal (WHO, 2013). Also from colposcopy, it can be determined whether to use cryotherapy or LEEP as treatment strategies (WHO, 2006).

3.6 Participant flow chart

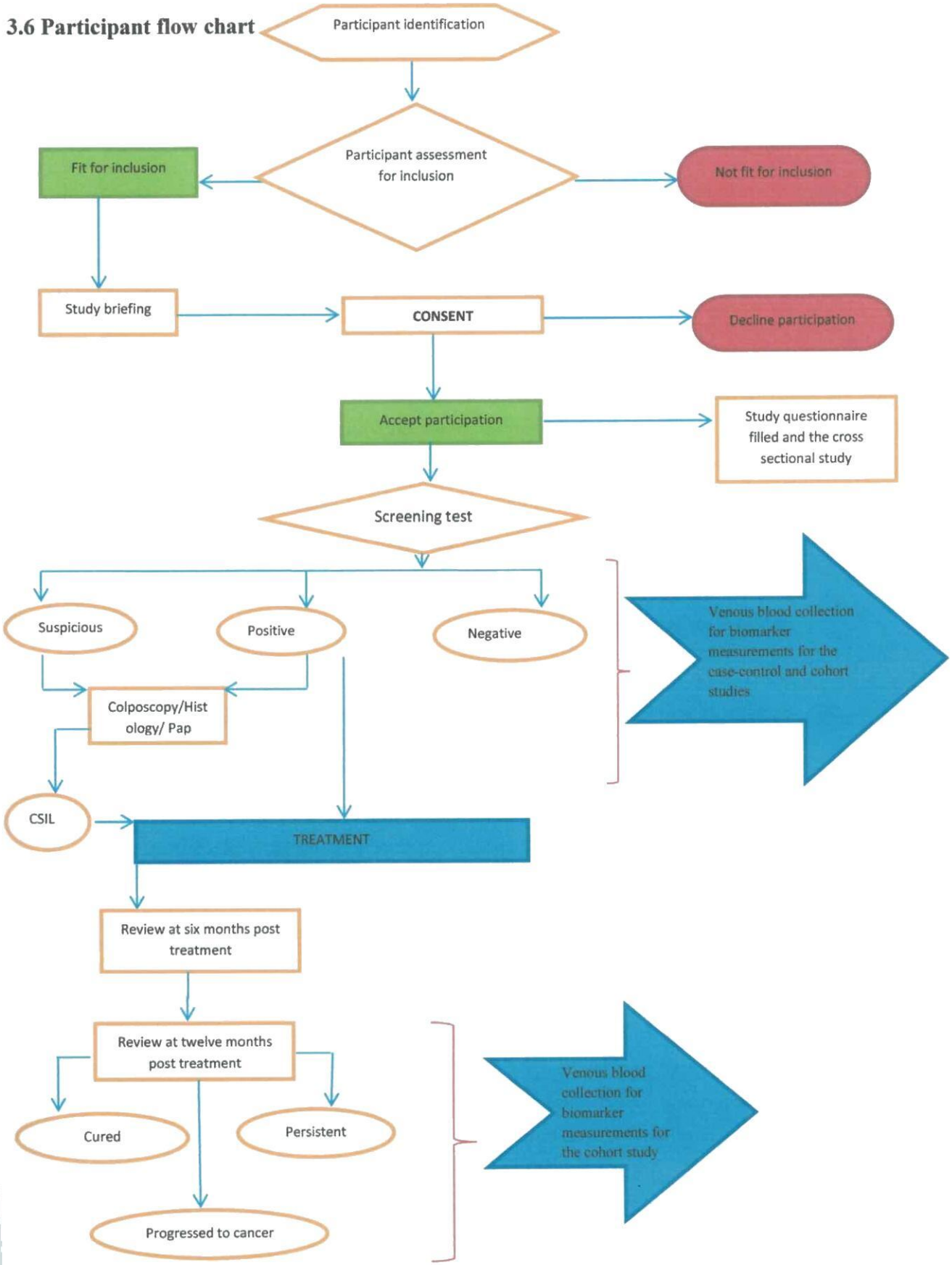


Figure 4: Participant flow chart

3.7 Target population

The study targeted women seeking cervical cancer screening and care, based on the current standard of care.

3.8 Study population

The study included women who attended the cervical cancer clinic at the time of the study for screening, diagnosis or treatment.

3.9 Participant sampling procedure

The study used a consecutive sampling method for the cross sectional study, in which all women at the cervical cancer clinic who consented to participate in the study were enrolled. This sampling was employed until the required sample size was attained. For the case control studies and the subsequent cohort, cases were recruited using purposive sampling. Then we employed the incidence density sampling method to obtain controls i.e. we recruited a control each time a case was identified. This applied for both CIN and CC case groups.

3.10 Inclusion criteria

The study included all women who reported to the cervical cancer clinic for cervical cancer care at MRRH during the time of the study following national guidelines; and consented to participate.

3.11 Exclusion criteria

Any woman that was too moribund to consent was excluded.

We excluded all those women who had already been started on treatment, those with insufficient data in their patient charts, as well as those with other secondary malignancies. We also planned to exclude or stop following up any woman that, at any time, developed any other malignancy.

3.12 Data collection

After routine care at the clinic, potential participants were explained to and taken through the consent process, filling the enrolment form and specimen collection. A suitable enrolment form (Appendix one) was used to collect the quantitative data from respondents, which was supplemented with more information from patient files or charts if needed. Recordable data on the enrolment form included patient's demographics including country, district, age, and other risk factors to cervical intraepithelial lesions. Also we captured the VIA, Pap test and colposcopy results together with histology results, if necessary, stating the exact grade of lesion. For all cytology and histology specimens, examination and grading of lesions followed the Bethesda grading system (2014). Cytological grading of cervical

lesions included LSIL, ASCUS, ASCH and HSIL while histological grading of cervical lesions included CIN1, CIN2, CIN3 and CIS.

Qualified and experienced research assistants collected blood specimens from every participant and these blood specimens were used to determine levels of all the biomarkers independently. Blood specimens were collected from participants at baseline and at 12 months after the initial visit.

Routinely, women with a positive VIA and found to have any precancerous lesion are treated with either cryotherapy, Loop Electrosurgical Excision Procedure (LEEP) or cold coagulation therapy. This is done depending on a number of factors which include mainly size of the lesion, age and HIV status. This is followed up with a first review after one month, second review at six months and third review at one year to determine whether a woman's lesions has been cured, persisted or progressed to a much higher grade. After that they can be subjected to normal screening once found to have recovered. It is at such clinical reviews that they receive their treatment and counselling.

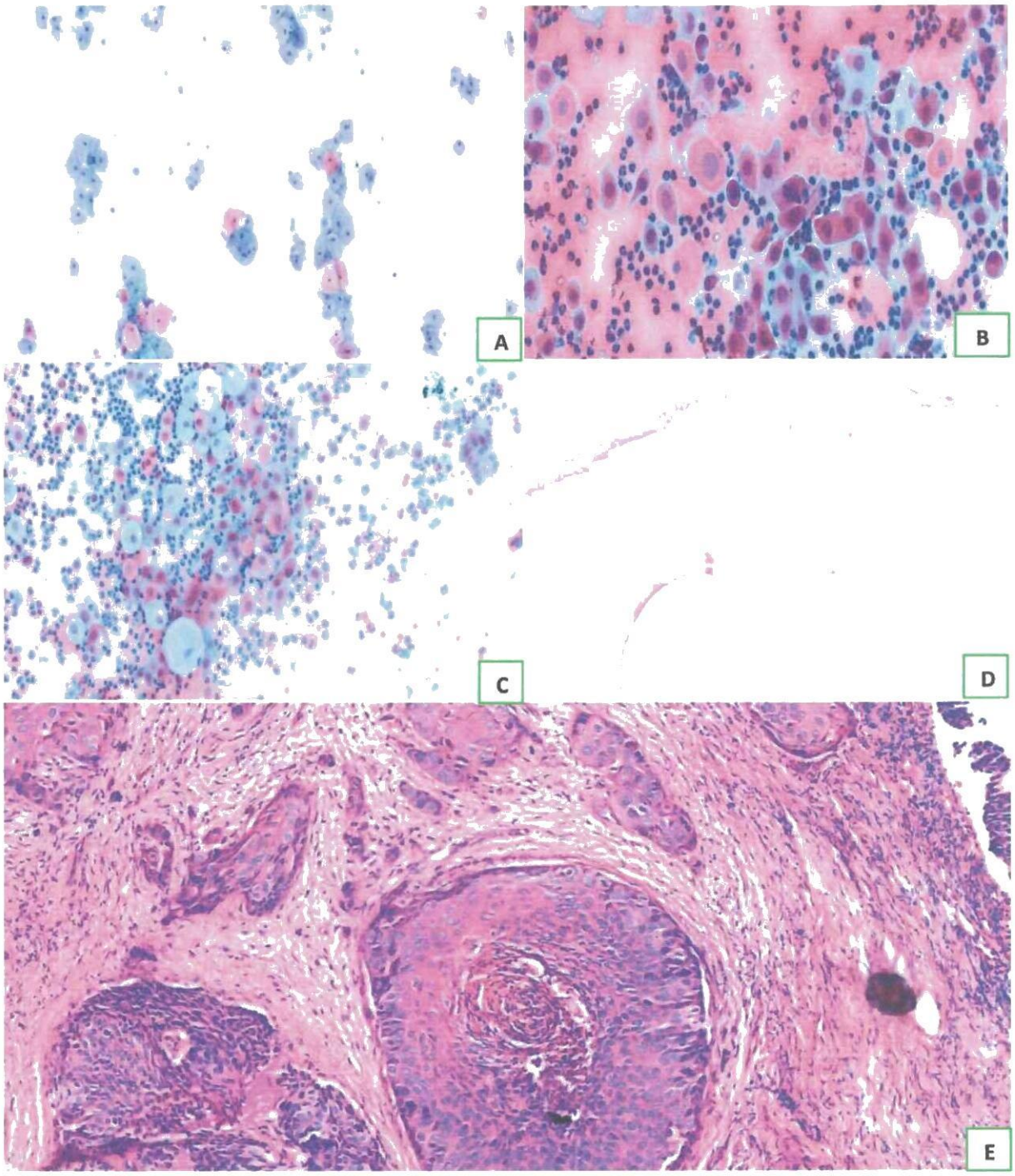


Figure 5: Photomicrographs of cytological and histological specimen preparations.

A-Pap smear negative of intraepithelial lesion or malignancy x400; B- Pap Smear Atypical squamous cells of undetermined significance x400; C- Pap Smear Low grade squamous intraepithelial lesion x400; D- Benign cervical tissue (Haematoxylin and Eosin) x400; E- Squamous cell carcinoma (Haematoxylin and Eosin) x400

3.13 Blood collection

Blood samples were requested by clinicians at the clinic, using the usual laboratory request form as usually used at the clinic. We collected 10ml of blood in two separate vacutainers, 5ml each. Blood specimens were collected by research assistants (clinic nurses) using the venipuncture procedure (appendix two), into serum separator vacutainers, and labeled with unique and corresponding study numbers.

A vacutainer needle was attached to its needle holder. A tourniquet was gently applied to the upper arm to enable the visualisation of vein. The participant was asked to make a fist. The suitable vein was then felt and identified. With an antiseptic, 70% ethanol, the puncture site was cleaned and allowed to dry. Touching the cleansed area was avoided.

With the thumb of the hand that is holding down the skin below the site, the venepuncture was performed with the bevel of the needle facing directly upwards in the line of the vein. The tourniquet was released and the vacutainer was pushed into the needle holder to allow it to fill.

When sufficient blood had been collected, the respondent was instructed to open the fist. The puncture site was covered with a piece of clean and dry and sterile cotton wool and the needle removed. The participant was instructed to continue applying pressure to the puncture site until bleeding ceased. The vacutainer was removed from the needle. The needle was discarded into the sharps container. Blood was mixed gently, labelled and all the used materials were discarded appropriately. The blood samples were collected at baseline and at 12 months follow up.

3.14 Blood specimen handling

Centrifugation, separation and storage of samples was performed by a Medical Laboratory scientist in the MUST Clinical and Research Laboratory. This Medical Laboratory scientist holds a masters degree in medical laboratory science and has handled specimens for many previous studies. For P16ink4A and FOXP3, the collected blood specimens were left to stand upright at room temperature to ensure complete clotting and clot retraction. Then samples were centrifuged (appendix three) at 1500g for 15 minutes at 4°c to separate the serum. Serum was then picked with an automatic pipette, and transferred into a new sterile cryovial tubes, leaving behind the cells and the buffy coat. The cryovial tubes with their contents were capped, and immediately frozen at -80°c until analysis.

3.15 Measurement of serum FOXP3 and P16ink4A concentrations.

This was done using P16ink4A (Human) ELISA Kit (appendix five) and FOXP3 (Human) ELISA Kit (appendix six) respectively, all from Elab sciences, following manufacturer's instructions (Figure 4). These blood samples were stored in MUST Clinical and Research Laboratory at -80^{0}c, and then analysed at the Makerere University College of Veterinary Medicine, Centre for Biosecurity and Global Health.

3.15.1 P16ink4A

All laboratory reagents, samples and standards were prepared accordingly. 100 µl of Sample, Standard, or Blank were added to each well and incubated for 2 hours at 37°C. 100 µl of Detection Reagent A were aspirated and added and incubated for 1 hour at 37°C. The wells were aspirated and washed 3 times. Then 100 µl of Detection Reagent B was added and incubated for 60 minutes at 37°C. Then the wells were aspirated and washed 5 times.

90 µl of TMB substrate solution was added and incubated for 10-20 minutes at 37°C. 50 µl of Stop solution was added. Optical densities were read immediately with a plate reader at 450nm. Concentrations were calculated using CurveExpert computer program.

3.15.2 FOXP3

All laboratory reagents, samples and standards were prepared accordingly. 100 µl of Sample, Standard, or Blank were added to each well and incubated for 90 minutes at 37°C. 100 µl of 1x Biotinylated detection antibody were added to each well and incubated for 1 hour at 37°C. The wells were aspirated and washed 3 times. 100 µl of 1x HRP conjugate were added to each well and incubated for 30 minutes at 37°C. Then the wells were aspirated and washed 5 times.

We added 90 µl of TMB Substrate solution to each well and incubated for 15 minutes at 37°C. 50 µl of Stop solution were added to the wells. Optical densities were read immediately using a plate reader at 450nm. Concentrations were calculated using CurveExpert computer program.

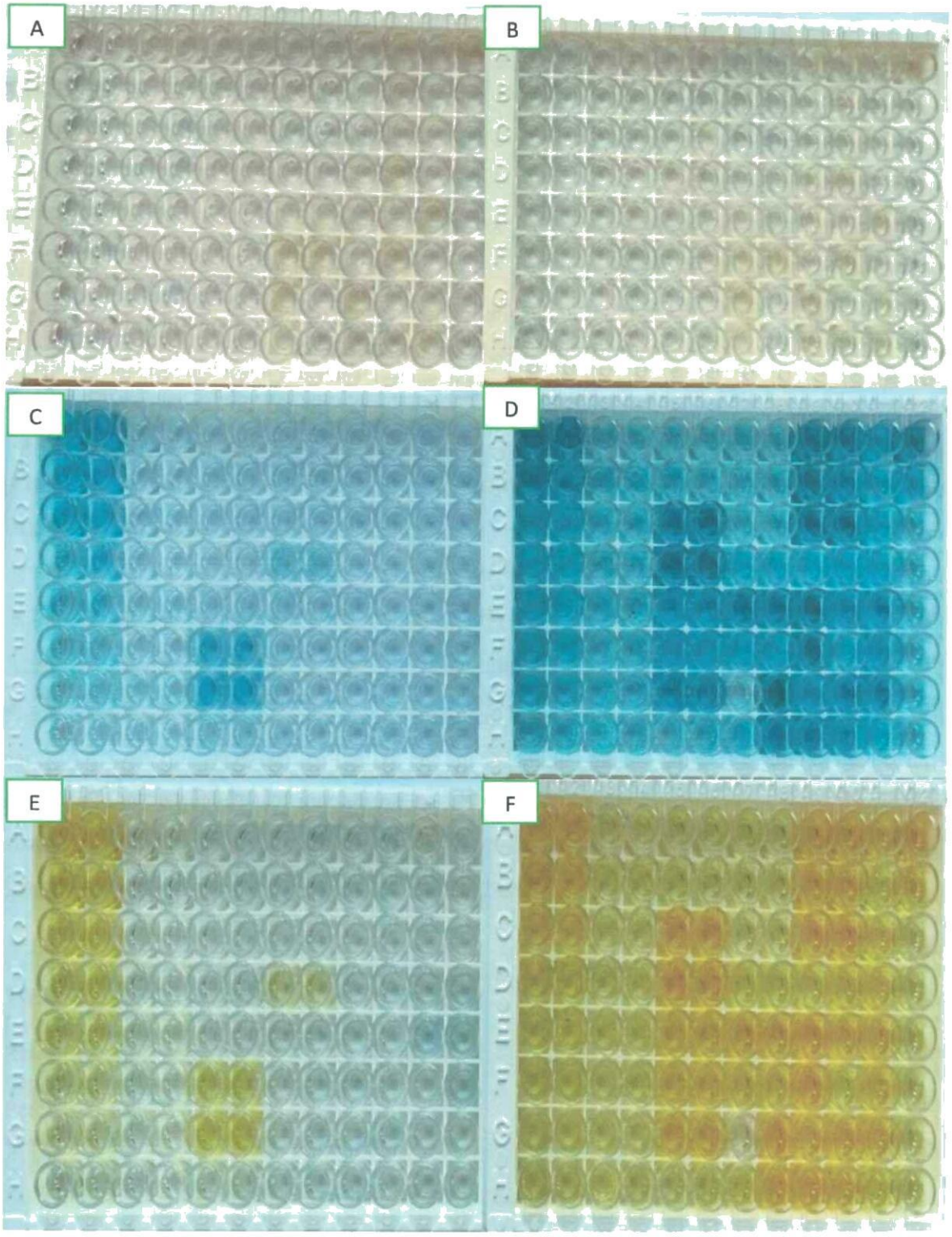


Figure 6: ELISA plate layout. A and B- after addition of sample, standard or blank; C and D-after addition of the substrate, and D and E- after addition of the stop solution.

3.16 Sample size

For objective one, the sample size was estimated using the formula by Kish Leslie 1965.

$$n = \frac{Z^2 \frac{\alpha}{2} p(1-p)}{d^2}$$

Where n is the required sample size,

Z is the Z score corresponding to the 95% confidence interval

d= 0.05, Level of precision which is assumed to be +/-0.05

$Z_{(1-\alpha)}$ =1.96 (Score of normal standard variance curve corresponding to 95% level of confidence) for a two sided test (1.96),

p is the prevalence of cervical intraepithelial neoplasia, 38.6% (Eilu et al., 2020), as a result of Pap smear cytology.

$$n = \frac{(1.96)2 \times 0.386\ (1-0.386)}{0.005}$$

364 participants

For objective two, the case control study included 270 women, of whom 90 were cervical cancer cases, 90 were cervical intraepithelial neoplasia cases and 90 were the unmatched controls (confirmed to be negative of intraepithelial lesion or malignancy) as shown below. This sample size was calculated using an online software, OpenEpi, Version 3, Open source calculator-SSCC. OpenEpi - Sample Size for Unmatched Case-Control Studies

Sample Size:X-Sectional, Cohort, & Randomized Clinical Trials

Two-sided significance level(1-alpha):	95
Power(1-beta, % chance of detecting):	80
Ratio of sample size, Unexposed/Exposed:	1
Percent of Unexposed with Outcome:	5
Percent of Exposed with Outcome:	21
Odds Ratio:	5.1
Risk/Prevalence Ratio:	4.2
Risk/Prevalence difference:	16

	Kelsey	Fleiss	Fleiss with CC
Sample Size - Exposed	70	69	81
Sample Size-Nonexposed	70	69	81
Total sample size:	140	138	162

References

Kelsey et al., Methods in Observational Epidemiology 2nd Edition, Table 12-15
Fleiss, Statistical Methods for Rates and Proportions, formulas 3.18 &3.19
CC = continuity correction
Results are rounded up to the nearest integer.

Fleiss, Statistical Methods for Rates and Proportions, formulas 3.18 &3.19
CC = continuity correction
Results are rounded up to the nearest integer.
Results from OpenEpi, Version 3, open source calculator—SS Cohort
Print from the browser with ctrl-P
or select text to copy and paste to other programs.

For objective three, the sample size for anticipated sensitivity and specificity was determined using the online version of the Burderer's formula (Buderer, 1996). wnarifin.github.io > Sample size calculator

Where:

n = required sample size,

Prevalence of CIN (p) = 6.6% (Ssedyabane et al., 2024a)

S N = anticipated sensitivity of P16 ELISA = 91.8% (Balasubramanian et al., 2009).

S P = anticipated specificity of P16 ELISA = 86.0% (Balasubramanian et al., 2009).

α = size of the critical region = 0.1 z $1-\alpha/2$ =1.96

L = absolute precision desired on either side of sensitivity or specificity = 0.1

This gave a sample size of 439 for sensitivity and 50 for specificity. Therefore, we considered the largest, 436 participants. After additional 10% to cater for dropout, we considered a sensitivity/specificity based minimum sample size of 480 participants. Therefore, we approached 480 participants who included 240 participants with a positive VIA with 240 respective unmatched controls. After confirmation of cases with histology, the final analysis was done with 93 CIN cases, 93 CC cases and 93 unmatched controls as shown in figure 5.

For objectives four, all the 186 cases from the case control study formed the cohort that was followed up for 12 months post treatment.

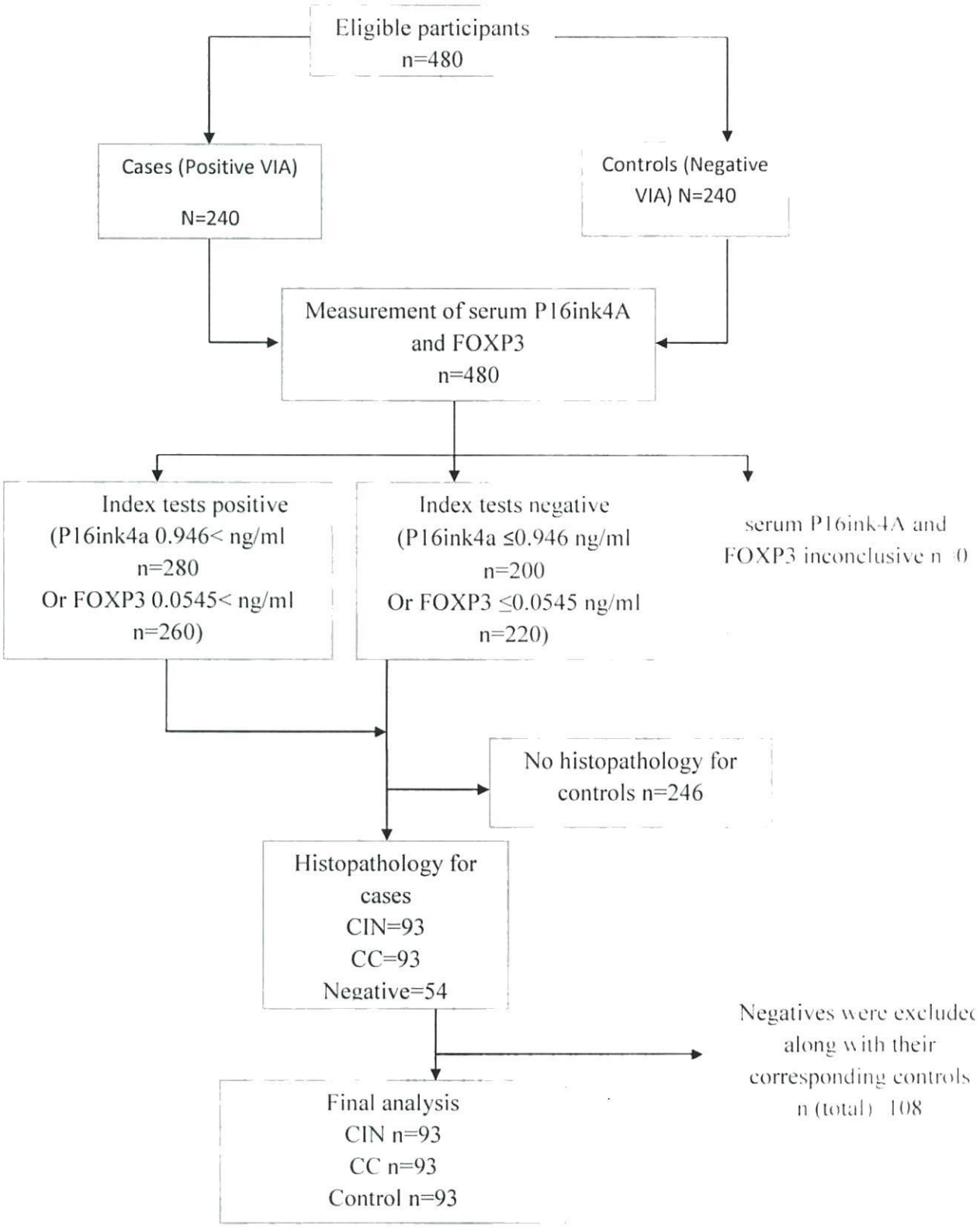


Figure 1: STARD flow diagram of participants through enrolment, eligibility, and outcomes of the study

3.17 **Data management and analysis**

Data was collected by the research assistants and the Principal Investigator. It was then entered into Microsoft Excel (Ms 2007) computer database, imported into and analysed using STATA 15 software. Descriptive statistics were used to characterize the population using frequencies, means ± standard deviations (SDs), median values and interquartile ranges (IQRs).

For objective one, the prevalence of cervical intraepithelial lesions was calculated as a fraction of those women with cervical intraepithelial lesions divided by the total number of study participants. Logistic regression analysis was performed to derive factors associated with cervical lesions. Variables with a p value of <0.2 at bivariate analysis were subjected to multivariate logistic regression to determine associated factors at ≤0.05 significance level. Prevalence and associations were presented as percentages and odds ratios with 95% confidence intervals. This was done after adjusting for age, family planning type and usage, HIV status, marital status, history of blood pressure, history of diabetes, and smoking status.

For objective two, mean serum levels of serum P16ink4A and FOXP3 concentrations were determined and compared across study groups using the students' t test. Bivariate and multivariate logistic regression analysis was employed to determine the associations between cervical lesions and serum P16ink4A and FOXP3 concentrations. After adjusting for age, family planning type and usage, HIV status, marital status, history of blood pressure, history of diabetes, and smoking status, multivariate logistic regression analysis was performed. Odd ratios and 95% confidence intervals are used to display associations using P-values of <0.05 as statistically significant.

For objective three, Receiver Operating Curves (ROC) were built to determine cut-off serum levels with the highest combined sensitivity and specificity for each biomarker for positive VIA and histologically/cytologically confirmed CIN2+ as determined by the area under the curve (AUC), taking p-values of <0.05 as significant.

For objective four, mean serum levels of each biomarker were determined at baseline and at the end of the follow up period, and compared using the students' t test, for all the histological or cytological grades of lesions, taking p-values of <0.05 as significant. We used multinomial logistic regression to determine association between treatment outcomes at 12 months and serum P16ink4A and FOXP3 concentrations. Data was presented as relative risk ratios with 95% confidence intervals and P-values of <0.05 were taken to be statistically significant.

3.18 Ethical consideration

With the help of research assistants, we sought informed consent (appendix Nine) from every participant before taking part in the study. Participants also consented to have their blood samples stored and files or chats reviewed, by research assistants, for more clinical and demographic information. We used study numbers not names on all collected data and blood specimens. We delinked all participant identifiable information during data analysis. A separate record was kept in the clinic, to help capture patient's telephone number that were used during follow up. All participant interaction with the research team took place in a private and comfortable room, free from other disturbances and only accessible to one participant at a time. All filled documents were filed before calling in the next participant.

Ethical review and approval was sought from the Department of Medical Laboratory Science, MUST Faculty of Medicine Research Committee, Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) (**MUST-2022-612**) and Uganda National Council for Science and Technology (UNCST) (**HS2722ES**). Administrative clearance will be sought from the Hospital Director, Mbarara Regional Referral Hospital, before commencement of the study. All women diagnosed with cervical intraepithelial lesions (cases) received the standard package of care at the cervical cancer clinic. Participants received their VIA results on spot. Pap smear results were ready in not more than a week's time after sample collection. Results on biomarkers were ready after one month from the time of sample collection. All results were delivered to participants by the nurse at the clinic. In cases where results returned with abnormal levels of biomarkers, participants were linked to relevant departments for appropriate management. Participants also received transport refund (Ushs 10,000/=) when they returned for follow up.

3.19 Quality assurance and control

A pre-study training was conducted for all the research assistants, midwives, cytopathologists, cytotechnologists and laboratory technologists before the study commenced. Participants were counselled, by a trained clinic nurse counsellor, to ensure psychological and physical preparedness prior to sample collection.

Furthermore, all wastes were disposed of appropriately as per the set infection control guidelines. Every collected data was cross checked by a second data clerk before entering it into the computer data base. While entering it into the data base, double entry method was used to correct any errors. SOPs were used in all laboratory testing procedures. All reagents, samples and consumables were stored as recommended by the manufacturer.

During measurement of FOXP3 and P16ink4A Mouse Anti-GAPDH Antibody was used to detect the internal positive controls for normalization of OD values of the target protein. The negative controls were HR Conjugated Anti-Rabbit IgG Antibody and HRP Conjugated Anti-Mouse IgG Antibody, and were used independently in different wells. Both positive and negative controls were run in the same plate with the rest of the samples.

3.20 Dissemination of results

All study participants received their results as well as a preliminary report of the study results. A written report was prepared and shared with MRRH administration. We also organised presentations for all MRRH staff to share our research findings. Research findings were summarised in a patient friendly the language, translated into the local language, provided to the cervical cancer clinic team and also pinned on hospital notice boards. Manuscripts were prepared and then published in peer reviewed journals. Presentations were made for conferences both local and international.

3.21 Covid-19 and Ebola Virus Disease risk management plan

All study participants, the entire research team as well as staff of the cervical cancer clinic adhered to the covid-19 and Ebola risk management plan (Appendix Thirteen).

CHAPTER FOUR: RESULTS

4.10 Prevalence and factors associated with cervical lesions

4.11 Demographic characteristics

In the cross-sectional study, we had a 100% response rate and attracted 364 people who met our inclusion criteria. Because their Pap smears were deemed unsatisfactory, thirteen (13) subjects were disqualified from the analysis due to missing data. 351 participants were ultimately included into the analysis. Participants in the study had a mean age of 41.9 (+/-12.2) years. Most of our participants were married (65.2%, 229/351), did not smoke (98.3%, 345/351), and lived in regions other than Mbarara city (63.8%, 224/351). The study participants who were positive for HIV constituted over half (58.1%, 204/351), with mean CD4 counts and viral loads of 493.392 cells per µl and 4719.833 copies per µl, respectively. Table 1

Table 1: Socio-Demographic Characteristics of Study Participants in the cross sectional study

Variables	Category	n/351	Percentage
Age		41.9 (12.2)	
Age group	18-29	59	16.8
	30-39	99	28.2
	40-49	92	26.2
	50 and above	101	28.8
Region of residence	Central Uganda	2	0.6
	Other parts of Western Uganda	224	63.8
	Mbarara city	125	35.6
History of High BP	No	312	88.9
	Yes	39	11.1
Diabetes History	No	333	94.9
	Yes	18	5.1
Marital status	Divorced	47	13.4
	Married	229	65.2
	Single	70	19.9
	Widowed	5	1.4
Highest educational level	Never studied	45	12.8
	Pre-primary	30	8.5
	Primary school	138	39.3
	Secondary school	87	24.8
	Tertiary institution	35	10
	University	16	4.6

HIV status	Unknown	1	0.3
	Negative	146	41.6
	Positive	204	58.1
HIV Viral load Category	N/A	146	41.6
	Not suppressed	18	5.1
	Suppressed	187	53.3
HIV Viral load copies per ul		4719.8 (16606.9)	
CD4 count		493.4 (234.5)	
Smoking	No	345	98.3
	Yes	6	1.7

Continuous variables, Age, HIV viral load and CD4 count are presented as mean (standard deviation)

4.12 Reproductive health characteristics

We also assessed our study participants for other reproductive characteristics **(Table 2)**. The most common gynecological complaint was vaginal discharge, with a prevalence of 12.8% (45/351). More than a third (37.6%, 132/351) were active contraceptive users, specifically hormonal contraceptives (87.1%, 115/132). A big proportion (80.3%, 282/351) of our participants reported to have had their sexual debut at an age between 12 and 20 years. Almost 88% (307/351) had an unknown HPV status, while 69.1% (242/351) reported to have had multiple sexual partners.

Table 2: Reproductive Characteristics of Study Participants.

Variables	Category	n/351	Percentage
Other gynecological conditions	None	264	75.21
	Cervicitis	35	9.97
	Vaginal discharge	45	12.82
	Warts	1	0.28
	Candidiasis	2	0.57
	Trichomoniasis	2	0.57
	Syphilis	2	0.57
Contraceptive use	No	219	62.4
	Yes	132	37.6
Contraceptive Type (N=132)	BTL	2	1.5
	Condom	2	01.5
	Hormonal	115	87.1
	IUD	13	9.9

Cervical cancer family history	No	331	94.6
	Yes	19	5.4
Age at sexual debut	31 and above	2	0.57
	21 to 30	67	19.09
	12 to 20	282	80.34
Lifetime sexual partners (number)	0 to 1	108	30.86
	2 and above	242	69.14
Full term births (Number)	0	12	3.42
	1 to 3	163	46.44
	4 and above	176	50.14
VIA test results	Negative	312	88.9
	Positive	37	10.5
	Suspicious for cancer	2	0.6
HPV status	Negative	14	4
	Positive	30	8.5
	Unknown	307	87.5

All data was categorical and the total number of participants was 351 except for Type of contraceptive (N=132)

4.13 Prevalence of cervical lesions

Out of 351 study participants, we observed a total of 23 with positive Pap smear results. This results in a 6.6% prevalence Figure 6.

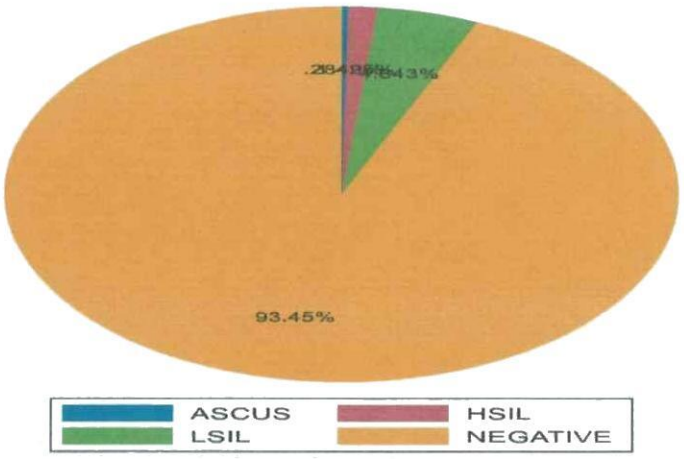


Figure 8: A pie chart showing prevalence of cervical lesions. LSIL-Low grade Squamous Intraepithelial Lesion; HSIL-High grade Squamous Intraepithelial Lesion; ASCUS-Atypical Squamous Cells of Undetermined Significance

More than half (73.9%, 17/23) of all positive cases were found to be low-grade squamous intraepithelial lesions (LSILs). All participants (23/23) with positive Pap smears reported no history of high blood pressure. A good number of participants (19/23) with cervical lesions were married; 100% for ACSUS, 80% for HSIL, and 82.4% for LSIL. More than half (52.9%, 9/17) of study participants with LSIL had attained no more than primary school education. The same proportion of participants (52.9%, 9/17) were HIV positive and had suppressed viral load (Table 3).

Table 3: Prevalence of cervical lesions across selected socio-demographic characteristics

Variable		ASCUS N=1	HSIL N=5	LSIL N=17	NILM N=328	Test	p-val
Age group	18-29	0(0.0%)	1(20.0%)	3(17.6%)	55(16.8%)	Fisher's exact	0.79
	30-39	0(0.0%)	2(40.0%)	6(35.3%)	91(27.7%)		
	40-49	1(100.0%)	0(0.0%)	5(29.4%)	86(26.2%)		
	50 and above	0(0.0%)	2(40.0%)	3(17.6%)	96(29.3%)		
Region of residence	Central Uganda	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)	Fisher's exact	0.71
	Other parts of Western Uganda	1(100.0%)	4(80.0%)	9(52.9%)	210(64.0%)		
	Mbarara city	0(0.0%)	1(20.0%)	8(47.1%)	116(35.4%)		
History of High BP	No	1(100.0%)	5(100.0%)	17(100.0%)	289(88.1%)	Fisher's exact	0.44
	Yes	0(0.0%)	0(0.0%)	0 0.0%)	39(11.9%)		
History of Diabetes	No	1(100.0%)	5(100.0%)	16(94.1%)	311(94.8%)	Fisher's exact	0.71
	Yes	0(0.0%)	0(0.0%)	1(5.9%)	17(5.2%)		
Marital status	Divorced	0(0.0%)	1(20.0%)	2(11.8%)	44(13.4%)	Fisher's exact	0.70
	Married	1(100.0%)	4(80.0%)	14(82.4%)	210(64.0%)		
	Single	0(0.0%)	0(0.0%)	1(5.9%)	69(21.0%)		
	Widowed	0(0.0%)	0(0.0%)	0(0.0%)	5(1.5%)		
Highest educational level	Never studied	0(0.0%)	2(40.0%)	2(11.8%)	41(12.5%)	Fisher's exact	0.97
	Pre-primary	0(0.0%)	0(0.0%)	1(5.9%)	29(8.8%)		
	Primary school	1(100.0%)	2(40.0%)	9(52.9%)	126(38.4%)		
	Secondary school	0(0.0%)	1(20.0%)	4(23.5%)	82(25.0%)		
	Tertiary institution	0(0%)	0(0.0%)	1(5.9%)	34(10.4%)		
	University	0(0.0%)	0(0.0%)	0(0.0%)	16(4.9%)		

HIV status	Unknown	0(0.0%)	0(0.0%)	0(0.0%)	1(0.3%)	Fisher's exact	0.70
	Negative	0(0.0%)	1(20.0%)	8(47.1%)	137(41.8%)		
	Positive	1(100.0%)	4(80.0%)	9(52.9%)	190(57.9%)		
Smoking	No	1(100.0%)	5(100.0%)	17(100.0%)	322(98.2%)	Fisher's exact	1.00
	Yes	0(0.0%)	0(0.0%)	0(0.0%)	6(1.8%)		
HIV Viral load Category	N/A	0(0.0%)	1(20.0%)	8(47.1%)	137(41.8%)	Fisher's exact	0.52
	Not suppressed	0(0.0%)	1(20.0%)	0(0.0%)	17(5.2%)		
	Suppressed	1(100.0%)	3(60.0%)	9(52.9%)	174(53.0%)		
CD4 count	200≤	0(0.0%)	2(11.8%)	0(0.0%)	49(14.9%)	Fisher's exact	0.54
	<200	0(0.0%)	1(5.9%)	0(0.0%)	4(1.2%)		
	Not Indicated	5(100.0%)	14(82.4%)	1(100.0%)	275(83.8%)		

Data for categorical variables were presented as frequency (proportion)

82.4% (14/17) of all participants with LSIL reported making their sexual debut at age 31 or older. Nevertheless, almost half of those with LSIL (58.8%, 10/17) were currently using contraceptives, particularly hormonal ones (52.9%, 9/17). Furthermore, more than half (70.69%, 12/17) of participants with LSIL reported having two or more lifetime sexual partners, compared to 60% (3/5) of those with HSIL. There was significant percentage of participants (80%, 4/5) with HSIL reported having four or more full-term births Table 4.

Table 4: Prevalence of Cervical Lesions Across Reproductive Characteristics

Variable		**ASCUS N=1**	**HSIL N=5**	**LSIL N=17**	**NILM N=328**	**Test**	**p-val**
Age at sexual debut	31 and above	1(100.0%)	5(100.0%)	14(82.4%)	262(79.9%)	Fisher's exact	0.8
	21 to 30	0(0.0%)	0(0.0%)	3(17.6%)	64(19.5%)		
	12 to 20	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)		
Contraceptive use	No	0(0.0%)	3(60.0%)	7(41.2%)	209(63.7%)	Fisher's exact	0.1
	Yes	1(100.0%)	2(40.0%)	10(58.8%)	119(36.3%)		

Type of contraceptive	BTL	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)	Fisher's exact	0.18
	Condom	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)		
	Hormonal	1(100.0%)	1(20.0%)	9(52.9%)	105(32.0%)		
	IUD	0(0.0%)	1(20.0%)	1(5.9%)	11(3.4%)		
	None	0(0.0%)	3(60.0%)	7(41.2%)	208(63.4%)		
Family History of cervical cancer	No	1(100.0%)	4(100.0%)	14(82.4%)	312(95.1%)	Fisher's exact	0.1
	Yes	0(0.0%)	0(0.0%)	3(17.6%)	16(4.9%)		
Number of lifetime sexual partners	1	1(100.0%)	2(40.0%)	5(29.4%)	100(30.6%)	Fisher's exact	0.5
	2 and above	0(0.0%)	3(60.0%)	12(70.6%)	227(69.4%)		
Number of full term births	0	0(0.0%)	0(0.0%)	0(0.0%)	12(3.7%)	Fisher's exact	0.6
	1 to 3	0(0.0%)	1(20.0%)	10(58.8%)	152(46.3%)		
	4 and above	1(100.0%)	4(80.0%)	7(41.2%)	164(50.0%)		
Other gynaecological conditions	None	1(100.0%)	4(80.0%)	14(82.4%)	245(74.7%)	Fisher's exact	0.6
	Cervicitis	0(0.0%)	1(20.0%)	3(17.6%)	31(9.5%)		
	Vaginal discharge	0(0.0%)	0(0.0%)	0(0.0%)	45(13.7%)		
	Warts	0(0.0%)	0(0.0%)	0(0.0%)	1(0.3%)		
	Candidiasis	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)		
	Trichomoniasis	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)		
	Syphilis	0(0.0%)	0(0.0%)	0(0.0%)	2(0.6%)		

Data for categorical variables were presented as frequency (proportion)

4.14 Factors associated with cervical lesions

When the bivariate analysis of several factors showed p values of less than 0.2, those variables were taken into consideration for multivariate analysis. The number of full-term births, lifetime sexual partners, age at first sexual experience, HIV status, type of contraception used, highest level of education attained, and family history of cervical cancer were some of these variables. The results of the multivariate analysis revealed that the likelihood of cervical lesions was three times higher in those who reported using hormonal contraceptives than in those who had not used any form of birth control, even after adjusting for the number of full-term births, type of contraceptive used, HIV status, age at sexual debut, CD4 counts, HIV viral load, highest educational level, and number of lifetime sexual partners. A statistically significant difference was observed (OR 3.032, p-value 0.025, 95% CI 1.150-7.989).

Additionally, compared to individuals who had not used any form of contraception, those who reported using IUDs as a method of birth control were six times more likely to acquire cervical lesions. This difference in risk was statistically significant (OR 6.284, p-value 0.039, 95% CI 1.094-36.095). Comparing participants with and without a family history of cervical cancer, those with a history of the disease were also four times more likely to have cervical lesions. Table 5 indicates that this observation was statistically significant (OR 4.144, P value 0.049, 95% CI 1.004-17.101). Table 5

Table 5: Logistic regression analysis showing association between cervical lesions and selected variables of study participants

Variables	Category	COR	P-value	95% CI	AOR	P-value	95% CI
Number of full term births	0	1			1		
	1 to 3	0.989	0.98	0.424 - 2.308	1.337	0.564	0.4982 - 3.5
	4 and above	1			1		
Number of life-time sexual partners	0 to 1	1			1		
	2 and above	0.826	0.674	0.339 - 2.011	0.849	0.748	0.312 - 2.31
Age at sexual debut	31 and above	1			1		
	21 to 30	0.614	0.442	0.177 - 2.130	0.980	0.976	0.257 - 3.73
	12 to 20	1			1		
HIV status	Negative	1			1		
	Positive	1.122	0.795	0.472 - 2.666	0.887	0.804	0.344 - 2.28
	Unknown	1			1		
Type of contraceptive	None	1			1		
	Hormonal	2.179	0.086	0.897 - 5.295	3.032	**0.025***	1.150 - 7.98
	IUD	3.782	0.111	0.737 - 19.395	6.284	**0.039***	1.094 - 36.0
	BTL	1			1		
	Condom	1			1		
Family history of cervical cancer	No	1			1		
	Yes	3.079	0.094	0.823 - 11.494	4.144	**0.049***	1.004 - 17.1
CD4 count	200≤	1			1		
	<200	6.125	0.173	0.451 – 83.116	5.053	0.234	0.351 – 72.6

Highest educational level	None	1			1		
	Pre-primary	0.353	0.363	0.038 - 3.328	0.272	0.269	0.027 - 2.73
	Primary school	0.976	0.968	0.299 - 3.193	0.811	0.743	0.231 - 2.84
	Secondary school	0.625	0.5	0.159 - 2.453	0.493	0.36	0.108 - 2.24
	Tertiary institution	0.302	0.294	0.032 - 2.826	0.191	0.172	0.018 - 2.05
	University	1			1		

COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; 95%CI: 95 percent confidence interval. Data were adjusted for Number of full term births, Type of contraceptive used, HIV status, Age at sexual debut, CD4 counts, HIV viral load, Educational Level, and Number of life-time sexual partners.

*-statistically significant ($p \leq 0.05$).

4.20 Association between serum P16ink4A and FOXP3 concentrations and cervical lesions

4.21 Demographic characteristics of participants in case-control study one

A total of 270 participants were included in the first case control study, out of whom 90 were positive for cervical cancer (CC), 90 positive for cervical intraepithelial neoplasia (CIN) as well as 90 participants who were negative for any cervical lesion (controls) **Table 6.** The mean age of participants among CC group was significantly ($p<0.001$) higher (51.1+/-13.1) than that in CIN group (34.9+/-7.8) as well as that of the control group (38.6+/-8.7). Majority of participants, 23%(21/90) in the CC group, 32%(29/90) of CIN group and 43%(39/90) of control group, aged between 40-49 years. This difference in distribution was also statistically significant ($p<0.001$). More than half of study participants, 57%(51/90) in the CC group, 60%(54/90) in the CIN group and 56%(50/90) in the control group, were married ($p<0.001$). The highest level of education among study participants was generally primary level and below, with 53%(48/90) in the CC group having attained a maximum of preprimary, 51%(45/90) in the CIN group and 44%(40/90) in the control group reported to have attained a maximum of primary school education. HIV positivity was reported in 76%(68/90) in the CC group, 52%(47/90) in the CIN group and 42%(38/90) in the control group and this difference in distribution was statistically significant ($p<0.001$). The most presenting complaint among study participants was cervicitis with a proportion of 58%(52/90) in the CC group, 71%(64/90) in the CIN group and 66%(59/90) in the control group and this difference in distribution was also statistically significant ($p<0.001$).

Table 6: Demographic characteristics of participants in case-control study one

Variable	Category	CC N=90 f(%)	CIN N=90 f(%)	Controls N=90 f(%)	Test	p-value
Age		51.1 (13.1)	34.9 (7.8)	38.6 (8.7)	ANOVA	<0.001
Age Group	19-29	2 (2%)	23 (26%)	18 (20%)	Fisher's exact	<0.001
	30-39	18 (20%)	37 (41%)	26 (29%)		
	40-49	21 (23%)	29 (32%)	39 (43%)		
	50-59	20 (22%)	1 (1%)	7 (8%)		
	60-max	29 (32%)	0 (0%)	0 (0%)		
History of high BP	No	71 (79%)	68 (76%)	71 (79%)	Chi-square	0.78
	Yes	19 (21%)	22 (24%)	19 (21%)		
History of Diabetes	No	70 (78%)	78 (87%)	75 (83%)	Chi-square	0.22
	Yes	20 (22%)	12 (13%)	15 (17%)		
Marital status	Divorced	4 (4%)	20 (22%)	19 (21%)	Fisher's exact	<0.001
	Married	51 (57%)	54 (60%)	50 (56%)		
	Single	28 (31%)	16 (18%)	20 (22%)		
	Widowed	7 (8%)	0 (0%)	0 (0%)		
Highest level of education					Fisher's exact	<0.001
	Never studied	32 (36%)	5 (6%)	11 (12%)		
	Pre-primary	48 (53%)	3 (3%)	6 (7%)		
	Primary school	10 (11%)	45 (51%)	40 (44%)		
	Secondary school	0 (0%)	23 (26%)	29 (32%)		
	Tertiary institution	0 (0%)	6 (7%)	2 (2%)		
	University	0 (0%)	7 (8%)	2 (2%)		
HIV status	Negative	22 (24%)	42 (47%)	52 (58%)	Fisher's exact	<0.001
	Positive	68 (76%)	47 (52%)	38 (42%)		
	Unknown	0 (0%)	1 (1%)	0 (0%)		
Smoking	No	90 (100%)	84 (94%)	89 (99%)	Fisher's exact	0.029
	Yes	0 (0%)	5 (6%)	1 (1%)		
Contraceptive use	No	57 (63%)	38 (44%)	56 (64%)	Chi-square	0.02

	Yes	33 (37%)	49 (56%)	32 (36%)		
Type of contraceptive	IUD	17 (45%)	9 (18%)	4 (13%)	Fisher's exact	0.002
	Hormonal	21 (55%)	39 (76%)	26 (81%)		
	BTL	0 (0%)	3 (6%)	2 (6%)		

CC: Cervical Cancer; CIN: Cervical Intraepithelial Neoplasia; IUD: Intra Uterine Device; BTL: Bilateral Tubal Ligation

4.22 Distribution of serum P16ink4A concentrations between study groups

The mean serum P16ink4A concentration among CIN cases was 1.11(+/-0.66) ng/ml and 1.13(+/-0.61) ng/ml among controls, and 1.45(+/-1.11) ng/ml among CC cases and this difference in means was statistically significant (p=0.008). Half of CIN cases (50%, 45/90) and controls (50%, 45/90) had P16ink4A concentration above 0.946ng/ml. Majority of CC cases (60%, 54/90) and 50% of controls (45/90) had P16ink4A concentration above 0.946ng/ml. This difference was also statistically significant as shown in table 7.

Table 7: Distribution of serum P16ink4A concentrations between cervical intraepithelial neoplasia cases, cervical cancer cases and controls

	Category	**Cancer N=90 f(%)**	**CIN N=90 f(%)**	**Control N=90 f(%)**	**Test**	**p-valu**
Baseline p16 (ng/ml)		1.45(1.11)	1.11(0.66)	1.13(0.61)	ANOVA	0.008
Baseline p16 categories (ng/ml)	≤0.946	36 (40%)	45 (50%)	51 (57%)	Chi-square	0.079
	0.946<	54 (60%)	45 (50%)	39 (43%)		

Baseline p16 (ng/ml) was presented as a continuous variable with mean (standard deviation)

4.23 Association between serum P16ink4A concentrations and cervical lesions.

From univariate analysis, there was increased odds of CIN (COR: 1.07, p-value: 0.78) and reduced odds of CC for serum P16ink4A (COR: 0.66, p-value: 0.03). After adjusting for smoking status, HIV status, History pf high blood pressure, History of diabetes, age, presenting complaint, contraceptive use, type of contraceptive and marital status, multivariate logistic regression analysis showed increased odds of CIN for serum P16ink4A though not statistically significant (AOR: 1.11, p-value: 0.70). There was also a statistically significant reduction in odds of CC for serum P16ink4A (AOR: 0.55, p-value: 0.01) as shown in table 8.

Table 8: Logistic regression analysis for association between serum P16ink4A concentrations and cervical lesions among study participants.

	Bivariate analysis			Multivariate analysis		
	COR	P Value	95% CI	AOR	P Value	95% CI
CIN	1.07	0.78	0.67-1.71	1.11	0.70	0.66-1.87
CC	0.66	0.03	0.46-0.97	0.55	0.01	0.34-0.88

COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence Interval; CIN: Cervical Intraepithelial Neoplasia; CC: Cervical Cancer.

All values were got after adjusting for smoking status, HIV status, History pf high blood pressure, History of diabetes, age, presenting complaint, contraceptive use, type of contraceptive and marital status.

4.24 Distribution of serum FOXP3 concentrations across study groups

The mean concentration of FOXP3 higher among CC cases compared with CIN cases and controls, and this difference was statistically significant (P value <0.001). More than half (52/90,58%) of CC cases had FOXP3 concentrations greater than 0.0545 ng/ml, and this proportion differed significantly from CIN cases and controls (P value <0.001) as shown in Table 9.

Table 9: Distribution of serum FOXP3 concentrations between cervical intraepithelial neoplasia cases, cervical cancer cases and controls

Variable	Categories	CONTROL N=90	CASES		Test	p value
			CIN N=90	CC N=90		
FOXP3 Concentration (ng/ml)		0.058(0.038)	0.055(0.019)	0.132(0.145)	ANOVA	<0.00
FOXP3 Categories	≤0.0545 ng/ml	62 (69%)	61 (68%)	38 (42%)	Chi-square	<0.00
	0.0545< ng/ml	28 (31%)	29 (32%)	52 (58%)		

FOXP3 concentration (ng/ml) is presented as a continuous variable with mean (standard deviation)

4.25 Association between serum P16ink4A concentrations and cervical lesions

After controlling for other factors which included smoking, HIV status, History of BP and Diabetes, age, presenting complaint, usage of contraceptives, type of contraceptive used and marital status, we observed that serum FOXP3 concentrations were significantly associated with cervical cancer, with a P value < 0.001. Increase in serum FOXP3 concentrations increases the chances of CC by 2 times (OR: 2.094, P value 0.038, 95% CI: 1.042 - 4.209). However, we also

observed increased odds of CIN for raised serum FOXP3 concentration (OR: 1.131, P value 0.719, 95% CI: 0.579 - 2.205), though not statistically significant as shown in Table 10.

Table 10: Logistic regression analysis for association between serum FOXP3 concentrations and cervical lesions among study participants

	Bivariate analysis			Multivariate analysis		
	COR	P VALUE	95% CI	AOR	P VALUE	95% CI
CIN	1.053	0.873	0.562 - 1.973	1.131	0.719	0.579 - 2.205
CC	3.031	<0.001	1.644 - 5.586	2.094	0.038	1.042 - 4.209

CI: Confidence interval, COR: Crude Odds Ratio, AOR: Adjusted Odds Ratio, CIN: Cervical Intraepithelial Neoplasia, CC: Cervical Cancer.

All values were got after controlling for factors including smoking, HIV, History of high BP and diabetes, age, presenting complaint, usage of contraceptives, contraceptive type as well as marital status.

4.30 Diagnostic accuracy of serum P16ink4A and FOXP3 for detection of cervical lesions

4.31 Demographic characteristics of participants in case-control two

We further recruited 279 participants in the second case control study, out of whom 93 were positive for cervical cancer (CC), 93 positive for cervical intraepithelial neoplasia (CIN) and 93 negative for any cervical lesion (controls). The mean age of CC cases was significantly ($p<0.001$) higher (51.1+/-13.1) than that in CIN cases (35.1+/-7.8) and 38.6+/-8.7 in controls. Majority of participants, 23% (21/93) of CC cases, 33% (31/93) of CIN cases and 43% (40/93) of controls, belonged to the 40-49 age bracket. This difference in distribution was also statistically significant ($p<0.001$). More than half of study participants, 57% (53/93) of CC cases, 61% (57/93) of CIN cases and 55% (51/93) controls, were married ($p<0.001$). The highest level of education among study participants was generally primary level and below, with 55% (51/93) of CC cases having attained a maximum of preprimary, 52% (48/93) CIN cases and 45% (42/93) controls reporting to have attained a maximum of primary school education. Positivity with HIV was report in 76% (71/93) CC cases, 54% (50/93) of CIN cases and 44% (41/93) of controls and this difference in distribution was statistically significant ($p<0.001$).

The most common presenting complaint among study participants was cervicitis with a proportion of 59% (55/93) among CC cases, 72% (67/93) among CIN cases and 67% (62/90) among controls and this difference in distribution was also statistically significant (p<0.001) as shown in table 11.

Table 11: Demographic characteristics of participants in case-control two

Variable	Category	CONTROL	CASES		Test	p-value
		N=93	CIN N=93	CC N=93		
Age		38.6(8.7)	35.1(7.8)	51.1(13.1)	ANOVA	<0.001
Age group	21-29	19 (20%)	23 (25%)	2 (2%)	Fisher's exact	<0.001
	30-39	27 (29%)	38 (41%)	20 (22%)		
	40-49	40 (43%)	31 (33%)	21 (23%)		
	50-59	7 (8%)	1 (1%)	20 (22%)		
	60-max	0 (0%)	0 (0%)	30 (32%)		
Region	Central	2 (2%)	2 (2%)	6 (6%)	Fisher's exact	<0.001
	Other districts	45 (48%)	53 (57%)	71(76%)		
	Mbarara	46 (49%)	38 (41%)	16 (17%)		
History of high BP	No	73 (78%)	70 (75%)	73 (78%)	Chi-square	0.82
	Yes	20 (22%)	23 (25%)	20 (22%)		
History of Diabetes	No	77 (83%)	81 (87%)	72 (77%)	Chi-square	0.28
	Yes	16 (17%)	12 (13%)	21 (23%)		
Marital status	Divorced	21 (23%)	20 (22%)	4 (4%)	Fisher's exact	<0.001
	Married	51 (55%)	57 (61%)	53 (57%)		
	Single	20 (22%)	16 (17%)	28 (30%)		
	Widowed	0 (0%)	0 (0%)	8 (9%)		
Highest level of education	Never studied	11 (12%)	5 (5%)	32 (34%)	Fisher's exact	<0.001
	Pre-primary	6 (6%)	3 (3%)	51 (55%)		
	Primary school	42 (45%)	48 (52%)	10 (11%)		
	Secondary school	30 (32%)	23 (25%)	0 (0%)		
	Tertiary institution	2 (2%)	6 (7%)	0 (0%)		
	University	2 (2%)	7 (8%)	0 (0%)		
HIV status	Negative	52 (56%)	42 (45%)	22 (24%)	Fisher's exact	<0.001
	Positive	41 (44%)	50 (54%)	71 (76%)		

	Unknown	0 (0%)	1 (1%)	0 (0%)		
Smoking	No	92 (99%)	88 (95%)	93 (100%)	Fisher's exact	0.027
	Yes	1 (1%)	5 (6%)	0 (0%)		
Presenting complaint	Vaginal discharge	12 (12%)	7 (7%)	14 (15%)	Fisher's exact	<0.001
	Back pain	2 (2%)	3 (3%)	0 (0%)		
	Cervicitis	62 (67%)	67 (72%)	55 (59%)		
	Candidiasis	5 (6%)	2 (2%)	0 (0%)		
	Painful micturition	2 (2%)	0 (0%)	10 (11%)		
	Valvular warts	6 (7%)	8 (9%)	14 (15%)		
	Syphilis	2 (2%)	3 (3%)	0 (0%)		
	Trichomoniasis	2 (2%)	0 (0%)	0 (0%)		
	Others	0 (0%)	3 (3%)	0 (0%)		
Contraceptive use	No	59 (65%	39 (43%)	59 (63%)	Chi-square	0.009
	Yes	32 (35%)	51 (57%)	34 (37%)		
Type of contraceptive	IUD	4 (13%)	11 (21%)	17 (44%)	Fisher's exact	0.007
	Hormonal	26 (81%)	39 (74%)	22 (56%)		
	BTL	2 (6%)	3 (6%)	0 (0%)		

CC: Cervical Cancer; CIN: Cervical Intraepithelial Neoplasia; IUD: Intra Uterine Device; BTL: Bilateral Tubal Ligation; BP: Blood pressure

Age is a continuous variable and was presented as mean (standard deviation)

4.32 **Individual and combined diagnostic accuracy of serum P16ink4A and FOXP3 concentrations**

A raised serum FOXP3 concentration showed moderate sensitivity of 32.22% and 57.78% for detection of CIN and CC from healthy controls respectively. It also showed a moderately high specificity of 68.89% for detection of both CIN and CC from healthy controls. The positive predictive values of raised serum FOXP3 concentration for the detection of CIN and CC from healthy controls were 50.88% and 50.41% respectively. Higher negative predictive values of 65.00% and 62.00% were observed for raised serum FOXP3 concentration for the detection of CIN and CC from healthy controls.

Raised serum P16ink4A concentration showed moderate sensitivities (50.00% and 60.00%) and specificities (56.67% and 55.56%) for the detection of CIN and CC from healthy controls respectively. Positive predictive values were 53.57% and 57.45% for the detection of CIN and CC from healthy controls respectively while negative predictive values were 53.13% and 58.14% for the detection of CIN and CC from healthy controls respectively.

However, a combination of raised serum FOXP3 and P16ink4A showed very low sensitivities of 18.89% in detecting CIN from healthy controls and 33.33% for detecting CC from healthy controls. This combination showed high specificity of 83.33% in detecting both CIN and CC from healthy controls with positive predictive values of 53.13% and 66.67%) and negative predictive values of 50.68% and 55.56% for detection of CIN and CC from healthy controls respectively as shown in table 12.

Table 12: Individual and combined diagnostic accuracy of serum P16ink4A and FOXP3 concentrations for **the detection of cervical intraepithelial neoplasia and cervical cancer from healthy controls**

Biomarker	CIN		CC	
	Percentage	95% CI	Percentage	95% CI
FOXP3				
Sensitivity	32.22%	25.40% - 39.05%	57.78%	50.56% - 64.99%
Specificity	68.89%	62.13% - 75.65%	68.89%	62.13% - 75.65%
Positive predictive value	50.88%	43.57% - 58.18%	65.00%	58.03% - 71.97%
Negative predictive value	50.41%	43.10% - 57.71%	62.00%	54.91% - 69.09%
P16ink4A				
Sensitivity	50.00%	42.70% - 57.30%	60.00%	52.84% - 67.16%
Specificity	56.67%	49.43% - 63.91%	55.56%	48.30% - 62.81%
Positive predictive value	53.57%	46.29% - 60.86%	57.45%	50.22% - 64.67%
Negative predictive value	53.13%	45.83% - 60.42%	58.14%	50.93% - 65.35%
COMBINATION OF FOXP3 and P16ink4A				
Sensitivity	18.89%	13.17% - 24.61%	33.33%	26.45% - 40.22%
Specificity	83.33%	77.89% - 88.78%	83.33%	77.89% - 88.78%
Positive predictive value	53.13%	45.83% - 60.42%	66.67%	59.78% - 73.55%
Negative predictive value	50.68%	43.37% - 57.98%	55.56%	48.30% - 62.81%

CI: Confidence Interval; CIN: Cervical Intraepithelial Neoplasia: CC: Cervical Cancer

The ROC analyses displayed the AUC of 0.6014 for raised serum FOXP3 in the detection of CIN from healthy controls (Fig 2A), and an AUC of 0.7679 for raised serum FOXP3 in the detection of CC from healthy controls (Fig 2B). The ROC also showed the AUC of 0.6085 for raised serum P16ink4A to detect CIN from healthy controls (Fig 2C) and an AUC of 0.7592 for raised serum P16ink4A to detect CC from healthy controls (Fig 2D). The combination of the two biomarkers showed an AUC of 0.5992 for the detection of CIN (Fig 2E) and 0.7642 for the detection of CC from healthy controls (Fig 2F).

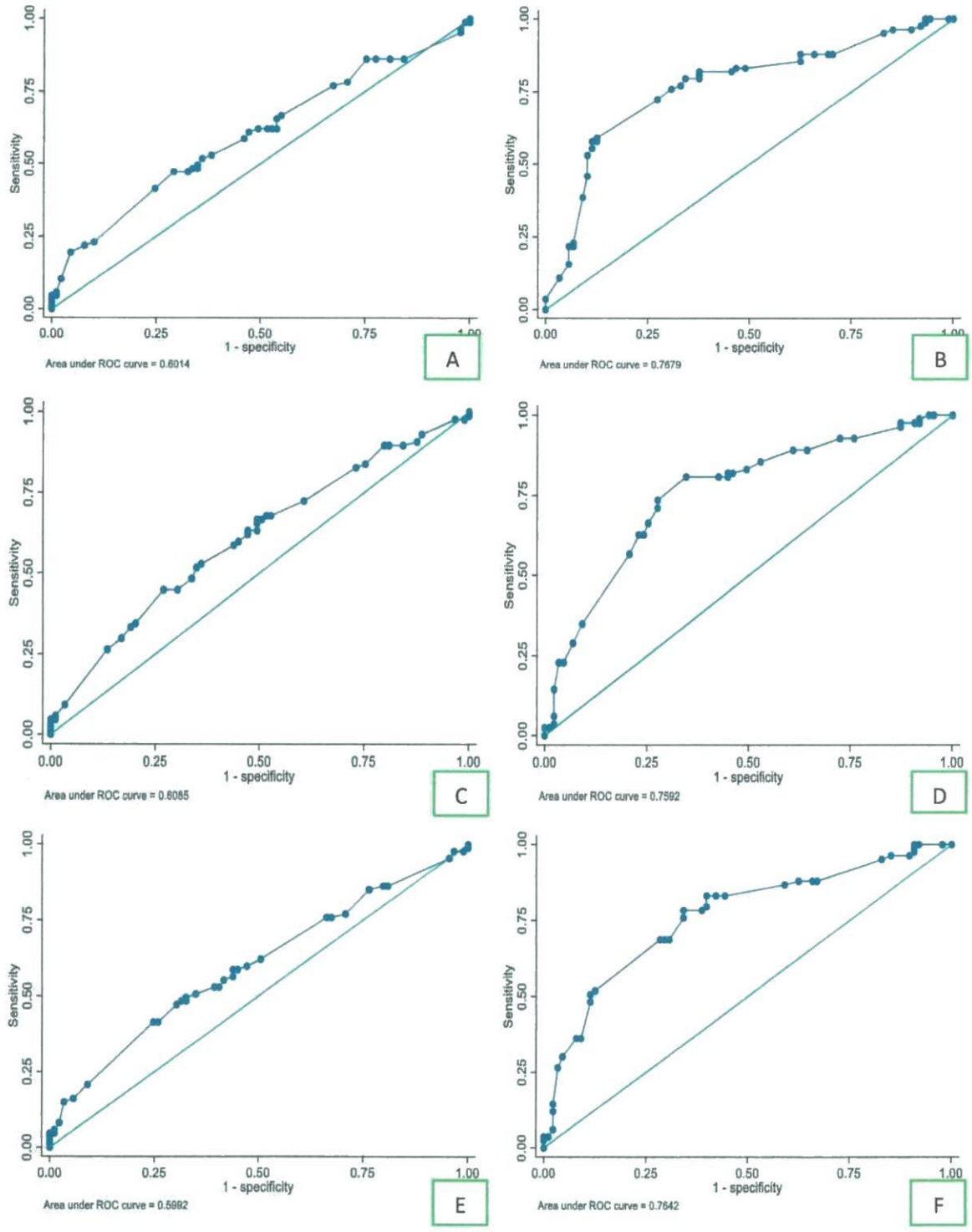


Figure 9: ROC curve showing individual and combined diagnostic accuracy of FOXP3 and P16ink4A for the detection of cervical intraepithelial neoplasia and cervical cancer.

4.40 Association between serum P16ink4A and FOXP3 and treatment outcomes of cervical lesions

4.41 Demographic characteristics of participants in the prospective cohort study

In the prospective cohort study, we followed a total of 180 participants from women who attended services at the cervical cancer clinic at MRRH, and these included 90 cases of CC and 90 cases of CIN. The mean age among the CC cases (51.13, +/-13.01) was significantly higher than that of CIN cases (35.06, +/-7.71) ($p<0.001$).

Most of the CC cases (79%, 71/90) and 57% (51/90) of CIN cases were residents of districts of Southwestern Uganda other than Mbarara and central Uganda ($p<0.001$). The majority of CC participants (57%, 51/90) and 60% (54/90) of the CIN participants were married while 4% and 22% of the CC and CIN respectively were single and this distribution in marital statuses was statistically significant ($p<0.001$).

The highest attained level of education among our study participants was generally primary level and below, with 53%(48/90) of CC cases having attained a maximum of preprimary and 52%(46/90) of CIN cases having attained a primary level of education ($p<0.001$). The HIV positivity among our study participants was generally high with 76%(68/90) of CC cases and 52%(47/90) of CIN cases reporting to a positive sero status, and this distribution across study groups was statistically significant ($p<0.002$). More than half of CC participants (63%, 57/90) and 44% (38/90) of CIN participants were not using contraceptives. Among those that reported usage of contraceptives, 57.6% (19/33) of the CC and 77.6% (38/49) of CIN participants used hormonal methods ($p<0.009$).

The mean FOXP3 baseline concentration (0.13 +/- 0.15) among CC participants was significantly higher than that of CIN participants (0.06 +/- 0.02) ($p<0.001$). Similarly, the baseline P16INKK4A mean concentration (1.45 +/- 1.11) among CC participants was significantly higher than that of CIN participants (1.09 +/- 0.66) ($p<0.01$) as shown in Table 13.

Table 13: Socio-demographic characteristics of study participants in the cohort study

Variable	Category	CANCER N=90	CIN N=90	Test	p-val
Age		51.13(13.01)	35.06(7.71)	Ind. t test	<0.00
Age group	21-29	2 (2%)	22 (24%)	Fisher's exact	<0.00
	30-39	18 (20%)	38 (42%)		
	40-49	21 (23%)	29 (32%)		
	50-59	20 (22%)	1 (1%)		
	60-max	29 (32%)	0 (0%)		
Region of residence	Central	4 (4%)	2 (2%)	Fisher's exact	<0.00
	Other districts	71 (79%)	51 (57%)		
	Mbarara	15 (17%)	37 (41%)		
History of high BP	No	71 (79%)	67 (75%)	Fisher's exact	0.6
	Yes	19 (21%)	23 (25%)		
History of Diabetes	No	70 (78%)	78 (87%)	Chi square	0.1
	Yes	20 (22%)	12 (13%)		
Marital status	Single	4 (4%)	20 (22%)	Fisher's exact	<0.00
	Married	51 (57%)	54 (60%)		
	Divorced	28 (31%)	16 (18%)		
	Widowed	7 (8%)	0 (0%)		
Highest level of education	Never studied	32 (36%)	5 (6%)	Fisher's exact	<0.00
	Preprimary	48 (53%)	3 (3%)		
	Primary	10 (11%)	46 (52%)		
	Secondary	0 (0%)	22 (25%)		
	Tertiary	0 (0%)	6 (7%)		
	University	0 (0%)	7 (8%)		
HIV status	Negative	22 (24%)	42 (47%)	Fisher's exact	0.00
	Positive	68 (76%)	47 (52%)		
	Unknown	0 (0%)	1 (1%)		
Smoking	No	90 (100%)	85 (94%)	Fisher's exact	0.0

	Yes	0 (0%)	5 (6%)		
Contraceptive use	No	57 (63%)	38 (44%)	Chi-square	0.00
	Yes	33 (37%)	49 (56%)		
Type of contraceptive (n=82)	IUD	14 (42.4%)	8(16.3%)	Fisher's exact	0.00
	Hormonal	19 (57.6%)	38 (77.6%)		
	Condom	0 (0%)	3 (6.1%)		
Presenting complaint	None	52 (58%)	61 (68%)	Fisher's exact	0.01
	Abnormal discharge	6 (7%)	4 (4%)		
	Others	8 (9%)	11 (12%)		
	Cervicitis	14 (16%)	14 (16%)		
	Painful micturation	10 (11%)	0 (0%)		
Age at sexual debut (years)		18.96(2.87)	18.79(2.77)	Ind. t test	0.6
Baseline FOXP3 concentration (ng/ml)		0.13(0.15)	0.06(0.02)	Ind. t test	<0.00
Baseline P16ink4A concentration (ng/ml)		1.45(1.11)	1.09(0.66)	Ind. t test	0.01

FOXP3- Forkhead-Box-Protein P3

*-statistically significant ($p \leq 0.05$).

Continuous variables, Age, Baseline FOXP3 concentration, Baseline P16ink4A concentration and Age at sexual debut are presented as mean (standard deviation).

4.42 Treatment outcomes for cervical lesions at 12 months post-treatment

Of the 180 at baseline, only 62 study participants returned for the post-treatment follow-up at 12 months, giving a loss to follow-up rate of 65.6%. Of those who returned for 12 months post-treatment follow-up, 47 participants had cleared lesions, 6 had persistent lesions and 9 participants had progressed lesions. Most of the participants with cleared cervical lesions 82.98% (39/47) had LSIL. However, all those participants who had persistent lesions also had LSIL 100%(6/6) and all those whose cervical lesions progressed were CC cases 100% (9/9). This distribution in the 12 months post-treatment follow-up was statistically significant ($p<0.001$).

For age distribution, more than a third (44%, 4/9) of all participants with progressed lesions were aged between 50 and 59 years. Also 33% (3/9) of participants with progressed lesions were aged 60 years and above. Among participants whose lesions persisted, 33% (2/6) were aged between 40 and 49 years. The same proportion was observed for those aged between 30 and 39 years.

The study found that 64% (30/47) of participants with cleared lesions and 78% (7/9) of those with progressed lesions were married. However, 50% (3/6) of those with persisted lesions were single (p=0.18). The majority of participants with cleared lesions (60%, 28/47), persistent lesions (50%, 3/6), and progressed lesions (56%, 5/9) were HIV positive though not statistically significant (p=0.91) as shown in table 14.

Table 14: Treatment outcomes for cervical lesions at 12 months' post treatment of Study Participants.

Variable	Category	CLEARED N=47	PERSISTED N=6	PROGRESSED N=9	Test	p-valu
Cervical Lesions	ASCUS	1 (%)	0 (%)	0 (%)	Fisher's exact	<0.001
	CANCER	2 (%)	0 (%)	9 (%)		
	HSIL	5 (%)	0 (%)	0 (%)		
	LSIL	39 (%)	6 (%)	0 (%)		
Age group	19-29	12 (26%)	2 (33%)	0 (0%)	Fisher's exact	0.061
	30-39	19 (40%)	2 (33%)	0 (0%)		
	40-49	14 (30%)	2 (33%)	2 (22%)		
	50-59	1 (2%)	0 (0%)	4 (44%)		
	60-max	1 (2%)	0 (0%)	3 (33%)		
Residence	Central	4 (9%)	2 (33%)	2 (22%)	Fisher's exact	0.27
	Other districts	42 (89%)	4 (67%)	7 (78%)		
	Mbarara	1 (2%)	0 (0%)	0 (0%)		
History of high BP	No	37 (79%)	4 (67%)	8 (89%)	Fisher's exact	0.66
	Yes	10 (21%)	2 (33%)	1 (11%)		
History of Diabetes	No	39 (83%)	5 (83%)	8 (89%)	Chi-square	0.91
	Yes	8 (17%)	1 (17%)	1 (11%)		
Marital status	Single	8 (17%)	3 (50%)	0 (0%)	Fisher's exact	0.18
	Married	30 (64%)	2 (33%)	7 (78%)		
	Divorced	9 (19%)	1 (17%)	2 (22%)		

Education level	Never studied	4 (9%)	0 (0%)	4 (44%)	Fisher's exact	0.027*
	Preprimary	0 (0%)	1 (17%)	0 (0%)		
	Primary	22 (47%)	2 (33%)	5 (56%)		
	Secondary	10 (21%)	3 (50%)	0 (0%)		
	Tertiary	6 (13%)	0 (0%)	0 (0%)		
	University	5 (11%)	0 (0%)	0 (0%)		
HIV status	No	19 (40%)	3 (50%)	4 (44%)	Fisher's exact	0.91
	Yes	28 (60%)	3 (50%)	5 (56%)		
Smoking	No	45 (96%)	5 (83%)	9 (100%)	Fisher's exact	0.31
	Yes	2 (4%)	1 (17%)	0 (0%)		
Contraceptive use	No	18 (40%)	3 (50%)	7 (78%)	Chi square	
	Yes	27 (60%)	3 (50%)	2 (22%)		
Type of contraceptive	IUD	6 (20%)	0 (0%)	7 (78%)	Fisher's exact	
	Hormonal	23 (77%)	3 (100%)	2 (22%)		
	Condom	1 (3%)	0 (0%)	0 (0%)		
Presenting complaint	None	1 (2%)	0 (0%)	0 (0%)		Fisher's exact
	Abnormal discharge	7 (17%)	0 (0%)	0 (0%)		
	Others	2 (5%)	1 (17%)	0 (0%)		
	Cervicitis	32 (76%)	5 (83%)	9 (100%)		
Age at sexual debut (years)		18.94(2.59)	19.17(2.71)	17.45(2.29)	ANOVA	0.26

IUD-Intrauterine Device; BP-Blood Pressure

Continuous variable Age at sexual debut is presented as mean (standard deviation)

*-statistically significant ($p \leq 0.05$).

4.43 Distribution of follow up serum Foxp3 and P16ink4A concentrations across treatment outcomes of cervical lesions

The 12 months follow-up mean serum FOXP3 concentration was found to be significantly ($p<0.001$) high among participants with progressed lesions (0.13 ng/ml +/-0.09) compared to mean concentrations among those with persistent (0.05ng/ml +/-0.01) and cleared lesions (0.05ng/ml +/-0.02). Similarly, the follow-up mean serum P16ink4A concentration (1.66ng/ml +/-0.99) was significantly ($p=0.006$) high among participants with progressed lesions compared with mean concentrations among participants with cleared lesions (0.98ng/ml +/-0.47) and persistent lesions (1.12ng/ml +/-0.35).

Considering the categorization of serum concentrations, high FOXP3 were observed in 78% (7/9) of participants with progressed lesions. In the same regard, 72% (34/47) of participants with cleared lesions and 67% (4/6) of participants with persistent lesions had low FOXP3 concentrations. Furthermore, raised P16ink4A was observed in 67% (6/9) of participants with progressed lesions. Also, 40% (19/47) of participants with cleared lesions, 67% (4/6) of participants with persistent lesions had a raised P16ink4A as shown in Table 15.

Table 15: Distribution of follow up serum Foxp3 and P16ink4A concentrations across treatment outcomes of cervical lesions of study participants.

Variable	Category	CLEARED N=47	PERSISTED N=6	PROGRESSED N=9	Test	p val
BASELINE P16ink4A (ng/ml)		1.04 (0.48)	0.82 (0.27)	1.51 (0.98)	ANOVA	0.041
BASELINE FOXP3 (ng/ml)		0.07 (0.02)	0.05 (0.01)	0.07 (0.06)	ANOVA	0.48
Follow UP FOXP3 (ng/ml)		0.05(0.02)	0.05 (0.01)	0.13 (0.09)	ANOVA	<0.00
Follow UP P16ink4A (ng/ml)		0.98(0.47)	1.12(0.35)	1.66(0.99)	ANOVA	0.006
Follow UP FOXP3 Categories	low Foxp3	34 (72%)	4 (67%)	2 (22%)	Fisher's exact	0.017
	high Foxp3	13 (28%)	2 (33%)	7 (78%)		
Follow UP P16ink4A Categories	Low p16ink4A	28 (60%)	2 (33%)	3 (33%)	Fisher's exact	0.26
	Raised p16ink4A	19 (40%)	4 (67%)	6 (67%)		

Continuous variables FOLLOW UP FOXP3 (ng/ml), Baseline FOXP3 (ng/ml) BASELINE P16ink4A (ng/ml) and Follow UP P16ink4A (ng/ml) are presented as mean (standard deviation)
*-statistically significand ($p \leq 0.05$).

4.44 Association between follow up serum FOXP3 concentrations and treatment outcomes of cervical lesions

For raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for persistence relative to clearance of cervical lesions would be expected to increase by a factor of 1.34 given the other variables in the model are held constant, though not statistically significant ($p=0.81$). Also, for raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for progression relative to clearance

of cervical lesions would be expected to increase by a factor of 27.82 given the other variables in the model are held constant, though not statistically significant (p=0.10) as shown in Table 16.

Table 16: Association between follow up serum FOXP3 concentrations and treatment outcomes of cervical lesions among study participants.

Variable	Relative risk	P Value	95% CI
Persisted lesion	1.34	0.81	0.12-15.18
Progressed lesion	27.82	0.10	0.52-1483.94

CI-Confidence Interval

All data was presented after controlling for age, region of residence, family planning usage and type, HIV status, highest education attained, marital status, smoking, history of blood pressure and history of diabetes

4.45 Comparison of baseline and follow up serum P16ink4A and FOXP3 concentrations among participants

After 12 months follow up, we observed that the mean difference between baseline and follow up serum concentration of P16ink4A and FOXP3 was not statistically significant based on t-test results as shown in **table 17**. However, we observed that serum concentrations of P16ink4A were higher than those of FOXP3. Also, follow up serum P16ink4A concentration was higher than baseline especially for persistent and progressed cervical lesions as shown in **figure 10**.

Table 17: Comparison of baseline and follow up serum P16ink4A and FOXP3 concentrations among participants

Variable	**Mean Difference**	**t-Statistic**	SE	SD	95% CI	**Pr(T < t)**	**Pr(|T| > |t|)**	**Pr(T > t)**
P16ink4A (ng/ml)	0.004	-0.084	0.048	0.379	-0.100 - 0.092	0.467	0.933	0.534
FOXP3 (ng/ml)	0.002	-0.327	0.005	0.041	-0.012 - 0.009	0.372	0.745	0.628

CI-Confidence Interval, SE-Standard Error, SD-Standard Deviation, **Pr(T < t)**- The p-value for the one-tailed test where the alternative hypothesis is that the mean P16ink4A or FOXP3 concentration at follow up is less than that at baseline, **Pr(|T| > |t|)**- The p-value for the two-tailed test where the alternative hypothesis is that the means of P16ink4A or FOXP3 concentration are different at baseline and at follow up, **Pr(T > t)**- The p-value for the one-

tailed test where the alternative hypothesis is that the mean P16ink4A or FOXP3 concentration at follow up is greater than that at baseline.

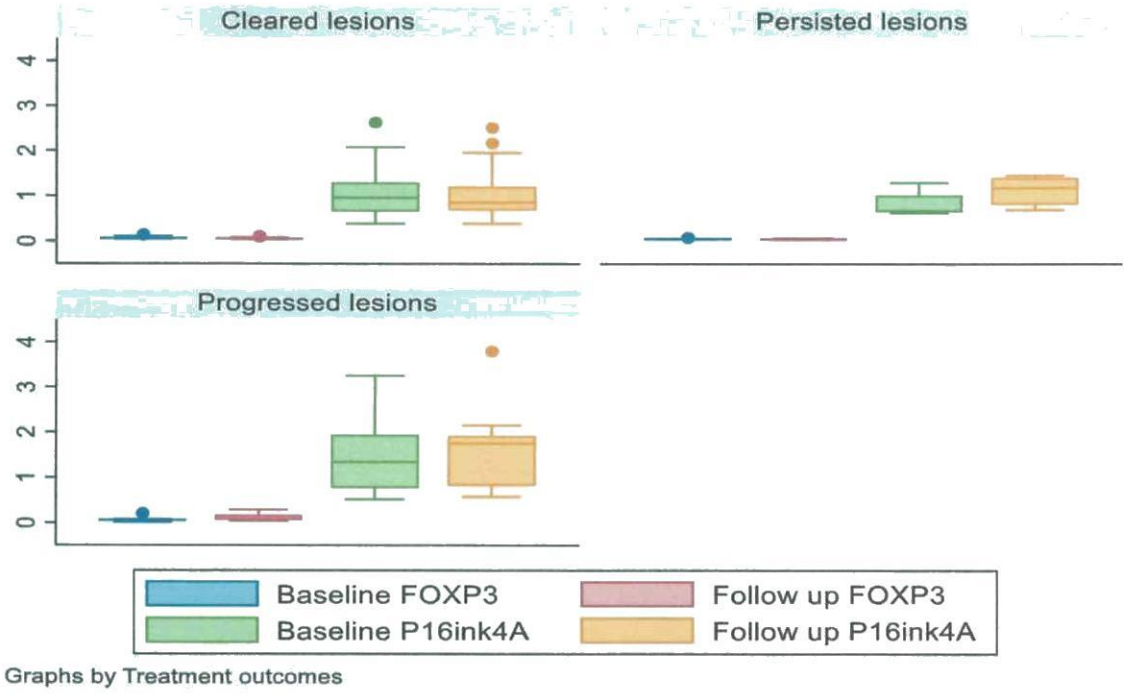


Figure 10: A box plot showing variation of serum P16ink4A and FOXP3 concentration at baseline and post follow up for all treatment outcomes.

4.45 Association between follow up serum P16ink4A concentrations and treatment outcomes of cervical lesions

For raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk for persistence relative to clearance of cervical lesions would be expected to increase by a factor of 5.16 given the other variables in the model are held constant, though not statistically significant (p=0.22). For raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk for progression relative to clearance of cervical lesions would be expected to reduce by a factor of 0.44 given the other variables in the model are held constant, though not statistically significant (p=0.63) as shown in Table 17.

Table 18: Association between follow up serum P16ink4A concentrations and treatment outcomes of cervical lesions among study participants.

Variable	Relative risk	P Value	95% CI
Persisted lesion	5.16	0.22	0.37-71.91
Progressed lesion	0.44	0.63	0.02-12.25

CI-Confidence Interval

All data was presented after controlling for age, region of residence, family planning usage and type, HIV status, highest education attained, marital status, smoking, history of blood pressure and history of diabetes

5.0 CHAPTER FIVE: DISCUSSION

This study was designed to assess prevalence and factors associated with cervical squamous intraepithelial lesions; association between serum levels of FOXP3 and P16ink4A and cervical lesions; the diagnostic utility of serum levels of FOXP3 and P16ink4A in diagnosis; and the association between serum levels of FOXP3 and P16ink4A and treatment outcomes of cervical squamous intraepithelial lesions among women attending the cervical cancer clinic at Mbarara Regional Referral Hospital. In this study, 6.6% of all participants recruited were positive for cervical lesions and usage of IUDs and hormonal contraceptives as well as having a family history of cervical cancer were significantly associated with cervical lesions. We also observed significant associations between serum P16ink4A and FOXP3 concentrations and CIN and CC. This study revealed that individual serum P16ink4A and FOXP3 concentrations have moderate accuracies for the detection of CIN and CC from healthy controls and their combination was more specific and sensitive. Increased serum concentrations of FOXP3 and P16ink4A also showed significant associations with persistence and progression of cervical lesions.

5.10 Prevalence and factors associated with cervical lesions

We found out that the prevalence of cervical lesions was 6.6%. This prevalence was lower than is reported in most studies. In other low-resource African countries, studies have reported higher prevalence rates, for instance 26.7-37% in Kenya (Njagi et al., 2021). There was a prevalence of 18.2% in Bangladesh (Bradford et al., 2012), and 22.1% in Ethiopia (Gedefaw et al., 2013). Previous studies especially among HIV-positive and negative populations have reported prevalence rates of 22.9% and 5.79%, respectively, in Swaziland (Jolly et al., 2017). The observed differences could be attributed to the fact that these studies were conducted among HIV-positive people, bearing in mind that HIV is a known risk factor for cervical lesions (Stelzle et al., 2021, Yuan et al., 2021, Adler et al., 2014). We take note of the fact that more than half of our study participants were HIV positive, but not immunocompromised, as shown by their CD4 counts and viral load results. However, a lower prevalence rate of 3.33% has been reported in Cameroon (Nkfusai et al., 2019). In Nigeria, a hospital based case control study documented a comparable prevalence of abnormal Pap cytology results (8.0%) (Ajah et al., 2015a). From this, we note the comparability of study populations in terms of socioeconomic status and sexual behaviour. An increased prevalence of cervical neoplasia in our study was found among married participants, HIV positive and those who were less educated (primary education). A similar pattern was earlier reported in related studies by Bayo et al in Mali (Bayo et al., 2002) and Palacio-Mejía et al in Mexico (Palacio-Mejía et al., 2003). Participants may have developed cervical neoplasia as a result of either early sexual

experience or multiple sexual partners, which are both significant risk factors for HPV infection.

We observed that all participants (23/23) with positive Pap smears had no history of high blood pressure. This is agreeing with other studies which have showed that cervical lesions are significantly associated with other metabolic syndrome components except high blood pressure (Reeves et al., 2007, Frumovitz et al., 2014, Calle et al., 2003, Bhaskaran et al., 2014, Song et al., 2008) (Poorolajal and Jenabi, 2016). Also from our recent studies in Ugandan populations, both urban and rural, we also observed significant associations between other metabolic syndrome components, except high blood pressure, with cervical lesions (Ssedyabane et al., 2023a, Mwangi et al., 2024).

This study also presents a higher prevalence of cervical lesions among married women. This contradicts the generally perceived notion about marriage as a protective factor against cervical lesions (Machida et al., 2017) and that unmarried women are at increased risk of cervical cancer (Zena et al., 2021). This is an indication that there is a likely exposure to HPV or other risk factors of cervical cancer among married women in this study population. The high prevalence of HIV among our study participants could be an indication that most of them practice high risk lifestyles which could include multiple sexual partners. In fact, population dynamics and trends show that 8.3% of all women in Uganda are married or cohabiting in polygamous relationships (Statistics, 2016). These observations could explain the increased prevalence of sexually transmitted infections like Chlamydia Trachomatis and HPV in this population, which are associated with cervical lesions (Ssedyabane et al., 2019). An equally high prevalence of cervical lesions among married women was reported from a study in a Cameroonian hospital population (Wabo et al., 2022).

We report that in this study population, usage of IUDs and hormonal contraceptives as well as having a family history of cervical cancer were significantly associated with cervical lesions. Although it deviates from widely accepted risk factors, this provides new information regarding risk factors for cervical intraepithelial lesions and possibly cervical cancer. For instance, many authors from previous studies reported smoking, HIV positivity, contraceptive use, and a family history of cervical cancer as possible risk factors for cervical cancer (Zheng et al., 2019, Xin et al., 2016, Yuan et al., 2021, Ghebre et al., 2017, Roura et al., 2016, Bruni et al., 2010, Stelzle et al., 2021, Cooper et al., 2007, Louie et al., 2009, Liu et al., 2015, Fonseca-Moutinho, 2011, Cancer, 2007). Furthermore, in a study on the risk factors for cervical

neoplasia by Kjellberg et al. (Kjellberg et al., 2000) observed a substantial correlation between higher grades of cervical intraepithelial neoplasia and HPV, smoking, as well as prolonged contraceptive usage. In addition, our previous study in this population showed that obesity is significantly associated with cervical intraepithelial lesions (Ssedyabane et al., 2023a) (Anufrieva et al., 2022).

We did not observe any association between smoking and cervical lesions. We observe that there were very few cases of smokers in the study population. In fact, smoking has been found not to be associated with any grade of cervical lesions as observed in a previous study by Castellsague´ et al. (Castellsague et al., 2014). However, to the contrary, smoking has been shown to be associated with cervical intraepithelial neoplasia (Wabo et al., 2022, Kjellberg et al., 2000) and other malignancies (Scherübl, 2021, Scherübl, 2023, Macacu et al., 2015). In this population, age was not associated with cervical lesions. However, it was earlier proposed by Bhatla et al. (Bhatla et al., 2013) that the age group of 21 to 40 years could be a risk factor for cervical lesions, based on the high prevalence of HPV and other risk factors in the same age group.

From logistic regression analysis, we found that usage of IUDs is significantly associated with cervical lesions among the study population. A number of previous studies have reported contradicting results in regard to IUDs and cervical lesions (Jensen et al., 2013, Shields et al., 2004, Zondervan et al., 1996, Castellsagué et al., 2011). A systematic review by Cortessis et al (Cortessis et al., 2017) reported less frequent cervical cancer cases in women who have used IUDs. Hence IUDs may be apparently protective against development of cervical lesions. This perceived protective role of IUDs against development of cervical lesions is based on observations that IUDs reduce HPV infection risk. It is also thought that IUDs trigger responses within the immune system that tend to fight viral infections including HPV (Hermanns and Heafez, 1981). From a case control study, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) Study, a decreased risk of both CIN3/CIS and ICC was observed among IUD users though not significant (Roura et al., 2016).

In agreement with our findings, IUD usage is associated with CIN2+ but not with CIN3+ as evidenced by a case-control study by Averbach et (Averbach et al., 2018). In some populations, especially those in less developed countries, usage of IUDs is thought to results in increased risk of sexually transmitted infections (STIs), including HPV, whose association with cancer of the cervix is well documented (Kiweewa et al., 2019). Similarly, a study among

Kenyan sex workers found that usage of IUDs was significantly associated with increased incidence of C. trachomatis infections (Masese et al., 2013). This is in agreement with our earlier observations in this population, which demonstrated that a co-infection of C. trachomatis and HPV is associated with cervical lesions (Ssedyabane et al., 2019). Certain types of IUDs, including LNG-IUS, though not be popular in our study population, tend to increase risk of HPV infection acquisition and persistence (Stensen et al., 2016). Lekovich et al.'s comparative study, (Lekovich et al., 2015) which examined the differences in cervical cytology and high-risk human papillomavirus (HPV) infection clearance between levonorgestrel- and copper-containing IUD users, also supported our findings. They showed that levonorgestrel-containing IUDs may be linked to higher risk of acquiring high-risk HPV infections and also lower clearance rates when compared to copper-containing IUDs.

In the same regard, we found a significant association between cervical lesions and usage of hormonal contraceptives. Results from a systematic review by Deese et al, provided evidence that is supports an increased risk of many other STIs, including HSV-2 infection among users of hormonal contraceptive methods including depot medroxyprogesterone acetate (DMPA) (Deese et al., 2018). Iversen et al.'s observations have also demonstrated that for women who have not received the HPV vaccine, their risk of cervical cancer remains high (Iversen et al., 2021). We note that HPV vaccination coverage among our study participants is very low (Patrick et al., 2022, Malande et al., 2019). The duration of usage of hormonal contraceptives could also have a significant correlation with cervical lesions. To further understand the potential impact of the usage duration of hormonal contraceptives on cervical lesions, a case control study by Kusmiyati et al (Kusmiyati et al., 2019) revealed that exposure to hormonal contraceptives for more than five years can increase one's risk of cervical cancer by 4.2 times.

Hormonal contraceptives alter cellular metabolism, which can lead to persistent HPV infections and the eventually development of cervical cancer. Oestrogen and progestin may encourage the expression of the oncogenes E6 and E7 of HrHPV genotypes. This process triggers the destruction of the p53 tumour suppressor gene and the incorporation of viral DNA, resulting in cellular transformation and the development of cancer (Comar et al., 2020, Bosch et al., 2002, Sasieni, 2007). Hormonal contraceptives tend to bind to certain HPV-DNA sequences within transcriptional regulatory areas, which may control cellular death, and to promote HPV DNA integration into the host cell genome (Gadducci et al., 2020, Castellsagué and Munoz, 2003, Moodley et al., 2003, Gadducci et al., 2011, Marks et al., 2011, Webster et al., 2001, de Villiers, 2003). With increased uptake of hormonal contraceptives in our

population, there may be an increased risk to cervical cancer as a result of hormonal contraception, moreover in a predominantly unvaccinated population (Patrick et al., 2022, Malande et al., 2019).

We also note that a recent review by Anastasiou et al showed no evidence of any association between hormonal contraceptive use and cervical lesions (Anastasiou et al., 2022). However, this study included very few included studies. Additionally, a case-control research conducted in Nigeria by Ajah et al. had also found no link between cervical neoplasia and hormonal contraception (Ajah et al., 2015a). This study did not reveal any significant association between oral contraceptive use and cervical cancer or any grade of cervical lesion. On the contrary, another systematic review showed that longer duration of oral contraceptive pills use has an associated risk for developing cervical cancer, especially for Adenocarcinoma (Asthana et al., 2020).

5.20 Association between serum P16ink4A and FOXP3 concentrations and cervical lesions

Our study also reveals a compelling association between serum P16ink4A concentrations and cervical lesions within our study population. Notably, our investigation marks a significant contribution to the existing literature as one of the pioneering studies in Uganda to explore the potential link between circulating P16ink4A concentrations and cervical lesions. Our findings align with prior research demonstrating a consistent association between P16ink4A immunohistochemistry and cervical lesions, as documented in previous studies (Zuberi et al., 2021, Sarwath et al., 2017, Omran and Alsheeha, 2015). Other studies have demonstrated that overexpression of P16ink4A increases with severity of lesions (Kanthiya et al., 2016, Cheah et al., 2016, Ding et al., 2020, Dixon et al., 2017, Peres et al., 2016). This explains the extensive usage of P16ink4A immunohistochemical staining in staging of cervical cancer due to its perceived diagnostic and prognostic potential (Ding et al., 2020, Peres et al., 2016, Shi et al., 2019, Pandey et al., 2018) especially among HPV infected women (Bergeron et al., 2015).

The significantly increased P16ink4A serum concentrations in cervical cancer could be resulting from inactivation of the retinoblastoma protein in HPV infected cervical cancer cells unlike in non-cancerous cells. Inactivation of retinoblastoma protein originates from increased expression and binding by viral oncoprotein E7, which eventually leads to increased expression of P16ink4A (Hellman et al., 2014). It has been demonstrated that P16ink4A exhibits oncogenic potential in HPV transformed cervical cells (Li et al., 2020b) as well as cell lines (McLaughlin-Drubin et al., 2013).

This study reported a statistically significant difference in age among our study groups. This collaborates well with previous studies in Tanzania (Zuberi et al., 2021) which showed increased expression of P16ink4A in older women (40-49 years) who are already considered a high risk group for cervical lesions. This same age group of women is said to have low cervical cancer screening rates (Weng et al., 2020) as well as low cervical cancer awareness (Black et al., 2019, Isabirye et al., 2020). This most likely leads to increased incidence of cervical cancer in such older women (Weng et al., 2020).

We observed a big proportion of cervical cancer and cervical intraepithelial neoplasia cases having P16ink4A above 0.56ug/ml. This is in agreement with previous studies which showed increased expression of P16ink4A in cervical intraepithelial neoplasia and cervical cancer (Cheah et al., 2016). However, a similarly big proportion of cases also showed raised P16ink4A serum concentration. This can be explained by the fact that big proportions of our study population reported numerous gynaecological conditions, mostly cervicitis, vaginal discharge and valvular warts; moreover, with significant distribution across CC, CIN and control groups. These are conditions that can be easily associated with low literacy, poor hygiene, limited cervical cancer knowledge and may lead to persistent genitourinary tract infections including HPV, which increases expression of P16ink4A (Dahiya et al., 2017). Previous HPV infections have been reported to increase the chances of acquiring another HPV infection. Most of our participants could hence been having transient HPV infections which are known to be very prevalent though most of them get cleared without causing any lesions (Shanmugasundaram and You, 2017).

We report an increased number of participants, especially among case groups who were married and with increased P16ink4A serum concentration. Being married is thought to increase one's chances of acquiring HR HPV infections which results in increased chances of developing cervical lesions and hence expression of P16ink4A (Diouf et al., 2020) though Yetimalar et al. (2012) reported otherwise.

Other factors including HIV, smoking, contraceptive use and level of education also varied significantly across cases and control groups. This shows their known role in acquisition or persistence of HPV infection and hence development of cervical lesions (Yu et al., 2016, Zhang et al., 2020, Ghebre et al., 2017, Stelzle et al., 2021, Asthana et al., 2020). Smoking is itself said to have the capacity to increase HPV viral load in infected cells (Xi et al., 2009). Other genetic and life style factors can also increase chances of HPV infection (Haukioja et al., 2014). This further explains the observation of raised P16ink4A serum concentrations in case

groups compared to controls, which aligns with earlier reports that increased P16ink4A expression is strongly associated with HPV mediated cervical pathology (Diouf et al., 2020).

Being a tumor suppressor protein, P16ink4A inhibits E2F dependent cell cycle progression in uninfected cells (Zhang et al., 1999). However, it gets overexpressed in HPV infected cells due to inactivation of pRb by HPV E7 (Keating et al., 2001, Klaes et al., 2001) and it accumulates in the nucleus and cytoplasm of any HPV infected cell (Rokitka et al., 2021, Pandey et al., 2018), hence its potential application in early detection of cervical cancer (Ding et al., 2020, Weng et al., 2020).

This study further presents a statistically significant association between serum FOXP3 concentration and cervical cancer. We present a two-fold likelihood of cervical cancer with an increase in serum FOXP3 concentration. This is in agreement with prior studies that have found increased FOXP3 expression in cervical cancer. For instance, Li et al found that FOXP3 imunohistochemical expression was significantly higher in cervical cancer compared with CIN and cervicitis (Wanyenze et al., 2022). In the same regard, Vattai et al also demonstrated that FOXP3 expression is significantly higher in higher CIN grades (Black et al., 2019). However, their studies were conducted on formalin fixed paraffin embedded cervical tissues. In a related study Xu et al studied the circulating autoantibody to FOXP3, in a case control study. They observed that the anti FOXP3 circulating autoantibody was significantly raised in cervical cancer group compared to the control group (Yeo et al., 2018b).

A case control study that included HPV-infected and HPV-uninfected women diagnosed with or without low or high-grade intraepithelial lesions of cervix revealed that increased FOXP3 expression was independently associated with the HPV infection (Baseman and Koutsky, 2005). A similar study that investigated the clinical significance of FOXP3 in cervical cancer also showed that Foxp3 had increased expression in cervical cancer cells and it was significantly associated with FIGO stage (Bast et al., 2005). These studies were also conducted on formalin fixed paraffin embedded cervical tissues, unlike ours which was conducted on serum.

FOXP3 is a transcriptional factor belonging to the forkhead/winged-helix family and it is widely recognized for its ability regulate T CD4+ CD25+ cells, hence fostering immunological tolerance and maintaining homeostasis in normal and non-diseased cells. About 700 genes and miRNAs linked to the TCR pathway, cell communication, and transcriptional control are activated or repressed transcriptionally to create these functions (Nelson et al., 2010). With

those roles, FOXP3 is able to control the lymphoid lineage effectively especially in malignancies including cervical cancer (Adams et al., 2006). It acts as a master regulator that modulates the genetic functional programming of regulatory T cells (Treg) and this promotes immunological tolerance (Ahn et al., 2015). Stable FOXP3 expression is hence necessary for Tregs to exert their suppressive effects (Ahn et al., 2015, Aitken et al., 2019, Ajah et al., 2015b). Specific isoforms of FOXP3 stimulate migration, cell division, and proliferation in nontumorigenic keratinocytes by transduction and also regulate critical pathways associated with the immunological response and the production of several proto-oncogenes (Akindele and Useh, 2021).

FOXP3 is thought to be a distinct marker of T cells and is crucial to the growth, development and function of T cells (Allan et al., 2008). It is suggested that FOXP3 facilitates the proliferation of cervical cancer cells through promoting cell cycle progression, promoting over proliferation and invasion of the cells and intensifying the malignant potential of cancer cells (Al-Daghri et al., 2015). In addition, FOXP3 activates CD4+ CD25+ Treg cells in some malignancies thus inhibiting the development and functional roles of Treg cells and this promotes tumor proliferation in the long run (Wanyenze et al., 2022, Allegra et al., 2012).

This study highlights the functional role of FOXP3 in cervical carcinogenesis. Research studies have revealed that FOXP3 has a number of isoforms. Notable is the isoform FOXP3Δ2Δ7 which is said to stimulate cell proliferation, migration and division in keratinocytes, hence exhibiting a protumorigenic activity (Adams et al., 2006). In the presence of the isoform FOXP3Δ2Δ7, there is increased expression of SATB1 gene, which itself is involved in epithelial-mesenchymal transition, one of the early steps in carcinogenesis (Bansil et al., 2015, Barrow-Laing et al., 2010). This isoform also induces the hypoxia signalling pathway, a very important pathway for carcinogenesis, through proliferation, survival, tumor invasion and then metastasis (Bartel, 2009, Bartholomew et al., 2011, Bartel, 2004).

In the current study, we included participants who were mostly HIV positive and most likely having HPV infections. There has been no clear assertion on the link between FOXP3+ Tregs and hrHPV (Anindo and Yaqinuddin, 2012). However, it is important to note that increased FOXP3 expression is dependent on hrHPV genotypes especially HPV 16 (Adams et al., 2006). The virus uses the FOXP3 increased expression to evade and control the immune system and hence persistence of the HPV infection (Baker et al., 1991). In the current study, we did not test for HPV and hence cannot make any assertion on this link.

We report that more than half of the cervical cancer participants, who were using contraceptives (hormonal contraceptives). Studies have associated long term use of hormonal contraceptives to development of cervical cancer especially among HPV positive women (Basu et al., 2015, Cancer, 2007, Asthana et al., 2020). Therefore, use of hormonal based contraceptives is likely to have resulted in increased expression of serum FOXP3 among these CC participants, though indirectly.

The association between serum FOXP3 concentrations and CIN was not statistically significant both at bivariate and multivariate analysis. These results are in agreement with those from earlier studies which also found that CIN had a weaker expression of FOXP3 and its association wasn't statistically significant (Al-Daghri et al., 2015). Some studies have reported a positive correlation between FOXP3 cellular expression and CIN. For instance, total FOXP3 expression was reported higher in more advanced CIN grades (Black et al., 2019). Hou et al also found higher proportions of FOXP3 expressing T cells among CIN patients (Hou et al., 2012). However, we did not stratify our analysis according to grades of cervical lesions.

When serum FOXP3 was used as one of the predictors for CIN, the prediction rate for CIN was 94.4% and this was suggestive of good and high prediction accuracy for FOXP3 (Yu Yang et al., 2018). FOXP3 expression levels were found to be high in the cytoplasm, nucleus, and cancer interstitium in cervical cancer, but low in cervical intraepithelial neoplasia (CIN). This suggests that FOXP3 may not play a role in the initiation of the malignancy, but rather promoting the growth of the tumor (Li et al., 2020a). We acknowledge that most of the studies in which there was a statistical significant association between FOXP3 and CIN, they categorised the CIN into different grades (that is to say CIN stage 1, 2 and three) whereas ours wasn't categorised. In addition to that, some of these studies that found out an association between serum FOXP3 and CIN used FOXP3 alongside other markers like glucocorticoid receptor (한관희 et al., 2022), VISTA (Wanyenze et al., 2022) Th17 cells (Hou et al., 2012), ZAP70 (Li et al., 2020a) among others.

5.30 Diagnostic accuracy of serum P16ink4A and FOXP3 for detection of cervical lesions

This study also provides evidence of the possible usage of blood-based biomarkers for the diagnosis of cervical lesions. We present the diagnostic utility of serum FOXP3 and P16ink4A as biomarkers of cervical precancerous and or cancerous lesions. In this study, raised FOXP3 serum concentration showed low sensitivity (32.22%) for the detection of CIN, and a moderate

sensitivity (57.78%) for the detection of CC. We also observed a moderately high specificity (68.89%) for the detection of both CIN and CC.

The low sensitivity and specificity observed in our study renders serum FOXP3 concentration as a test with low value as a screening as well as a diagnostic tool in our population. The ROC for raised serum FOXP3 in our population showed an AUC of 0.6014 for detection of CIN and 0.7679 for CC. This further demonstrated its limitations in detection of pre-cancerous and cancerous cervical lesions in our study population. This may be attributed to the demography of our study population.

It is worth noting that 70% of our study participants with CC were 40 years and above. FOXP3 expression diminishes with advancing age and hence in the elderly, the proportion of resting T-reg drops while activated T-reg and FOXP3^{low} expressing T cells increases (Kumar et al., 2018). Our findings were not in agreement with Xu et al who postulated that circulating autoantibody to FOXP3 reflects continuous development of the cervical lesion and this may be a potential biomarker with early prognostic values for cervical cancer (Xu et al., 2015)

Raised serum P16ink4A concentration has shown potential to be an effective cervical cancer screening test in low-resource settings based on its molecular function and performance on IHC and in prior studies in general populations (Wu et al., 2016). From our study, raised P16ink4A serum concentration had moderate sensitivity (50.00% and 60.00%) and specificity (56.67% and 55.56%) for detection of CIN and CC. This demonstrates its low diagnostic utility in the screening and diagnosis of cervical pre-cancerous or cancerous lesions. Our findings underscore earlier reports from immunohistochemical studies which presented P16ink4A as a potential biomarker of high grade cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (Mao et al., 2007). Several other studies on cervical specimens agree with our finding. For instance, Wu et al (Wu et al., 2016) reported that P16ink4A ELISA on cervical samples did not perform well as a screening tool with only 30.8% cases of correct P16ink4A prediction to detect or rule out CIN2+, and 69.2% cases of incorrect P16ink4A prediction. They also mentioned that P16ink4A was a promising biomarker in measuring progression of cervical disease but also citing difficulties in choosing a numerical threshold to distinguish CIN1 from CIN2+ in a population of women with high rates of HPV positivity and low-grade dysplasia. (Wu et al., 2016). Earlier reports by Balasubramanian et al (Balasubramanian et al., 2009) disagreed with our study. They reported that P16ink4A enhanced ELISA had a sensitivity of 91.8% and specificity of 86.0% demonstrating its diagnostic utility in the screening for cervical

lesions (Balasubramanian et al., 2009). This could however be attributed to the difference in sample types as they employed liquid based cytology samples. Leung et al (Leung et al., 2021) also demonstrated the feasibility of quantifying P16ink4A by ELISA in fresh cervical samples, and its potential as an adjunct to existing screening strategies in the identification of high grade dysplastic lesions using liquid based cytology (Leung et al., 2021).

A combination of FOXP3 and P16ink4A displayed very low sensitivity of 18.89% in detecting CIN and 33.33% for CC but rather high specificity of 83.33% in detecting both CIN and CC among our study participants. This presents their combined effects as a relatively good diagnostic tool but poor screening tool for cervical lesions.

5.40 Association between serum P16ink4A and FOXP3 and treatment outcomes of cervical lesions

We also report results of a likely association between raised serum concentrations of FOXP3 and P16ink4A and progression of cervical lesions. This shows the potential usage of serum FOXP3 and P16ink4A serum concentrations in predicting the outcomes of cervical cancer and cervical intraepithelial neoplasia in our study population. To the best of our knowledge, this is the first study to predict treatment outcomes of cervical lesions using blood based biomarkers. In this study, baseline serum concentrations of FOXP3 and P16ink4A following initial diagnosis and 12 months post treatment for cervical cancer and cervical intraepithelial neoplasia were measured.

We observed that most of the cervical cancer participants with progressed lesions were aged 50 years and above, were married, HIV positive, using IUDs, less educated and presented with cervicitis. It has been demonstrated earlier that HIV positivity and being married likely increase rates of HPV infection and probably persistence (Stelzle et al., 2021, Adler et al., 2014, Yuan et al., 2021). Also, we take note of the fact that majority of women in Uganda reside it polygamous relationships (Statistics, 2016), which likely increases their chances of HPV infection and hence persistence. The fact that cervicitis was a common complaint among those with progressed lesions, this highlights the importance of STIs in development of cervical lesions as well as treatment outcomes. In this population, we earlier on demonstrated a high prevalence of Chlamydia (Ssedyabane et al., 2019), one of the most important STIs in cervical cancer, which could be responsible for cervicitis. Low level of education, as shown in this study is strongly linked with low socio-economic status as well as reduced knowledge on cervical cancer (Akinyemiju, 2012) and they could be responsible for progression of cervical lesions.

In this study, we demonstrated the likely importance of FOXP3 and P16ink4A in predicting treatment outcomes of cervical lesions. FOXP3 and P16ink4A genes display co-expression while FOXP3 significantly regulates P16ink4A expression (Ramirez et al., 2022, Xie et al., 2015). FOXP3 is a member of forkhead/winged-helix family and a transcription factor specifically expressed on Tregs. It plays crucial roles in the development and functions of Treg cells significantly in the inhibition of immune response (Devaud et al., 2014, Van Gool et al., 2019). Recent studies revealed that FOXP3 expression is not only specifically linked to Tregs naturally generated in the thymus, but also in the cytoplasm and/or nucleus of cancer cells and it promotes tumor progression and consequently poor disease outcomes characterized with metastases (Sarkar et al., 2022, Whiteside, 2018). P16ink4A, a cyclin dependent kinase inhibitor 2A is a tumor suppressor protein which downregulates cell multiplications and play important roles in cell cycle arrest in malignancies, and hence loss of this tumor suppressor biomolecule in malignancies has been associated with tumor progression (Witkiewicz et al., 2011).

The baseline and follow up distribution of serum FOXP3 and P16ink4A across treatment outcomes were significantly different among our study participant groups. This finding was in line with our earlier observations in this population which reported the likely beneficial use of circulating P16ink4A in detection of cervical lesions (Ramirez et al., 2022). FOXP3 expression was significantly high ($p<0.001$) among participants with progressed lesions 0.13(0.09) than those with persistent lesions 0.05(0.01) as well as those with cleared lesions 0.05(0.05).

Similarly, serum P16ink4A concentrations across treatment outcomes were significantly high ($p=0.009$) among participants with progressed lesions 1.66(0.99) compared to those with persisted 1.12(0.35) and cleared lesions 0.98(0.47). This demonstrates the extend of gene expression as it has been shown to be highest in CC cases followed by CIN II-III and CIN I (Ngcobo et al., 2021). It is worth noting that all participants with progressed lesions were from the cervical cancer group. These findings uphold the fact that FOXP3 and P16ink4A expressions are upregulated in cervical cancers (Hu and Wang, 2017, Ramirez et al., 2022, Szylberg et al., 2016).

Although not statistically significant ($p=0.10$), we observed that in the cases of raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for progression relative to clearance of cervical lesions would be expected to increase by a factor of 27.82. This demonstrates that elevated FOXP3 expression would likely result in progression of cervical cancer. This is in line with the roles of FOXP3. FOXP3 elevation has been demonstrated as a mechanism of immune evasion by tumor cells as it down regulates

chronic inflammation in the tumor microenvirinments thereby promoting tumorigenesis (Chen et al., 2019, Fontenot et al., 2003, Yamagami et al., 2011).

The prognostic role of FOXP3 has also been shown in other cancer. For instance, Lee et al. studied the prognostic impact of FOXP3 expression in triple-negative breast cancer and reported that FOXP3-positive Treg cells had stronger prognostic significance than FOXP3-negative Treg cells, and concluded that FOXP3-positive Tregs are independent prognostic factor for overall survival (hazard ratio 2.4) and progression free survival with hazard ratios of 2.0 (Lee et al., 2013).

Similar observations were recorded for the association between follow up serum P16ink4A concentrations and treatment outcomes. For raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk of persistence relative to clearance of cervical lesions was expected to increase by 5.16, although this observation was not statistically significant (p=0.22). Conversely, in cases of raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk for progression relative to clearance of cervical lesions was expected to reduce by a factor of 0.44. This justifies the upregulation of P16ink4A in cancer progressions and its down regulation in cleared cervical cancer cases. P16ink4A has been reported to be a major antitumor biomolecule (a potent inhibitor) that arrest cytokinesis at G1 or S stage of the cell cycle (D'Arcangelo et al., 2017, Ramirez et al., 2022). Raised serum FOXP3 and P16ink4A concentrations are thus associated with cervical cancer progression. However, they are not only specific to cervical cancers as they have been reported as prognostic indicators in several other cancers like breast cancers, colorectal cancers, squamous cell carcinomas (Park et al., 2013, Sznurkowski et al., 2017, Zeng et al., 2013).

In this population, we take note of other potential factors that could likely be associated with treatment outcomes. From our earlier observations, we reported that lipids and insulin resistance markers are associated with poor treatment outcomes in this same population (Ssedyabane et al., 2023d). Other factors that could have led to poor treatment outcomes include obesity, a condition that is not only prevalent in this population but also associated with cervical lesions (Ssedyabane et al., 2023b).

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSIONS

We report a prevalence of cervical intraepithelial lesions of 6.6%, which is lower than global estimates. Use of hormonal and IUD contraceptives as well as family history of cervical cancer are significantly associated with cervical intraepithelial lesions among our study population.

Serum P16ink4A and FOXP3 concentrations are associated with cervical lesions especially cervical cancer, among our study population. Quantitative measurement of circulating P16ink4A may be beneficial in diagnosis of cervical cancer based on objectivity in their measurement and the relatively low cost compared to current testing methods.

Serum P16ink4A and FOXP3 concentrations showed moderate accuracy for the detection of CIN and CC from healthy controls. Their combination was more specific than sensitive and better applicable for diagnosis than screening for cervical lesions.

Serum concentrations of FOXP3 and P16ink4A are associated with persistence of cervical intraepithelial neoplasia and progression of cervical cancer. Quantitative measurement of circulating P16ink4A may as well be beneficial in monitoring prognosis of cervical cancer based on objectivity in their measurement and the relatively low cost compared to current testing methods.

6.2 RECOMMENDATIONS

Based on the observed associations, we recommend prospective studies to further understand associations and underlying biochemical processes between different types of IUDs and hormonal contraceptives and cervical lesions.

Because a combination of P16ink4A and FOXP3 improved diagnostic accuracy, additional studies could be conducted to assess the performance of P16ink4A and FOXP3 in combination with other classes of biomarkers in detection and predicting the prognosis of cervical lesions. Other biomarkers could include circulating microRNAs whose diagnostic accuracy has also been studied, though not in a Ugandan population (Ssedyabane et al., 2024b, Ssedyabane et al., 2023c)

More population based studies are recommended to further refine the prognostic value of these biomarkers. Larger prospective studies are also recommended to further understand the

biological mechanisms behind FOXP3 and P16ink4A and clearance, persistence or progression of cervical lesions.

6.3 LIMITATIONS AND STRENGTHS

For objective one, the study is limited by the inherent weaknesses of a cross-sectional study. This design is non-ideal for studying associations between population variables. Therefore, associations presented here may not be easily extrapolated to the wider population. However, we applied a mitigation in the form of bivariate and multivariate logistic regression. We also acknowledge that classic Pap smear testing could have lowered the diagnostic power compared to liquid cytology. We mitigated this by ensuring all Pap smears are read by multiple cytotechnologists and cytopathologists. However, the major strength of this study is its statistical power, which we considered while calculating the sample size. We also employed standard methods during collection, processing, examining, and reporting methods for Pap smear specimens.

Another limitation to this study is that we did not test for HPV. Though being recommended as part of cervical cancer screening, HPV DNA is not yet fully available in resource limited settings including Uganda. Therefore, we could not exactly ascertain whether increased serum P16ink4A and FOXP3 concentrations arose from HPV positivity, though it is known that HPV leads to increased expression of P16ink4A and FOXP3. We mitigated this by collecting as much data as possible on all other factors that depict sexual behaviour. These could potentially show one's sexual behaviour. Also, in the case control studies, we think there might have been selection bias while recruiting participants, and hence some variables may not have been distributed randomly across the study population. The possible mitigation was in the form of recruiting all participants from the same pool. In this we ensured that all study variables are equally distributed across study groups.

We did not analyse associations between P16ink4A and FOXP3 concentrations in terms of grades of cervical lesions. This could have masked significant associations between P16ink4A and FOXP3 concentrations and specific grades of cervical lesions. We however considered all other variables in logistic regression models. Also, we did not determine presence of specific FOXP3 isoforms. This is a missed opportunity for determination of the exact FOXP3 isoforms that could be associated with cervical lesions in our study population. We mitigated this by using objective and accurate measurement methods for FOXP3 regardless of the isoform.

The case control and cohort studies might have suffered from misclassification bias, especially during selection and allocation of controls. Therefore, there might have been some participants who were actually not meant to be controls, and this might have negatively affected statistical inferences. We did our best to apply the currently acceptable standard tests to ascertain case and control status of the study participants. We did not calculate predictive values as the nature of the study design does not allow. The prevalence/incidence, as seen in the column sum in the 2 × 2 table of participants classified as positive and negative with the reference test, is an artificial result of the study design. We did our best to explicitly describe sensitivity and specificity values in relation to clinical practice.

All serum samples used in this study were stored at recommended temperatures and then run as a batch. We believe this storage time may have led to some minimal reductions in the concentrations of FOXP3 and P16ink4A. We also report that we experienced a very high loss to follow-up rate, especially among participants in the cancer group and this could have lowered the power of our study by some degree. However, this study was designed with an adequate sample size to take care of such rates of loss to follow up.

6.4 PUBLISHED PAPERS

1. Ssedyabane F, Ngonzi J, Tusubira D, Nambi Najjuma J, Kajabwangu R, Okeny C, Nuwashaba D, Namuli A, Niyonzima N. Association between serum P16ink4A concentration and CIN and cervical cancer among women attending a cervical cancer clinic in western Uganda: A case control study. Gynecol Oncol Rep. 2024 Mar 31;53:101388. doi: 10.1016/j.gore.2024.101388. PMID: 38590932; PMCID: PMC10999470.

2. Ssedyabane F, Niyonzima N, Nambi Najjuma J, Birungi A, Atwine R, Tusubira D, Randall TC, Castro CM, Lee H, Ngonzi J. Prevalence of cervical intraepithelial lesions and associated factors among women attending a cervical cancer clinic in Western Uganda: results based on Pap smear cytology. SAGE Open Med. 2024 May 16;12:20503121241252265. doi: 10.1177/20503121241252265. PMID: 38764539; PMCID: PMC11100407.

APPENDIX ONE: Questionnaire

TITTLE: **Association of FOXP3 and P16ink4A serum expression with stages, treatment strategies and outcomes for cervical squamous intraepithelial lesions**

Respondent's identity number .....Date .../...../.....

Demographics

Age .....

Address: Country..... District ..... Village .....

History of high blood pressure: Yes ☐ No

History of Diabetes: Yes ☐ No

Marital status: Single ☐ Married ☐ Divorced

Highest level of education attained:

University ☐

Tertiary institution ☐

Secondary school ☐

Primary school ☐

Pre-primary ☐

Never studied ☐

Risk factors to cervical intraepithelial lesions

HIV status: pos ☐ neg ☐ Don't know ☐

If positive, HIV VL copies/ml ..... CD4 count c/UL .....

Smoking: Yes ☐ No ☐

Any other STI..... ....

Contraceptive use: Yes ☐ No ☐ Family history of cervical cancer: Yes No

Type of contraceptive: IUD ☐ Hormonal ☐ Others. .....

Age at sexual debut ☐ Number of sexual partners ever had

Number of full term births ☐

BASELINE RESULTS

VIA TEST RESULTS: Negative ☐ Positive ☐ Suspicious for Cancer ☐

HPV: Negative ☐ Positive ☐

CONFIRMED LESION (tick appropriately)

NILM ☐ ASCUS ☐ ASCH ☐ LSIL ☐ HSIL

Negative ☐ CIN1 ☐ CIN2 ☐ CIN3 CIS

BASELINE SERUM LABORATORY RESULTS

Test		Result
	P16INK4A(ng/ul)	
	FOXP3(ng/ul)	

TREATMENT OPTION (tick appropriately)

Cryotherapy ☐
Thermocoagulation ☐
LEEP ☐
OTHERS ☐

LABORATORY RESULTS AT TWELVE MONTHS POST TREATMENT

VIA TEST RESULTS: Negative ☐ Positive ☐ Suspicious for Cancer
HPV: Negative ☐ Positive ☐

CONFIRMED LESION (tick appropriately)

NILM ☐ ASCUS ☐ ASCH ☐ LSIL ☐ HSIL ☐

Negative ☐ CIN1 ☐ CIN2 ☐ CIN3 ☐ CIS ☐

FOLLOW UP SERUM LABORATORY RESULTS

Test		Result
	P16INK4A(ng/ul)	
	FOXP3(ng/ul)	

TREATMENT OUTCOME AT 12 MONTHS

Cleared ☐ persisted ☐ progressed ☐

PPENDIX TWO: SOP for Venipuncture

TANDARD OPERATING PROCEDURE FOR COLLECTING VENOUS BLOOD

pparatus: Red top vacutainer, vacutainer needle, alcohol swab, gloves, cotton, tourniquet, puncture resistant ntainer.

rocedure

1. Assemble equipment and include needle and syringe or vacuum tube, depending on which is to be used.
2. Perform hand hygiene using soap and water and dry hands with single-use towels
3. Identify and prepare the patient.
4. Select the site, preferably at the antecubital area (i.e. the bend of the elbow). Warming the arm with a hot pack, or hanging the hand down may make it easier to see the veins. Palpate the area to locate the anatomic landmarks.
5. Apply a tourniquet, about 4–5 finger widths above the selected venipuncture site.
6. Ask the patient to form a fist so that the veins are more prominent.
7. Put on well-fitting, non-sterile gloves.
8. Disinfect the site using 70% isopropyl alcohol for 30 seconds and allow it to dry completely (30 seconds).DO NOT touch the site once alcohol or other antiseptic has been applied.
9. Anchor the vein by holding the patient's arm and placing a thumb below the venipuncture site.
10. Enter the vein swiftly at a 30 degree angle.
11. Once sufficient blood has been collected, release the tourniquet before withdrawing the needle.
12. Withdraw the needle gently and then give the patient a clean gauze or dry cotton-wool ball to apply to the site with gentle pressure.
13. Discard the used needle and syringe or blood-sampling device into a puncture resistant container.
14. Check the label and forms for accuracy.
15. Discard sharps and broken glass into the sharps container. Place items that can drip blood or body fluids into the infectious waste bags.
16. Remove gloves and place them in the general waste. Perform hand hygiene using soap and water and dry hands with single-use towels (WHO, 2010)

APPENDIX THREE: SOP for Centrifugation
STANDARD OPERATION PROCEDURE FOR CENTRIFUGATION

- Inspect the centrifuge to ensure it is working properly, there is no damage, and that the centrifuge is able to move freely.
- Select the appropriate centrifuge tubes or containers; inspect them to make sure there are no cracks or flaws. Discard any tubes/containers that are cracked or flawed.
- Fill tubes with the desired liquid. Never overfill or under fill the tubes or containers (follow the manufacturer's limits).
- Make sure that the centrifuge tubes are balanced; use a balance to ensure that the weights of both tubes match. Do not balance based purely on volume! This is especially true for solutions with different samples or different concentrations of the same sample.
- Tightly secure the lids on the centrifuge tubes.
- Make sure that the outside of the centrifuge tubes are clean and dry before placing them in the centrifuge.
- Balance the tubes within the centrifuge. Tubes should be weighted to ensure that they balance properly.
- Close the lid and make sure it has a tight fit. Set the run speed and run time. (Never use the rotor's maximum run speed).
- Do not leave the centrifuge until it is operating at full speed and the machine seems to be running smoothly. Ensure that there is no abnormal noise or vibration.
- If you notice an unusual noise or vigorous shaking, immediately turn the centrifuge off and remove it from the power source.
- Typically, this is caused by the centrifuge not being balanced properly. If the problem does not resolve and the centrifuge is balanced properly, most likely the centrifuge is broken; do not use the centrifuge until it is fixed.
- Once the centrifuge has completed its run, allow the centrifuge to stop its rotation before opening the lid. Never open the lid or touch the centrifuge before it has stopped its rotation. NEVER TRY TO HASTEN THE STOPPING PROCESS! Stopping the machine prematurely can lead to injury and mechanical failure.
- Preferably do not open the lid for at least 10 minutes after the rotation has stopped to allow the aerosols that were released during centrifugation to settle.
- If centrifuging hazardous materials use the centrifuge in a fume hood.

- If centrifuging biosafety level 2 materials the centrifuge must be used in a biosafety cabinet or rotors must have aerosol containment.
- If centrifuging radioactive materials, use the appropriate shielding during this process.
- After the centrifuge has completely stopped moving, you may remove your samples from the centrifuge.

APPENDIX FOUR: SOP for P16ink4A ELISA

STANDARD OPERATING PROCEDURE FOR QUANTITATIVE DETERMINATION OF HUMAN P16ink4A CONCENTRATION IN SERUM

Requirements

- The ELISA kit
- Microplate reader with 450 nm wavelength filter
- High-precision transfer pipette, EP tubes and disposable pipette tips
- Incubator capable of maintaining 37°C
- Deionized or distilled water
- Absorbent paper
- Loading slot for Wash Buffer
- Gloves

t principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been re-coated with an antibody specific to Human **P16INK4A**. Samples (or Standards) and biotinylated etection antibody specific for Human **P16INK4A** are added to the micro ELISA plate wells. Human **16INK4A** would combine with the specific antibody. Then Avidin-Horseradish Peroxidase (HRP) njugate are added successively to each micro plate well and incubated. Free components are washed vay. The substrate solution is added to each well. Only those wells that contain Human **P16INK4A**, otinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-bstrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical nsity (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is portional to the concentration of Human **P16INK4A**. You can calculate the concentration of Human **5INK4A** in the samples by comparing the OD of the samples to the standard curve.

l

Add 100 µL of standard or sample to each well.

Incubate for 90 min at 37°C.

Remove the liquid.

dd 100 µL of Biotinylated Detection Ab.

ncubate for 1 hour at 37°C.

- Aspirate and wash 3 times.
- Add 100 µL of HRP Conjugate.
- Incubate for 30 min at 37°C.
- Aspirate and wash 5 times.
- Add 90 µL of Substrate Reagent.
- Incubate for 15 min at 37°C.
- Add 50 µL of Stop Solution.
- Read at 450 nm immediately.

alculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

APPENDIX FIVE: SOP for FOXP3 ELISA

STANDARD OPERATING PROCEDURE FOR QUANTITATIVE DETERMINATION OF HUMAN FOXP3 CONCENTRATION IN SERUM

Requirements

- The ELISA kit
- Microplate reader with 450 nm wavelength filter
- High-precision transfer pipette, EP tubes and disposable pipette tips
- Incubator capable of maintaining 37°C
- Deionized or distilled water
- Absorbent paper
- Loading slot for Wash Buffer
- Gloves

Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human **FOXP3**. Samples (or Standards) and biotinylated detection antibody specific for Human **FOXP3** are added to the micro ELISA plate wells. Human **FOXP3** would combine with the specific antibody. Then Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human **FOXP3**, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human **FOXP3**. You can calculate the concentration of Human **FOXP3** in the samples by comparing the OD of the samples to the standard curve.

Method

- Add 100 µL of standard or sample to each well.
- Incubate for 90 min at 37°C.
- Remove the liquid.
- Add 100 µL of Biotinylated Detection Ab.
- Incubate for 1 hour at 37°C.

- Aspirate and wash 3 times.
- Add 100 µL of HRP Conjugate.
- Incubate for 30 min at 37°C.
- Aspirate and wash 5 times.
- Add 90 µL of Substrate Reagent.
- Incubate for 15 min at 37°C.
- Add 50 µL of Stop Solution.
- Read at 450 nm immediately.

:ulation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

APPENDIX SIX: Informed consent form English version

MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY

RESEARCH ETHICS COMMITTEE

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E-mail: irc@must.ac.ug, mustirb@gmail.com

INFORMED CONSENT FORM

This document outlines the research study and expectations for potential participants. It should be written in laymanterms and typed on MUST-REC letterhead.

Instructions

1. The wording of this document should be directed to the potential participant not MUST-REC.
2. If a technical term must be used, then define it the first time it is used and any acronyms or abbreviationsused should be spelled out the first time they are used.
3. All the sections of this document must be completed without any editing or deletions.
4. Please use a typing font that is easily distinguishable from the questions of this form. Preferably the font sizeshould be 12.

Study title – This should be the same as on all other documents related to the study.

Association between foxp3 and p16ink4a serum expression with grades,treatment strategies and outcomes for cervical squamous intraepithelial lesions

Principal Investigator(s)

Mr Frank Ssedyabane

Introduction

What you should know about this study:

1. You are being asked to join a research study.
2. This consent form explains the research study and your part in the study.
3. Please read it carefully and take as much time as you need.
4. You are a volunteer. You can choose not to take part and if you join, you may quit at any time. There willbe no penalty if you decide to quit the study.

Brief background to the study

Cancer of the mouth of the uterus is a common but easily preventable form of cancer in women. Over 270 000 women die of it every year. About 7 of all women that die of this cancer in leveloping countries including Uganda.

lowever, these deaths can be prevented if cancer of the mouth of the uterus is detected in its early tages. Laboratory testing is key in detection of these early stages. With laboratory tests that are heap and easy to perform, this cancer can be detected earlier.

Uganda, testing for this cancer is done with methods, such as visual inspection with acetic acid /IA) and the Pap test, which are not accurate. Therefore, we need to look for new tests that may be ed as stand-alone tests or in combination with the older ones to enhance accuracy in testing for rvical cancer at an early stage.

is against such a background that some particles should be studied for their usefulness for tecting cancer of the mouth of the uterus and its early stages.

ose of the research project

ude a statement that the study involves research, estimated number of participants, an explanation ie purpose(s) of the research procedure and the expected duration of the subject's participation.

study seeks to provide evidence on the usage of blood amounts of these particles in testing for stagesof cancer of the mouth of the uterus. The Results of this study will guide decision making whether these tests should be used in testing for cancer of the mouth of the uterus either as stand- tests of in combination with older ones.

We plan to recruit atleast 272 participants, whom we intend to follow for a maximum of 12 months. At the start, as participants get tested for cancer of the mouth of the uterus, we shall take off a blood sample and measure these particles. The same particles will be measured at 12 months, for those that will have undergone treatment for early stages of this cancer.

Why you are being asked to participate?

Explain why you have selected the individual to participate in the study.

You have been chosen because you have come to this clinic to test for cancer of the mouth of the uterus, and could benefit if we measure these particles in your blood.

Procedures

Provide a description of the procedures to be followed and identification of any procedures that are experimental, clinical etc. If there is need for storage of biological (body) specimens, explain why, and include statement requesting for consent to store the specimens and state the duration of storage.

For this study, we are asking for your permission to collect 10 milli liters of your venous blood and also ask you a few questions. We may also review your patient chart/records in case some information cannot be provided by you. We are also asking for your permission to record your cancer of the mouth of the uterus testing results.

The collected blood sample is going to be used for measurement of these particles that we think are related to cervical cancer. These measurements are going to be done now, and repeated at 12 months, for those that will have undergone treatment for cancer of the mouth of the uterus or its early stages.

Because you undergo treatment for cancer of the mouth of the uterus or its early stages, the clinic nurse may request you to come back to the clinic for review earlier than 12 months. This will depend on the grade of cancer of the mouth of the uterus or the treatment you will have received. In such a situation, that will be a regular review and not for this study. For this study, we shall only require you to return after 12 months.

We need to compare results which we will get at the start with those at 12 months. We also plan to perform some other unforeseeable, but study-related, tests in the future. We therefore ask you to allow us to store these samples in our laboratory (MUST clinical and research laboratory).

Risks or discomforts

Describe any reasonably foreseeable risks or discomforts-physical, psychological, social, legal or other associated with the procedure, and include information about their likelihood and seriousness. Discuss the procedures for protecting against or minimizing any potential risks to the subject. Discuss the risks in relation to anticipated benefits to the subjects and to society.

There is no medical risk linked to the study. The only discomfort is the little pain you will feel while collecting venous blood specimen for the purpose of the study. The methods involved in testing for cancer of the mouth of the uterus are the ones routinely used in clinical practice and they come along with a discomfort of specimen collection. We will try our best to perform those tests as fast as possible such that the

discomfort is minimised. For those that will be asked to return to the clinic for follow up, you will be informed in advance such that you prepare yourself. We will also try our best to be fast during your review such that you spend little time at the clinic.

Benefits

Describe any benefits to the subject or other benefits that may reasonably be expected from the research. If the subject is not likely to benefit personally from the experimental protocol note this in the statement of benefits.

The main focus of this study is the amount of these blood particles and cancer of the mouth of the uterus or its early stages for which you will get results. So we are going to test your blood for amounts of each of them since they are thought to be related to cancer of the mouth of the uterus. We are also going to ensure that you get tested for cancer of the mouth of the uterus or its early stages. So your participation in the study will allow you to know your status concerning the mentioned conditions/diseases and to inform your doctor about their usage in diagnosis and treatment accordingly. In addition there will be a benefit for the entire population in that, if we show that the blood amounts of these molecules are related to cervical cancer early stages and their treatment outcomes, we shall recommend their usage at every cervical cancer clinic. You will know your cancer of the mouth of the uterus testing (VIA) results on spot. Other tests (Pap smear or histology) results will be ready in not more than a month's time after sample collection. Results on the new particles will be ready aftertwo months from the time of sample collection. All results will be given to you by your nurse a the clinic. In case your results return with abnormal levels, you will be linked to respective departments fo appropriate management. Also, in case you are found to have any cervical abnormality, you will be treated straight away at this clinic.

Incentives or rewards for participating

It is assumed that there are no costs to subjects enrolled in research protocols. Any payments to be made to th subject, e.g., travel expenses, token of appreciation for time spent, must also be stated, including when th payment will be made.

There will be no financial expenses for you to participate in this study and you will not be paid eithe However, when you return for your review at 12 months, you will be given a transport refundof ugx 10,000/=

Protecting data confidentiality

Provide a statement describing the extent, if any, to which confidentiality or records identifying the subjec will be maintained. If data is in form of tape recordings, photographs, movies or videotapes, researcher shou describe period of time they will be retained before destruction. Showing or playing of such data must disclosed, includinginstructional purposes.

Information about you and your health status that will be collected during the study will be confidential. V will not record your name on the study enrolment form but only a study number. Only research assistants a medical staff involved in the study will have access to the information you will give us and to your biologi results.

We shall store all research data that has been collected on questionnaires and consent forms. They cont vital information regarding the different variables in this research. In the future, we may need to come back

:m as requested by the relevant authorities. Documents will be stored in the histopathology unit of the partment of medical laboratory science for a maximum of ten years after which they will be burnt.

otecting subject privacy during data collection

scribe how the privacy of the participant will be ensured during the process of data collection.

uring the study, we will use identification codes to ensure that the data is anonymous. No names will be used the laboratory results records. In addition, Informed consent process will be made in a private room.

ght to refuse or withdraw

clude a statement that participation is voluntary and that refusal to participate will involve no penalty or s ofbenefits to which the subject is otherwise entitled.

is up to you to decide whether or not to take part in the study. If you decide to take part you willbe given s information sheet to read, understand, keep and be asked to sign a consent form. This will not affect the ndard of the care you would receive.

hat happens if you leave the study?

clude a statement that the subject may discontinue participation at any time without penalty or loss of nefits.

you decide not to participate in the study or to withdraw after signature, you will receive standard care fered in this hospital without any prejudice.

ho do I ask/call if I have questions or a problem?

clude contact for the researcher and Chairperson, MUST-REC.

In case you have any questions regarding conduct of the study, please address them to the chairman UST-REC. For questions regarding the technical details of the study, please contact the principal vestigator.

1. Associate Professor Paul
   Alere
   Chairman, MUST-REC
   P.O. Box 1410 Mbarara Tel: 0485433795

2. Mr. Frank Ssedyabane
   Principal Investigator
   P. O. Box 1410 Mbarara

   Tel: 0777031163

hat does your signature or thumbprint on this consent form mean?

Your signature on this form means

- You have been informed about this study's purpose, procedures, possible benefits and risks

- You have been given the chance to ask questions before you sign
- You have voluntarily agreed to be in this study

I have understood the objectives and purposes of this study, as well as the risks and benefits of participating in this study. I do understand that participation in this study is voluntary and that no consequences arise from my withdrawal from it. My signature below indicates that I have voluntarily accepted to participate in this study.

Name of adult participant	Signature/Thumbprint of participant/ Parent/Guardian/Next of Kin	Date
Name of person obtaining consent	Signature	Date
Print Name of witness	Signature or thumbprint or mark	Date

APPENDIX SEVEN: Informed consent form Runyankore version

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E-mail: irc@must.ac.ug, mustirb@gmail.com

EKIHANDIIKO KY'OKWIKIRIZA OMANYISIIBWE

Engyenderwaho

1. Ebigambo by'omukihandiiko eki bishemereire kwetegyeresesa omuntu orikubaasa kwejumbira omu mushom' ogu beitu ti kakiiko karikushwijuma eby'okucondooza.
2. Ekigambo ky'obukugu kyaba eky'okukozesibwa kishemereire kubanza kushoborirwa omurundi gw'okubanza ogu kirakozesibwe kandi n'ebihandiiko by'omubugufu bishemereire kubanza kuhandiikwa omu bwijwiire omurundi gw'okubanza gw'okukozesibwa.
3. Ebicweka byona ebiri omu kihandiiko eki bishemereire kwijuzibwa hatariho kuhindurwa nari kushazamu ekintu kyona.
4. Reeba ngu wakozesa obuhango bw'enyuguta oburi kutaan' ahari obu obukozeseibwe omukuhandiika ebibuuzo by'ekihandiiko eki.

Omutwe gw'omushomo

Akakwate akari ahagati y'entumwa z'obutafari bw'omubiri foxp3 na p16inka4a n'emitindo 'okweyoreka kw'obundi bucweka bwentumwa obutoreinwe omu shagama, entebekanisa 'okuragura n'ebirikuruga omu butafari bw'aha bironda bw'aha munwa gwa nyinenda.

Omucondooza Mukuru

Omw. Frank Ssedyabane

Okwanjura

Eky' oshemereire kumany' aha kucondooza oku

1. Noshabwa kwejumbira omu mushomo gw'okucondooza
2. Ekihandiiko ky'okwikiriza eki nikishoborira aha mushomo gw'okucondooza oku noku oragwejumbiremu.
3. Nyaburawe kishome n'obwegyendesereza kandi otware obwiire bwona obu orikwenda.
4. Ori nyekundiire. Nobaasa kusharamu obutagwejumbiramu, kandi waba wagutahiremu nobaasa kugurugamu ah'orikwendera. Tiharikwiija kubaho kibonerezo ky'okuruga omu kucondooza.

Ekirikugyemesereza okucondooza oku

Ekookoro y'aha munwa gwa nyinenda n'emwe aha kookoro ezirikukizayo kwita omu nsi yoona omu bakazi kwonka kandi erikukizayo kubaasa kwerindwa. Neyita abakazi abari kukira 270,000 buri mwaka kandi ahari abo, 85% nibaruga omu nsi ezirikugyezaho kutunguuka. Okukyebera kwa labu nikikuru omu bikwatireine n'okuragurira kandi n'okucendeeza akabi k'ekookoro y'aha munwa gwa nyinenda n'obutandikiro bwayo. Haaba hariho okukyebera kwa labu kurikumara, kuhikire, kandi kurikubasika, ekookoro ya nyinenda nebaasa kumanywa kare kandi eragurirwe gye. Omuri Uganda, okukyebera ekookoro y'aha munwa gwa nyinenda nikukorwa omu miringo ebiri, okusiiga omubazi aha munwa gwa nyinenda reero okareeba erangi y'omubazi oku yahinduka hamwe n'okwiiha amaizi/akashushu aha munwa gwa nyinenda gakakyeberwa kureeba haaba harimu obutafari bw' ekookoro. Obwesigye bukye bw'omuringo gw'okukyeber' amaizi ga nyinenda buretsire okucondoozibwa kw'endiijo miringo y'okukyebera ekookoro y'aha munwa gwa nyinenda. Emiringo egyo ebaasa kukozesibwa yonka nari erikukwatanisibwa n'endiijo miringo enkuru kwenda ngu ekookoro y'aha munwa gwa nyinenda yarahuka kumanywa juba. N'okugyemeserezibwa okwo, entumwa z'obutafari bw'omubiri ezimwe nka (FOXP3, P16inka4a, Cmyc, n'obundi bucweka bw'entumwa omu shagama obutoreinwe) zishemereire kucondoozibwa kureeba omugasho gwaazo omu kubaasa kukwata ekookoro y'aha munwa gwa nyinenda neekitandika.

Ekigyendererwa ky'okucondooza oku

Omushomo ogu nigwenda kumanyisa sayansi eriho aha kukozesa entumwa z'obutafari bw'omubiri (fox p16inka4a, cmyc, n'obundi bucweka bw'entumwa omu shagama obutoreinwe) omu kubaasa kureeba y omukazi ayine ekookoro y'aha munwa gwa nyinenda bukiri kare.

Ebirarugye omu kucondooza oku nibiija kuhabura omu kucwamu yaba kukozesa emiringo y'okukyebera yonka omu kukyebera ekookoro y'aha munwa gwa nyinenda nari kugikozesa hamwe n'endiijo miring butoosha. Nitwetebekanisa kutoorana abantu abarikuhika 272 kwejumbira omu mushom' ogu, abu turiky kukuratira kumar' ameezi 12. Aha kutandika kw'omushomo, abarikuza kugwetabiramu baaba nibakyeb ekookoro y'aha munwa gwa nyinenda, nitwiija kwihaho eshagama tupime obwingi bw' ezo ntu z'obutafari bw'omubiri. Entumwa z'obutafari bw'omubiri ezo nizo zimwe ezirikwiija kupimw' aha mu gw' ameezi 12, ahabw' abo abaraabe baraguriirwe ahabw'okugira obumanyiso bw'okubanza bw'ekoc y'aha munwa gwa nyinenda.

Ahabwenki noshabwa kwejumbira omu kucondooza oku?

Natoranw' ahabwokuba waija omu irwariro eri kukyebeza ekookoro y'aha munwa gwa nyinenda. nahabw'ekyo obaasa kuba ori omu kabi k'okurwaara ekookoro y'aha munwa gwa nyinenda ninga oburonda oburikwiija arugwamu ekookoro.

birikuza kukorwa:

ukushaba orusa kupima obureingwa bwawe n'oburemeezi bwawe kandi n'okukwihaho ec ikumi shagam' aha mukono. Kandi nitukushaba orusa kwiih'amaizi/akashushu aha munwa gwa nyinenda agakyebera kureeba gaba garimu obutafari bw'ekookoro. n'okusiiga omubazi aha munwa gwa nyinenda ureeba oku erangi y'omubazi erahindukye. Okusiiga omubazi n'okwiih' amaizi aha munwa gwa nyinenda niringo erikwiija kukozesibwa kukukyebera ekookoro y'aha munwa gwa nyinenda ninga kumany' ahi okoro yahika reero ekateebwa aha rureng' orweshemereire kuteebwaho.

gama ei turakwiheho neija kukozesibwa kupima obwingi bw'ebishaju omu shagama nebindi cworeka oku omubiri gwawe guri kukuuma shukari ekaguma omu buteeka. Okupima oku nikuza 'wa hati kagarukye kukorwe aha muheru gw'ameezi 12 ahabw'abo abraabe batungire okuragurirw' v'obumanyiso bw'okubanza bw'endwara y'ekookoro y'aha munwa gwa nyinenda.

taaga kugyeragyeranisa ebirarugye omu kukyeber' aha kutandika kw'omushomo n'ebirarugye omu ber' aha muheru gw'ameezi 12. Kandi nituteekateeka kwija kukyebera ebindi bintu ebitakamanyirwe hati kwonka ebiine akakwate n'omushom' ogu omu biro by'omumaisho. Nahabwekyo nitukushaba iriza kubiika eshagama yaawe omu lab yeitu erikukyebera eby'amagara n'okucondooza omu univasite rara.

nari okuguubwa kubi

kabi kari kumanywa kureetwa omu kwejumbira omu kucondooza oku. Okuguubwa kubi kwonka baasa kubaho n'obusaasi bukye obu orikuhurira baaba nibakwihaho eshagam' aha mukono. Ebindi kukukorwaho omu kukukyebera ekookoro y'aha munwa gwa nyinenda n'ebituura nibikorw' butoosha kandi buriijo habah' obutaguubwa gye baaba barikwihaho eky'okukyebera Ntwija we kugaruka omu irwariro kureeba ngu tiwaguubwa kubi kumara obwiire bureingwa. Abo teeka. Naitwe nitwiija kugyezaho nk'okuturikubaasa kukoresa obwiira twaba nitubakorah' zi mubaase kumara obwiire bukye omu irwariro.

Amagoba

Omushom' ogu ekirikukira obukuru ekigworekyereire n'obwingi bw'ebishaju omu shagama hamwe n'ezindi ntumwa z'obutafari bw'omubiri, kandi n'okukyebera ekookoro y'aha munwa gwa ninenda. ebi orikwiija kuheebwa ebirarugye omu kukyebera.

Nahabw' ekyo, nituza kukyebera eshagama yaawe kureeba obwingi bw'ebishaju ebirimu, kandi tukyebere n'oku omubiri gwaawe gurikukuuma shukari kandi tupime obureingwa bwaawe n'oburemeezi bwaawe ahabwokuba nibiteekateekwa kugir' akakwate n'ekookoro y'aha munwa gwa nyinenda n'ebirikuruga omu kugiragurira. Kandi nituza kukukyebera kureeba yaba oyine obumanyiso bw'okubanza bw'ekookoro y'aha munwa gwa nyinenda. Nahabwekyo, okwejumbira kwawe omu mushom' ogu nikwiija kukureetera kumanya oku oyemereire omu kikwatireine n'ebi twakugambira/n'endwara kandi n'okugambira omushaho waawe oku yakubaasa kukozesa ebyaruga omu kukyebera kumanya eky'orweire kandi akakuragurira nk'oku kishemereire. Kandi okwongyer' ahari ebyo, nihaija kubaho okugasirwa omu bantu boona twaheza kworek' akakwate akari aha bwingi bw'entumwa z'obutafari bw'omubiri omu shagama n'okugira obumanyiso bw'okubanza bw'ekookoro y'aha munwa gwa nyinenda kandi n'ebirarugye omu kuragurirwa. nitwiija kuhamisiriza okukoresibwa kwabwo omu marwariro g'ekookoro y'aha munwa gwa nyinenda. Noija kumanyiraho ebirarugye omu kukyeberwa kw'ekookoro y'aha munwa gwa nyinenda twaba tukoreise omuring' ogw'okusiiga omubazi aha munwa gwa nyinenda reero tukareeba empinduka erikubaho aha rangi y'omubazi ogwo. Endiijo miringo y'okukyebera ekookoro y'aha munwa gwa nyinenda nk'okwiih'amaizi/akashushu aha munwa gwa nyinenda tukagakyebera, n'okupima obwingi bw'ebishaju omu shagama, nikiija kutwara esande nk'emwe kumanya ebyarugamu. Ebirarugye omu kukyebera oku omubiri guri kurinda obwingi bwa shukari omu shagama nibiija kutungwa hahwah' okwezi kumwe. Ebirarugye omu kukyeberwa kwona noija kubiheebwa nansi waawe aha irwariro. Ebirarugye omu kukyebera obwingi bw'ebishaju na shukari kubyakuba bitashoborokire, noija kukwatanisibwa n'abashaho abashemereire kukuragurira. Kandi nabwo kuwashangwa oyine oburweire bwon' aha munwa gwa nyinenda, noija kuragurirwa aho naho aha irwariro ry'ekookoro y'aha munwa gwa nyinenda.

Ebihembo ahabw'okwejumbira omu mushomo

Torikwiija kuhebwa sente zoon' ahabw'okwejumbira omu mushom' ogu. Kwonka ku orahindukye aha muhe gw'ameezi 12, noija kuheebwa shs 20,000 za Uganda z'okukugarurayo.

Okurinda ebihama

birikukwata aha magara gaawe ebi abashaho barakwiheho omu mushomo nibiija kurindwa nk'ebihama iturikwiija kuhandiika iziina ryawe aha kihandiiko kyona kureka enamba yaawe y'omushomo. Abashaho umwe nabacondooza bonka nibo barikwiija kumanya ebirikukukwataho ebi oratugambire hamwe nebirarugye nu kukyebera.

kurinda ebihama by' abarikucondoozibwaho baaba nibabuuzibwa

mushomo gwaba nigugyenda omu maisho, nitwiija kukoresa obumanyis' oburikwiija kureetera irikukukwataho byabura kumanywa buri omwe. Tihariho maziina goon' agarateebwe aha kihandiiko kya). Kandi okwongyer' ahari ebyo, ekihandiiko ky'okwikiriza omanyisiibwe nikiija kwijuzibwa omu mwanya vehereire.

)ugabe bw'okwanga nari okurugamu

ri ahariiwe kusharamu kwejumbira nari kubura kwejumbira omu mushomo. Ku oraikirize kwejumbira omu condooza oku noija kushabwa kuta omukono gwawe aha kihandiiko eki. Ekyo tikirikwiija kuhindur' aha itindo gw'obuhereza obu oyine kutunga.

habaho ki waruga omu kucondooza?

orasharemu obutejumbira omu kucondooza oku nari washaramu kukurugamu nobu wakuba watetreh ukono gwawe, noija kutunga obuhereza obu kushemereire omu irwariro eri hatariho kusosorwamu kwona.

nbuuza oha nari ninterera oha kunakuba nyin' ekibuuzo nari ekizibu?

1. Associate Professor Paul Alere
   Mukuru w'entebe y'eby'okucondooza omuri Mbarara Univasite.
   P.O. Box 1410 Mbarara
   Tel: 0485433795

2. Mr. Frank Ssedyabane
   Omucondooza Mukuru
   P. O. Box 1410 Mbarara
   Tel: 0777031163

ukono gwaawe nari ekinkumu kyaawe aha kihandiiko ky'okwikiriza eki nikimanyisa ki?

- Wamanyisibwa ekigyendererwa ky'okucondooza oku, ebirikuza kukorwa, ebi wakugobamu kandi n'ebihikirizi
- Waheebwa omugisha gw'okubuuza ebibuuzo otakateireho mukono
- Wayekundira kwejumbira omu kucondooza oku

Nayetegyereza ekigyendererwa ky'omushom' ogu hamwe n'akabi n'ebihembo ebyakuba biri omu kukwejumbiramu. Ninyetegyereza ngu okwejumbira omu mushom' ogu n'okwekundira kwangye kandi ku hatariho kirikwiija kumbaho nasharamu kugurugamu. Omukono gwangye nigworeka ngu nasharamu kwejumbira omu mushom' ogu nyekundiire.

Amaziina g'orikwejumbiramu omukuru	Omukono gw'orikwejumbiramu nari/ Ori kumwemererera oheirwe obushoborozi	Ebiro
Amaziina g'orikubuuza	Omukono	Ebiro
Amaziina ga kareebi	Omukono/ Ekinkumu	Ebiro

APPENDIX EIGHT: Covid-19 and EVD risk management

1) Purpose

- The purpose of this plan is to protect the health and safety of the research team, research participants and the entire community at the research site from COVID-19 and Ebola virus disease.

2) Scope

- This plan will apply to everyone on site including the principal investigator, . research assistants, gynaecologists and all the participants. A risk assessment has been completed to identify COVID-19 and Ebola virus disease hazards and inform the actions in this plan.

3) Responsibilities

- This plan has been developed by the Researcher and will be implemented by Researcher and the entire research team. The researcher is responsible for on-site detection temperature monitoring, ensuring proper ventilation disinfection, distribution of personal protective equipment, communication and training where necessary. Anyone on the site must comply with the requirements of this plan at all times. Any breaches of the requirements of this plan must be reported immediately to the Researcher.

4) Recruitment of participants

- This will be done one at a time or separate stations that are far apart to avoid congregating of people and help manage physical distancing, which is all about keeping a safe distance from others. This will be 2 meters.
- The research team will conduct body temperature checks and any one with COVID-19 and Ebola virus disease signs and symptoms will be denied access.
- Access to the research site will be granted to those wearing face masks.

5) Managing physical interaction

- Congregating will be discouraged at all costs. Once a participant has received all the care that they came for and have been recruited and samples collected, they will be advised to depart from the premises. If involving more than one team member, we shall ensure physical separation/distancing.
- All procedures will be properly planned to minimize contact between participants.
- Meetings will be limited to "when necessary" and will be held in a well-ventilated area, with physical separation of all those present.

- In situations where it will not be possible or safe for participant and research team to distance themselves from each other by 2 meters, face masks and appropriate Personal Protective Equipment (mask, face shield and gowns) shall be worn.
- Visitors (those neither part of the research team nor participants) and customers will not be allowed in the research site.

Sample collection.

- Participants shall avoid contact with others as they await their turn for sample collection procedures. they will practice physical distancing (at least 2 meters).
- Sample collection area and waiting area shall be disinfected at the beginning of the day and at the end.
- Where possible, the principle of "one person- one equipment" will be adopted.
- For equipment operated by more than one person, surfaces or components with high contact frequency, such as pens, surfaces and handles, will be disinfected regularly.
- The team will avoid of sharing tools/equipment. If required, tools/equipment will to be cleaned thoroughly between shifts and before being used.

Workplace ventilation, cleaning and disinfection

- The work environment will be kept clean and tidy, and disinfected regularly.
- The buildings need to be kept well ventilated with fresh air where possible.
- Air conditioning units if present, will be regularly cleaned, and the air conditioning return air filter checked and replaced regularly.

Personal health and hygiene

- All the team members and participants shall be required to follow the Personal Health Guidelines (coughing with mouth covered, sneezing using a clean cloth and proper disposal of used tissue paper).
- Everyone at the research site shall regularly sanitize their hands with clean soapy water or hand sanitizer before entry onto site.
- Any personal items brought to site by team members shall be segregated (kept separate from other member items).
- Any work-specific PPE, other than base clothing should be kept at site and donned on site– individual PPE for team member must be kept separate from other member PPE.
- No sharing of dishes, drinking utensils or cutlery.
- All eating and drinking utensils shall be cleaned by the user.
- Face masks shall be worn all the time

- All staff and the study team shall be required to be vaccinated.

9) Training

- All the participants and research team will be trained in this COVID-19 and Ebola virus disease Management Plan.
- Site inductions will include this Management Plan.

10) Review and inspections

- Regular research site inspections will be conducted to ensure this plan is being followed and that it is effective.
- This plan will be reviewed weekly in line with Ministry of Health guidelines on the spread of COVID-19 and Ebola virus disease.

Covid-19 and Ebola virus disease mitigation plan

1. Physical distance shall be maintained, at least 2 meters to the greatest extent possible. The number of persons in a research space at one time will be limited to that which permits physical distancing of at least 2 meters between individuals.
2. Face coverings will be REQUIRED. Choice of face covering will be appropriate for the study procedures. Cloth face coverings will be appropriate for some interactions; face masks or face shields will be necessary in others.
3. There will be frequent hand-washing/sanitizing. Research staff shall wash their hands before interacting with research participants. If this will not be possible, hand sanitizer shall be used.
4. Signage will be posted to promote hand washing.
5. Hand sanitizers will be readily available and visible in the research space.
6. Surfaces/touch points, equipment, supplies, and materials will be Cleaned and disinfected prior to and between each participant visit.
7. A visitor's log documenting individuals who enter the research space will be kept to facilitate contact tracing, should it be needed. Researchers will use this template. Information will be used ONLY to facilitate contact tracing. Visitor logs will be securely destroyed after 60 days if they will not be used.

PPENDIX NINE: Ethical clearance letters

MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
02 NOV 2023
APPROVED
VALID UNTIL DATE SHOWN ABOVE

02/11/2022

To: SSEDYABANE FRANK

+256777031163

Type: Initial Review

Re: MUST-2022-612: ASSOCIATION OF FOXP3, P16INK4A, CMYC AND SELECTED MICRO RNA SERUM EXPRESSION WITH GRADES, TREATMENT STRATEGIES AND OUTCOMES FOR CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS , VERSION THREE, 2022-10-31

I am pleased to inform you that at the **148th** convened meeting on **05/10/2022**, the MUST Research Ethics Committee, committee meeting, etc voted to approve the above referenced application.
Approval of the research is for the period of **02/11/2022** to **02/11/2023.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **02/11/2023** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents approved in this application by MUST Research Ethics Committee:

No.	Document Title	Language	Version Number	Version Date
1	TRANSLATED CONSENT FORM FOR STORAGE OF MATERIALS	RUNYANKORE	VERSION TWO	2022-11-10
2	EDITED CONSENT FORM FOR STORAGE OF MATERIALS	ENGLISH	VERSION TWO	2022-10-31
3	Protocol	English	VERSION THREE	2022-10-31
4	Informed Consent forms	ENGLISH	VERSION TWO	2022-10-29
5	Informed Consent forms	RUNYANKORE	VERSION ONE	2022-09-09
6	Data collection tools	RUNYANKORE	VERSION ONE	2022-09-09
7	Data collection tools	ENGLISH	VERSION ONE	2022-09-09

Yours Sincerely

Assoc. Prof. Paul Alele
For: MUST Research Ethics Committee

Telegrams
Telephones

Dirs' Office: 048521806
Others : 048520007/21086/20024
Dirs mobile: 0772 415875
In any correspondence on
This subject please quote ref: No.....

THE REPUBLIC OF UGANDA

Ministry of Health
MBARARA REGIONAL REFERRAL HOSPITAL
P.O. Box 40,
Mbarara - Uganda

February 10, 2022

Mr. Ssedyabane Frank
2021/PHD/045/PS
Mbarara University of Science and Technology

Re: Administrative Clearance of Protocol on "Association of FOXP3, P16NK4A, CMYC and selected Micro RNA serum expression with grades, treatment strategies and outcomes for Cervical Squamous Intraepithelial Lesions".

Mbarara Regional Referral Hospital has received and reviewed your proposal referenced above and hereby grants approval to conduct this study. This clearance covers the proposal and the accompanying documents listed below:

- Approval from MUST Research Ethics Committee (In English only)
- Appendix 1: Questionnaire (In English only)
- Appendix 2: Venipuncture (In English only)
- Appendix 3: Centrifugation (In English only)
- Appendix 4: SOP for CMYC Elisa (In English only)
- Appendix 5: SOP for P161NK4A Elisa (In English only)
- Appendix 6: SOP for FOXP3 Elisa (In English only)
- Appendix 7: SOP for Total MRNA Extraction (In English only)
- Appendix 8: SOP for Micro RNA Quantification (In English only)
- Appendix 9: Consent Form (In English and Runyankore)
- Appendix 10: Blood Specimen handling for miRNA Analysis (In English only)
- Appendix 13: Covid-19 and EVD Risk Managemenly (In English only)

This approval is subjected to the following conditions:

1. That you will abide by the regulations governing research in the country as set by the Uganda National Council for Science and Technology.
2. That any changes to the protocol and study documents that you may find necessary to make will be reviewed and approved by relevant authorities.
3. That the conduct of your study shall be monitored by the Mbarara Regional Referral Hospital supervisors in the relevant departments/units.
4. That you provide a copy of the final document upon completion of the study, including a summary of the results and any publications to Mbarara Regional Referral Hospital.
5. That you will include Mbarara Regional Referral Hospital in your acknowledgements in all your publications.

My best wishes to you.

13 FEB 2023

Dr. Barigye Celestine
HOSPITAL DIRECTOR

Uganda National Council for Science and Technology

Our Ref: HS2722ES

26 April 2023

SSEDYABANE FRANK
MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
Mbarara

Re: Research Approval: **ASSOCIATION OF FOXP3, P16INK4A, CMYC AND SELECTED MICRO RNA SERUM EXPRESSION WITH GRADES, TREATMENT STRATEGIES AND OUTCOMES FOR CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS**

I am pleased to inform you that on **26/04/2023**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **26/04/2023** to **26/04/2025**

Your research registration number with the UNCST is **HS2722ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project. As the Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. Keeping all co-investigators informed of the status of the research
2. Submitting all changes, amendments, and addenda to the research protocol or the consent form (where applicable) to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the implementation of the changes. UNCST must be notified of the approved changes within five working days
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review and copies to the National Drug Authority and a notification to the UNCST
4. Unanticipated problems involving risks to research participants or others must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST notification after review by the REC
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

Please note that this approval includes all study related tools submitted as part of the application as shown below

No.	Document Title	Language	Version Number	Version Date
1	CONSENT FOR STORAGE OF SPECIMENS	ENGLISH	TWO	[illegible]
2	TRANSLATED CONSENT FOR STORAGE OF SPECIMENS	RUNYANKORE	TWO	[illegible]
3	Project Proposal	English	THREE	
4	Approval Letter	English		
5	Administrative Clearance	English		
5	DATA COLLECTION FORM RUNYANKORE	RUNYANKORE	ONE	[illegible]
6	English consent form	ENGLISH	TWO	[illegible]

Yours sincerely,

Dr. Christopher Ddamulira
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
P.O. Box 6884
KAMPALA, UGANDA

COMMUNICATION

TEL: (256) 414 705500
FAX: (256) 414 234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
14 DEC 2024
APPROVED
VALID UNTIL DATE SHOWN ABOVE

To: SSEDYABANE FRANK

14/12/2023

+256777031163
Type: Protocol Amendment

Re: ASSOCIATION OF FOXP3, P16INK4A, CMYC AND SELECTED MICRO RNA SERUM EXPRESSION WITH GRADES, TREATMENT STRATEGIES AND OUTCOMES FOR CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS

I am pleased to inform you that at the convened meeting on **28/11/2023**, the **MUST Research Ethics Committee** voted to approve the changes to the study titled **ASSOCIATION OF FOXP3, P16INK4A, CMYC AND SELECTED MICRO RNA SERUM EXPRESSION WITH GRADES, TREATMENT STRATEGIES AND OUTCOMES FOR CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS** reference Number **MUST-2022-612** .

Please note that the approval of the research is valid until **14/12/2024**. The approved changes to the study include;

To remove c-MYC and microRNAs from the list of biomarkers

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC eight weeks prior to the expiration date of **14/12/2024** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to notify the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents reviewed in this application by **MUST Research Ethics Committee**:

No.	Document Title	Language	Version Number	Version Date

Yours Sincerely

Dr. Paul Alele
For: MUST Research Ethics Committee



To: SSEDYABANE FRANK

08/12/2023

+256777031163

Type: Annual Renewal

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
06 DEC 2024
APPROVED
VALID UNTIL DATE SHOWN ABOVE

Re: ASSOCIATION OF FOXP3, P16INK4A, CMYC AND SELECTED MICRO RNA SERUM EXPRESSION WITH GRADES, TREATMENT STRATEGIES AND OUTCOMES FOR CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS

I am pleased to inform you that at the **160** convened meeting on **01/01/1970**, the MUST Research Ethics **Committee** reviewed the progress report to the above study reference number **MUST-2022-612** and found it satisfactory. In this respect, annual renewal of the study is granted. The study was initially approved on **02/11/2022** and expired on **02/11/2023**.

The Approval of the research is for the period of **08/12/2023** to **08/12/2024.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC eight weeks prior to the expiration date of **08/12/2024** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to notify the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.
8. **All approved study documents should be stamped by the REC before commencement of data collection.**

The following is the list of all documents reviewed in this application by MUST Research Ethics Committee

No.	Document Title	Version	Date

Yours Sincerely

Dr. Paul Alele
For: MUST Research Ethics Committee



REFERENCES

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Research Report

Association between serum P16ink4A concentration and CIN and cervical cancer among women attending a cervical cancer clinic in western Uganda: A case control study

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ABSTRACT

Introduction: Tissue expression of P16ink4A is correlated with cervical lesions. In this study we determined the association between serum P16ink4A concentrations and cervical lesions among women attending the cervical cancer clinic at Mbarara Regional Hospital (MRRH) South Western Uganda.

Material and Methods: We recruited 90 cervical intraepithelial neoplasia (CIN) cases, 90 cervical cancer (CC) cases before treatment and 90 controls. Clinical and demographic data were recorded. Serum P16ink4A concentrations were measured by quantitative Elisa. Cases were confirmed with cytology and/or histology. Descriptive statistics and logistic regression were done with STATA 17 and P-values of <0.05 were considered statistically significant.

Results: The mean serum P16ink4A concentration among CIN cases, CC cases and controls was 1.11(−0.66) ng/ml, 1.45(+/−1.11) ng/ml and 1.13(+/−0.61) ng/ml respectively (p 0.008). 50 % of CIN cases and controls as well as 60 % of CC cases had P16ink4A concentration above 0.946 ng/ml. There were increased odds of CIN for serum P16ink4A though statistically insignificant (AOR: 1.11, p-value: 0.70) There was also a statistically significant reduction in odds of CC for serum P16ink4A (AOR: 0.55, p-value: 0.01).

Conclusion: Serum P16ink4A may likely be associated with cervical lesions especially CC in our study population and this may aid detection of such lesions. Diagnostic utility studies for circulating P16ink4A in detection of cervical cancer are recommended.

1. Introduction

There are over 770,828 estimated incident cases of cervical cancer worldwide (Sung, 2021) making it the second most common cancer among women aged 15 to 44 years (Sung, 2021; Arbyn, 2020; Bruni, 2019). It accounts for more than 270 000 deaths annually, mostly in developing countries (WHO, 2020), particularly in sub-Saharan Africa. Across East Africa, incident cases of cervical cancer have been reported at 43/100,000 ( ). The reported age-standardized cervical cancer incidence in Uganda is higher than the global average of 56.2 per 100,000 women ( ). Early diagnosis is key to cervical cancer elimination (2021).

Many diagnostic modalities have been developed in relation to cervical cancer with the most popular being the cytological staining technique developed by G. Papanicolaou. In addition, population wide

Abbreviations: AOR, Adjusted Odds Ratio; COR, Crude Odds Ratio; CI, Confidence Interval; CIN, Cervical Intraepithelial neoplasia; CC, Cervical Cancer; HIV, Human Immunodeficiency Virus; HPV, Human Papilloma Virus; HrHPV, High Risk Human Papilloma Virus; LEEP, Loop Electrosurgical Excision Procedure; OR, Odds Ratio; PAP, Papanicolaou; SD, Standard Deviation; STATA, Statistical Software for Data Science; VIA, Visual Inspection with acetic acid.

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eening programs have promoted early detection of preneoplastic le-
ns among asymptomatic patients, and thus reducing the incidence
l mortality of cervical cancer in developed countries. In spite of the
cess of these programs, cervical cancer cases still occur, even in the
eloped countries, and also among screened women (Abdia, 2021).
erefore, the Pap test is hampered by a number of factors thus being
ne to false negative results, even in laboratories with highly effective
l rigorous control procedures (Abdia, 2021). In addition to that, the
iance of normal epithelia or benign inflammatory conditions like
erve cell hyperplasia is capable of producing false positive results,
ich leads to repeated cytological testing, unnecessary treatments for
ients and other related tremendous costs. Furthermore, cytology is
o affected by a substantial rate of interobserver discrepancies even
ong a panel of expert pathologists. These factors could be some of the
ses of low cervical cancer screening rates in Uganda (Nakisige, Schiffman, & Maria, 22; Isabirye et al., 2020; Wanyenze, 2022). This therefore calls for re sensitive and specific biomarkers for identification of dysplastic ls (Sankaranarayanan, 2014). These biomarkers could be user ndly and less costly tests for screening and diagnosis of cervical ncer especially in low resource settings.

Biomarkers including P16ink4A have been used severally, though in munohistochemical studies. A number of studies have suggested that 6ink4A may be used for the identification of abnormal cells in cyto-ic cervical specimens. For instance, Yang et al (Yang, 2016) innova-ely demonstrated the importance of P16ink4A in detecting cervical ions using a piezoelectric immunosensor in liquid based preparations. 6ink4A is a cyclin dependent kinase inhibitor whose over expression closely associated with high risk HPV infection and Cervical Intra-thelial Neoplasia (CIN) (Pientong, 2004). This therefore suggests that 6ink4A could be used in cervical cancer screening and this can reduce ambiguity from other diagnostic techniques (Pientong, 2004; Anu-eva, 2022; Chuerduangphui, 2018). It is hypothesized that, the tgrowth of dysplastic lesions is enhanced by increasing expression of al oncogenes E6 and E7 whose interaction with the various cell cycle gulating proteins like the retinoblastoma gene product, inhibit tran-iption of P16ink4A. This creates a direct proportionality, where by, rease in expression of viral oncogenes in dysplastic cervical cells re-cts an increased expression of P16ink4A. A significant linear rela-nship is thought to exist between lesion grade and intensity of 6ink4A immunohistochemical staining, ($p = 0.0001$) with the pression of P16ink4A reported to have a 100 % specificity for CIN and .5 % sensitivity in detecting HR- HPV. Although P16ink4A can be jectively quantified and measured in blood using methods that can be ndardised, this has never been demonstrated in serum samples. This dy therefore aimed at describing the association between serum 6ink4A concentration and cervical lesions among women attending cervical cancer clinic of Mbarara Regional Referral Hospital, South stern Uganda.

Materials and methods

1. Study design

We conducted an unmatched case control study that purposively mpled all women seeking cervical cancer care at the cervical cancer nic of Mbarara Regional Referral Hospital (MRRH) between April 22 and June 2023. Cases were defined as women with a confirmed agnosis of CIN or cervical cancer prior to treatment and controls were those negative for intraepithelial lesions or malignancy. Our outcomes of interest were cervical intraepithelial lesions/cervical cancer ile the exposure was serum concentration of P16ink4A.

2. Study setting

MRRH, is a tertiary hospital located in rural south western Uganda, ose catchment area is approximately four million people (Uganda Ministry of Health, 2016) across 13 districts of south western Uganda plus neighbouring countries including Rwanda, Burundi, Tanzania and the Democratic Republic of Congo. The clinic operates five days a week and receives an average of 15 women per day. Staff at the clinic include several nursing officers, senior residents and gynaecologists, who are headed by a gynaecologic oncologist. Visual inspection with acetic acid, colposcopy, conventional cytology and HPV DNA (for HIV positive women) are the screening tests routinely done at the clinic while confirmation of cervical lesions is achieved on histology ( ). Women with pre-malignant lesions are treated with cryotherapy and thermocoagulation; those with confirmed cervical cancer either undergo gynecologic cancer surgery or are referred to Uganda Cancer Institute for radiotherapy and chemotherapy, according to their cancer stage and clinical findings.

2.3. Sampling method

Cases were selected through purposive sampling and controls enlisted based on the incidence density sampling method for each identified case, a corresponding control was recruited in real time. This prospective process continued until we achieved the necessary sample size.

2.4. Sample size determination

This sample size was calculated using an online software, OpenEpi, Version 3, Open source calculator-SSCC. OpenEpi - Sample Size for Unmatched Case-Control Studies. We considered a two-sided confidence level (1-alpha) of 95 %, a study power of 80 %, a case to control ratio of 1, an average proportion of cervical cancer or cervical intraepithelial neoplasia cases with P16ink4A expression of 30.0 and a proportion of controls with P16ink4A expression of 10. We also used the least extreme odds ratio of 3.86. Considering the module of Fleiss with continuity correction, the calculated sample size came to 72 participants in each of the case and control groups. Factoring in an expected 25 % attrition rate, this gave a total of 270 participants (90 CIN cases, 90 CC cases and 90 unmatched controls).

2.5. Informed consent statement

We sought written informed consent from every participant before taking part in the study. We also used study numbers, not names, on all data collection tools as well as on serum specimens. During data analysis, we delinked all participants identifiable information. All participant interaction with research assistants including consenting and specimen collection took place in a private and comfortable side room in the clinic, free from disturbances; and only accessible to one participant at a time.

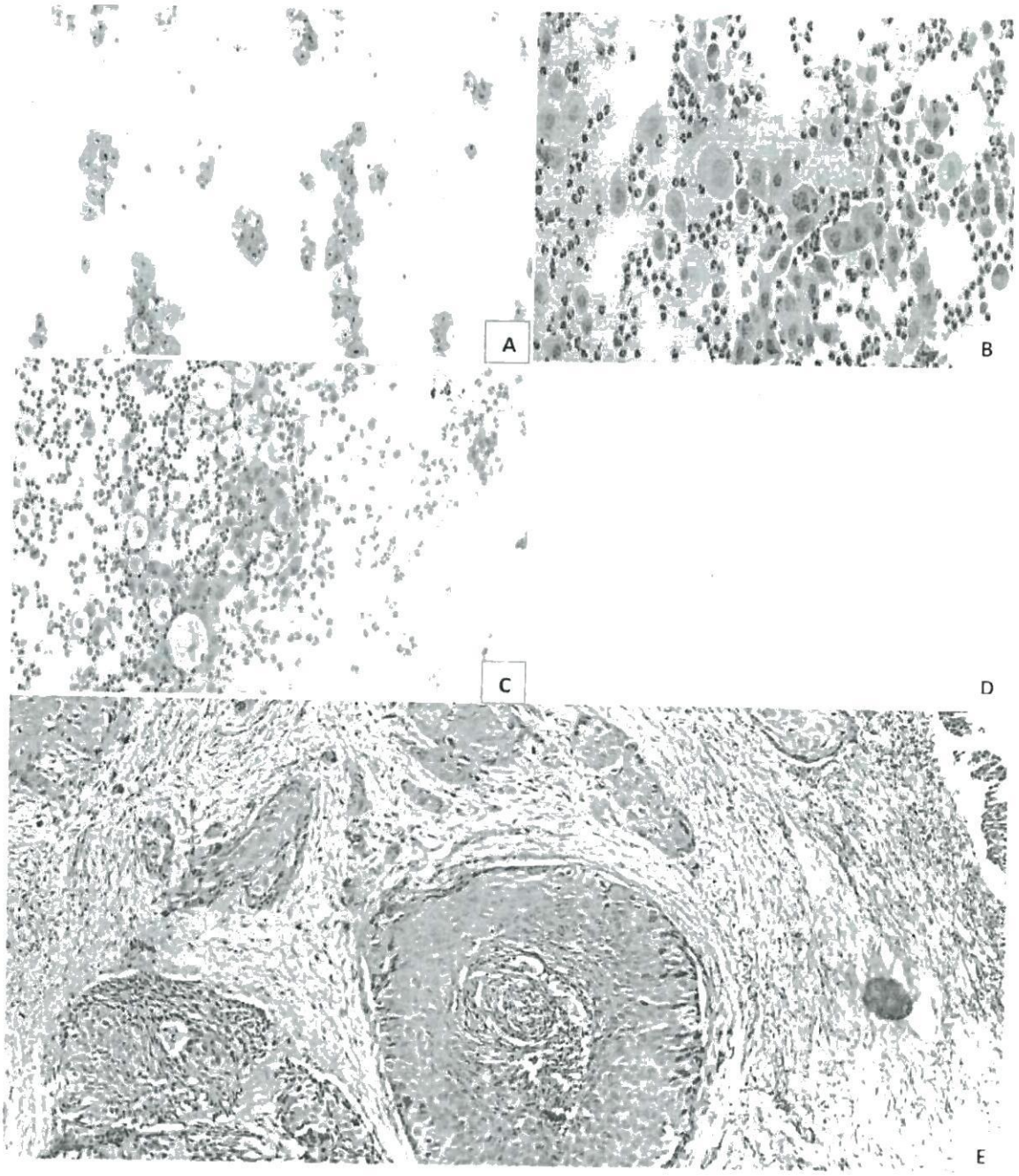
2.6. Institutional review board statement

For this study we secured ethical approval from the Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) (MUST-2022–612). Our study was also registered by the Uganda National Council for Science and Technology (UNCST) (HS2722ES). We also sought administrative clearance from the Hospital Director, Mbarara Regional Referral Hospital, before commencing the study. All women diagnosed with cervical lesions received the standard package of care, following national guidelines at the cervical cancer clinic.

2.7. Data collection

2.7.1. Demographic data collection

Demographic data were gathered using a previously validated questionnaire, administered by a proficient research assistant who was a qualified midwife stationed at the cervical cancer clinic. The data



1. Photomicrographs. A-Pap smear negative of intraepithelial lesion or malignancy ×400; B- Pap Smear Atypical squamous cells of undetermined significance ×400; C- Pap Smear Low grade squamous intraepithelial lesion ×400; D- Benign cervical tissue (Haematoxylin and Eosin) ×400. E- Squamous cell carcinoma (Haematoxylin and Eosin) ×400. : A box plot showing serum concentration of P16ink4A across different groups of study participants. CC- Cervical Cancer, CIN- Cervical Intraepithelial Neoplasia. : A-Pap smear negative of intraepithelial lesion or malignancy ×400; B- Pap Smear Atypical squamous cells of undetermined significance ×400; C- Pap Smear Low grade squamous intraepithelial lesion ×400; D- Benign cervical tissue (Haematoxylin and Eosin) ×400; E- Squamous cell carcinoma (Haematoxylin and Eosin) ×400.

ompassed key variables such as age, residential region, family ining practices and methods, HIV status, educational attainment, ital status, history of blood pressure, and history of diabetes. owing standard care procedures and upon obtaining written rmed consent, each participant was guided by the research assistants ompleting the questionnaire.

2.7.2. Blood collection

Four millilitres (4 mL) of venous blood were aseptically drawn from the mid-cubital vein by venepuncture and collected into plain-vacutainers. Each specimen was meticulously labelled with a unique identification number (code) and left to clot at room temperature for two hours. Subsequently the specimens were transported to the laboratory, where centrifugation was performed at 1000 × g for 15 min at 2 – 8°C to separate serum from blood cells. The resultant serum was then

arefully transferred into cryovial tubes using a micropipette. All serum amples were stored at −80°C until analysis.

.7.3. Measurement and interpretation of serum P16INK4A concentration

P16ink4A was measured using Human CDKN2A (Cyclin Dependent inase Inhibitor 2A) ELISA Kit, Elabscience Biotechnology Inc. This kit is esigned for in vitro quantitative determination of Human CDKN2A oncentrations in serum, plasma and other biological fluids with a nsitivity of 0.38 ng/mL and a detection range of 0.63–40 ng/mL. ctual measurements using this ELISA kit were based on the Sandwich-LISA principle. The micro ELISA plates were pre-coated with an anti-ody specific to Human CDKN2A. Standards or samples were added to e micro ELISA plate wells and combined with the specific antibody. nen a biotinylated detection antibody specific for Human CDKN2A and vidin-Horseradish Peroxidase (HRP) conjugate were added succes-vely to each micro plate well and incubated. Free components were ashed away. The substrate solution was added to each well. Only those ells that contained Human CDKN2A, biotinylated detection antibody d Avidin-HRP conjugate appeared blue in colour. The enzyme-bstrate reaction was terminated by the addition of stop solution and e colour turned yellow. The optical density (OD) was measured using a croplate reader at a wavelength of 450 nm ± 2 nm. The OD value was oportional to the concentration of Human CDKN2A. Prior to running, samples were diluted 1:3 and this was considered while making the al calculations for concentration. We ran samples along with refer-e standards. We created two categories of P16ink4A concentrations calculated using the cutpt by Phil Clayton which determines a cut-off nt (0.946) that maximises sensitivity and specificity. This method ls cutpoint on the ROC curve closest to the point with perfect sensi-ty and specificity. The first category was named low P16ink4A centration (≤0.946 ng/ml) while the second category was named ed concentration (0.946 < ng/ml).

4. Data management and analysis

Data was collected by the Principal Investigator together with arch assistants into an excel spread sheet (Microsoft Office Profes-al Plus 2013, version 15.0.4675.1003, Microsoft Inc, USA) and then orted into STATA 17 software (StataCorp LLC, College Station, as, United States). Descriptive statistics were used to describe the ulations using frequencies, means ± standard deviations (SDs) or ian values for continuous variables as well as frequencies and pro-ions to describe categorical variables. We used bivariate and ivariate logistic regression analysis to derive associations between n P16INK4A and cervical lesions. Multivariate logistic regression ysis was done after controlling for age, family planning usage and . HIV status, marital status, history of blood pressure, history of etes and smoking status. Associations are presented as odds ratios 95 % confidence intervals and a P-value of < 0.05 was considered tically significant.

. Eligibility criteria

ll women aged 21 years and above, who presented at the MRRH cal cancer clinic during the study period, and provided written ned consent for participation in the study were included. We ded all those women that were too ill and those currently under-treatment for cervical lesions.

esults

Population characteristics

otal of 270 participants were included in the study out of whom 90 positive for cervical cancer (CC), 90 positive for cervical intra-lial neoplasia (CIN) and 90 negative for any cervical lesion (con-The mean age of CC cases was significantly (p < 0.001) higher -/-13.1) than that of CIN cases (34.9+/-7.8) and that of controls (38.6+/-8.7). Majority of participants, 23 %(21/90) of CC cases, 32 %(29/90) of CIN cases and 43 %(39/90) of controls, belonged to the 40–49 age bracket. This difference in distribution was also statistically significant (p < 0.001). More than half of study participants in each group, i.e. 57 %(51/90) of CC cases, 60 %(54/90) of CIN cases and 56 %(50/90), were married. However, 31 %(28/90) of CC cases, 18 %(16/90) of CIN cases and 22 %(20/90) of controls were single (p < 0.001). The highest level of education among study participants was generally primary level and below, with 53 %(48/90) of CC cases having attained a maximum of preprimary, and 51 %(45/90) CIN cases and 44 %(40/90) controls also reporting to have attained a maximum of primary school education. Positivity with HIV was reported in 76 %(68/90) CC cases, 52 %(47/90) of CIN cases and 42 %(38/90) of controls and this difference in distribution was statistically significant (p < 0.001). The most common presenting complaint among study participants was cervicitis with a proportion of 58 %(52/90) among CC cases, 71 %(64/90) among CIN cases and 66 %(59/90) among controls and this difference in distribution was also statistically significant (p < 0.001) as shown in Table 1.

3.2. Distribution of serum P16ink4A concentrations between cervical intraepithelial neoplasia, cervical cancer and controls

[illegible] shows the distribution of serum P16ink4A across participant groups. CC cases had a higher serum P16ink4A concentration compared to CIN cases and controls. The mean serum P16ink4A concentration among CIN cases was 1.11(+/− 0.66) ng/ml and 1.13(+/− 0.61) ng/ml among controls, and 1.45(+/− 1.11) ng/ml among CC cases and this difference in means was statistically significant (p = 0.008). Half of CIN cases (50 %, 45/90) and controls (50 %, 45/90) had P16ink4A concentration above 0.946 ng/ml. Majority of CC cases (60 %, 54/90) and 50 % of controls (45/90) had P16ink4A concentration above 0.946 ng/ml. This difference was also statistically significant as shown in

3.3. Association between serum P16ink4A concentrations and cervical lesions

From univariate analysis, there was increased odds of CIN (COR: 1.07, p-value: 0.78) and reduced odds of CC for serum P16ink4A (COR: 0.66, p-value: 0.03). After adjusting for smoking status, HIV status, History of high blood pressure, History of diabetes, age, presenting complaint, contraceptive use, type of contraceptive and marital status, multivariate logistic regression analysis showed increased odds of CIN for serum P16ink4A though not statistically significant (AOR: 1.11, p-value: 0.70). There was also a statistically significant reduction in odds of CC for serum P16ink4A (AOR: 0.55, p-value: 0.01) as shown in Table 3.

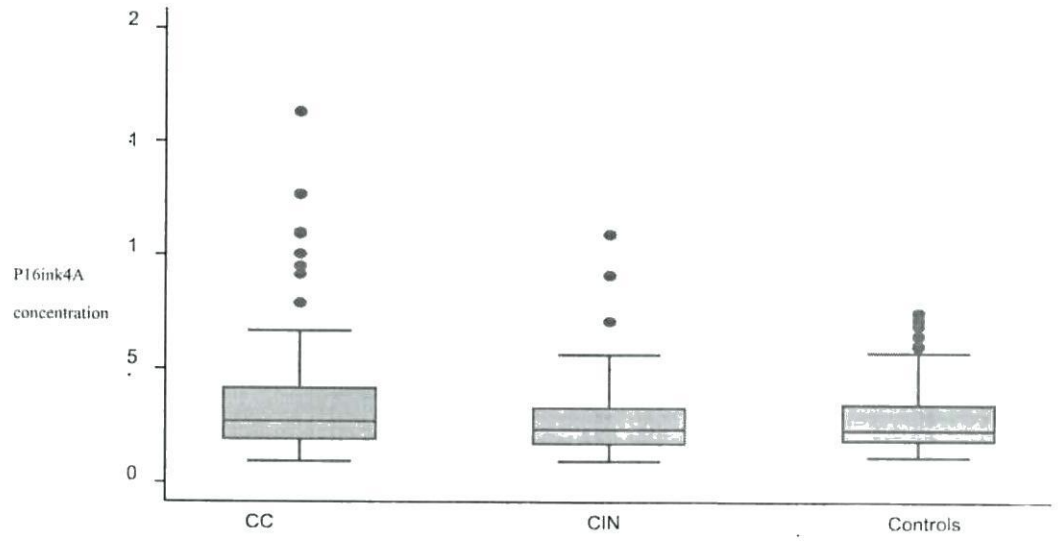
4. Discussion

This study reveals a compelling association between serum P16INK4A concentrations and both cervical cancer and cervical intraepithelial neoplasia within our study population. Notably, our investigation marks a significant contribution to the existing literature as one of the pioneering studies in Uganda to explore the potential link between circulating P16INK4A concentrations and cervical lesions. Our findings align with prior research demonstrating a consistent association between P16INK4A immunoexpression and cervical lesions, as documented in previous studies ([illegible]). Other studies have demonstrated that overexpression of P16ink4A increases with severity of lesions ([illegible] 2016; [illegible]). This explains the extensive usage of P16ink4A immunohistochemical staining in staging of cervical cancer due to its perceived diagnostic and prognostic potential ([illegible] 2020; Peres, 2016; Shi, 2019; Tejedo, 2018) especially among HPV infected women ([illegible]).

ble 1
mographic characteristics of participants at Mbarara Regional Referral Hospital between April 2022 and June 2023.

ariable	Category	CC N = 90 f(%)	CIN N = 90 f(%)	Controls N 90 f(%)	Test	p-value
ge		51.1 (13.1)	34.9 (7.8)	38.6 (8.7)	ANOVA	0.001
ge Group	19–29	2 (2 %)	23 (26 %)	18 (20 %)	Fisher's exact	0.001
	30–39	18 (20 %)	37 (41 %)	26 (29 %)		
	40–49	21 (23 %)	29 (32 %)	39 (43 %)		
	50–59	20 (22 %)	1 (1 %)	7 (8 %)		
	60-max	29 (32 %)	0 (0 %)	0 (0 %)		
listory of high BP	No	71 (79 %)	68 (76 %)	71 (79 %)	Chi-square	0.78
	Yes	19 (21 %)	22 (24 %)	19 (21 %)		
listory of Diabetes	No	70 (78 %)	78 (87 %)	75 (83 %)	Chi-square	0.22
	Yes	20 (22 %)	12 (13 %)	15 (17 %)		
larital status	Divorced	4 (4 %)	20 (22 %)	19 (21 %)	Fisher's exact	0.001
	Married	51 (57 %)	54 (60 %)	50 (56 %)		
	Single	28 (31 %)	16 (18 %)	20 (22 %)		
	Widowed	7 (8 %)	0 (0 %)	0 (0 %)		
ighest level of education	Never studied	32 (36 %)	5 (6 %)	11 (12 %)	Fisher's exact	<0.001
	Pre-primary	48 (53 %)	3 (3 %)	6 (7 %)		
	Primary school	10 (11 %)	45 (51 %)	40 (44 %)		
	Secondary school	0 (0 %)	23 (26 %)	29 (32 %)		
	Tertiary institution	0 (0 %)	6 (7 %)	2 (2 %)		
	University	0 (0 %)	7 (8 %)	2 (2 %)		
IV status	Negative	22 (24 %)	42 (47 %)	52 (58 %)	Fisher's exact	<0.001
	Positive	68 (76 %)	47 (52 %)	38 (42 %)		
	Unknown	0 (0 %)	1 (1 %)	0 (0 %)		
noking	No	90 (100 %)	84 (94 %)	89 (99 %)	Fisher's exact	0.029
	Yes	0 (0 %)	5 (6 %)	1 (1 %)		
ontraceptive use	No	57 (63 %)	38 (44 %)	56 (64 %)	Chi-square	0.02
	Yes	33 (37 %)	49 (56 %)	32 (36 %)		
ype of contraceptive	IUD	17 (45 %)	9 (18 %)	4 (13 %)	Fisher's exact	0.002
	Hormonal	21 (55 %)	39 (76 %)	26 (81 %)		
	BTL	0 (0 %)	3 (6 %)	2 (6 %)		

Cervical Cancer; CIN: Cervical Intraepithelial Neoplasia; IUD: Intra Uterine Device; BTL: Bilateral Tubal Ligation
is a continuous variable and it is presented as mean (standard deviation).
other variables are categorical and are presented as frequency (proportion).



2. A box plot showing serum concentration of P16ink4A across different groups of study participants. CC: Cervical Cancer; CIN: Cervical Intra helial Neoplasia.

The significantly increased P16ink4A serum concentrations in cer- l cancer could be as a result of inactivation of retinoblastoma protein HPV infected cervical cancer cells unlike in non-cancerous and mal cells. Inactivation of retinoblastoma protein results from over ression and binding by viral oncoprotein E7, which eventually leads to increased expression of P16ink4A ( ) It has been demonstrated that P16ink4A exhibits oncogenic potential in HPV transformed cervical cells ( ) as well as cell lines (Dreyfus).

We also observed a statistically significant difference in age among

Table 2
Distribution of serum P16ink4A concentrations between cervical intraepithelial neoplasia cases, cervical cancer cases and controls at Mbarara Regional Referral Hospital between April 2022 and June 2023.

	Category	Cancer N = 90 f(%)	CIN N = 90 f(%)	Control N = 90 f(%)	Test	p-value
Baseline p16 (ng/ml)		1.45 (1.11)	1.11 (0.66)	1.13 (0.61)	ANOVA	0.008
Baseline p16 categories (ng/ml)	≤0.946	36 (40 %)	45 (50 %)	51 (57 %)	Chi-square	0.079
	0.946<	54 (60 %)	45 (50 %)	39 (43 %)		

Baseline p16 (ng/ml) is presented as a continuous variable with mean (standard deviation).

Table 3
Logistic regression analysis for association between serum P16ink4A concentrations and cervical lesions among study participants at Mbarara Regional Referral Hospital between April 2022 and June 2023.

	Bivariate analysis			Multivariate analysis		
	COR	P Value	95 % CI	AOR	P Value	95 % CI
CIN	1.07	0.78	0.67–1.71	1.11	0.70	0.66–1.87
CC	0.66	0.03	0.46–0.97	0.55	0.01	0.34–0.88

COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence Interval; CIN: Cervical Intraepithelial Neoplasia; CC: Cervical Cancer.
All values were got after adjusting for smoking status, HIV status, History pf high blood pressure, History of diabetes, age, presenting complaint, contraceptive use, type of contraceptive and marital status.

our study groups. Among CC cases, the mean age was 51.1 (13.1), which was statistically different compared to controls. This collaborates well with previous studies in Tanzania (Cohen, 2021) that showed increased expression of P16ink4A in older women (40–49) years who are considered a high risk group for cervical lesions. This is likely due to the fact that this group of women could have low screening rates for cervical cancer (Weng, 2020) as well as low cervical cancer awareness (Isabirye et al., 2020; Black et al., 2019) which translate into increasing incidence of cervical cancer in older women (Weng, 2020).

We observed a big proportion of cervical cancer and cervical intraepithelial neoplasia cases having P16ink4A above 0.946 ng/ml. This is in agreement with previous studies which show increased expression of P16ink4A in cervical intraepithelial neoplasia and cervical cancer (Cheah, 2016). However, a similarly big proportion of cases also showed raised P16ink4A serum concentration. This can be explained by the fact that big proportions of our study population reported numerous gynaecological conditions, mostly cervicitis, vaginal discharge and valvular warts; moreover, with significant distribution across CC, CIN and control groups. These are conditions that can be easily associated with low literacy, poor hygiene, limited cervical cancer knowledge and may lead to persistent genitourinary tract infections including HPV, which increases expression of P16ink4A (Dahiya, 2017). Previous HPV infections have also been reported to increase the chances of acquiring another HPV infection. Most of our participants, especially cases, could have been having transient HPV infections which are known to be very prevalent though most of them get cleared without causing any lesions (Shanmugasundaram and Friedman, 2014).

We report an increased number of participants, especially cases who were married and with increase P16ink4A serum concentration. Being married is thought to increase one's chances of acquiring hrHPV infections which results in increased chances of developing cervical lesions and hence expression of P16ink4A (Deshpande, 2020) although Yetimalar et al (Yetimalar, 2012) reports otherwise.

Other factors including HIV, smoking, contraceptive use and level of education varied significantly across cases and control groups. This emphasizes their known role as factors associated with acquisition or persistence of HPV and hence cervical lesions ( ). For instance, smoking is itself said to increase HPV viral load in infected cells ( ) Other genetic and life style factors can increase chances of HPV infection ( ). HIV has been categorically proven to be a risk factor for HPV persistence ( ) which in turn increases expression of P16ink4A. This further explains the observation of raised P16ink4A serum concentrations in control groups, who were mostly HIV positive, compared to controls. This confirms reports that P16ink4A is strongly associated with cervical pathology ( ).

Considering the fact that P16ink4A, a tumor suppressor protein, can become an oncogene, and accumulates in the nucleus and cytoplasm of HPV infected cells (Pandey, 2015; Ro , 2021), studies have reported its potential application in early diagnosis of cervical cancer (2020; Weng, 2020). Also considering the fact that a non-subsidized HPV DNA single test can cost as much as 20 dollars in Uganda, P16ink4A quantification in serum presents an opportunity for a cheaper alternative. P16ink4A ELISA test can cost as low as 8 dollars for a single test and this is likely to improve cervical cancer screening uptake and eventually reduce cervical cancer mortality and morbidity. Basing on this, P16ink4A ELISA could be evaluated more for usage along with routine tests such as VIA, HPV DNA, or Pap cytology, in an algorithm. The observed significant association between serum P16ink4A concentration and cervical lesions points to its likely clinical utility. P16ink4A ELISA could as well be studied more for its application in triage of CIN, monitoring prognosis of cervical cancer after treatment and monitoring risk of recurrence of cervical lesions.

A major strength of this study is the adequate statistical power which is reflected in the sample size that was calculated using scientifically accepted methods. We also measured P16ink4A using scientifically accepted methods. A major limitation to this study is that we did not test for HPV. Therefore, we could not exactly ascertain whether increased serum P16ink4A concentrations arise from HPV positivity, though it is known that HPV leads to increased expression of P16ink4A. Also, this being a case control study, we think there must have been selection bias while recruiting participants, and hence some variables may not have been distributed randomly across the study population. We did not stratify our analyses based on grades of lesions for cases (CIN and CC). This could have masked statistically significant associations between P16ink4A concentrations and specific grades of cervical lesions.

5. Conclusion

Serum P16ink4A concentrations may likely be associated with cervical lesions especially cervical cancer, among our study population. Quantitative measurement of circulating P16ink4A may be beneficial in diagnosis of cervical cancer. Prospective studies are recommended to evaluate the diagnostic utility of circulating P16ink4A in diagnosis of cervical cancer.

Availability of data and materials

All data from which this article was generated is available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Frank Ssedyabane: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joseph Ngonzi:** Writing

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Declaration of competing interest

The authors declare that they have no known competing financial nterests or personal relationships that could have appeared to influence he work reported in this paper.

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Prevalence of cervical intraepithelial lesions and associated factors among women attending a cervical cancer clinic in Western Uganda; results based on Pap smear cytology

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Abstract

Introduction: There are high incidence and mortality rates of cervical cancer among females in East Africa. This is exacerbated by limited up-to-date data on premalignant lesions and associated factors in this setting. In this study, we determined the prevalence of cervical intraepithelial lesions and associated factors among women attending the Mbarara Regional Referral Hospital cervical cancer clinic in Southwestern Uganda.

Methods: In this cross-sectional study, 364 participants were recruited from among women attending the Mbarara Regional Referral Hospital cervical cancer clinic from 1 April to 30 June 2023. On consent, the study nurse collected demographic data and Pap smears, which were microscopically examined and reported by a laboratory scientist and a pathologist following the Bethesda grading system (2014). Statistical analyses were done in STATA version 17, using proportions, Chi-square, bivariate, and multivariate logistic regression analysis to determine associated factors at α 0.05 significance level.

Results: The mean age of participants was 41.9 years. A third of all study participants (37.6%, 132/351) were contraceptive users, mostly hormonal contraceptives (87.1%, 115/132). Almost 88% (307/351) had an unknown Human Papilloma Virus status. The prevalence of cervical intraepithelial lesions among our study participants was 6.6% (23/351), of which 73.9% (17/23) were low-grade squamous intraepithelial lesions. More than half (9/17, 52.9%) of low-grade squamous intraepithelial lesions were active hormonal contraceptive users. Use of hormonal contraceptives (OR: 3.032, *p*: 0.0253), use of intrauterine devices (OR: 6.284, *p*: 0.039), and any family history of cervical cancer (OR: 4.144, *p*: 0.049) were significantly associated with cervical intraepithelial lesions.

Conclusion: The prevalence of cervical intraepithelial lesions was 6.6%, lower than global estimates. Use of hormonal and intrauterine device contraceptives, as well as family history of cervical cancer, were significantly associated with cervical intraepithelial lesions among our study population. Prospective studies are recommended to further understand associations between different types of intrauterine devices and hormonal contraceptives, and cervical lesions.

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Keywords
Cervical intraepithelial neoplasia, risk factors, cervical cancer, LSIL, HSIL, ASCUS, Uganda

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Introduction

Globally, cervical cancer is number four among the most common malignancies affecting women.[1] About a million incident cervical cancer cases and 307,000 mortalities were reported in Sub-Saharan Africa in 2020,[2] with low and middle-income countries recording increasing incidence and mortality rates.[3,4] Incidence and mortality rates stand at 40.1/100,000 and 28.6/100,000, respectively among females in East Africa.[2] Cervical cancer is, however, ranked as the commonest cancer among women, responsible for death of more women than any other cancer in Sub-Saharan Africa, Asia (including India), as well as some countries in Central and South America.[5] On a similar note, 569,847 incident cases of cervical cancer were reported worldwide in 2018 by Bray et al.,[6] with an age-standardised incident rate in East Africa being 40.1 per 100,000. South Africa had the highest age-standardised incidence rate of 43.1 per 100,000. Majority of African countries, including Uganda, have been reported to have an age-standardised incidence rate of at least 40 per 100,000.[1] In 2019, it was reported that 2784.9 million women worldwide aged 15 years and older were at risk of developing cancer of the cervix.[7] The prevalence of cervical precancerous lesions around Africa has been reported to be in the range between 3.7% and 22.6%.[8–16] From the Ugandan perspective, Eilu et al.[17] showed that the prevalence of cervical intraepithelial neoplasia stands at 38.6%.

Human papillomavirus (HPV) is a well-established necessary but not sufficient cause of cervical lesions.[18,19] Development of cervical cancer occurs through several stages, starting with chronic infection with high-risk HPV (HrHPV) genotypes, through premalignant stages (cervical intraepithelial neoplasia (CIN)), and finally culminates into invasive cervical cancer (ICC).[20,21] HPV16 and HPV18 have been implicated to be HrHPV subtypes highly associated with around 70% of all cervical cancers.[22] After a persistent infection with HrHPV genotypes, epithelial cells undergo a series of changes, typically epithelial-mesenchymal transition,[23] turning epithelial cells into mesenchymal cells which can metastasise; a property that is thought to contribute to the metastatic nature of human cancer cells.[24] During the productive stage of infection, the viral genome is relatively maintained at a low number of copies in the basal cells.[25–28] The viral episomes remain maintained in basal cells by being replicated alongside the host cell chromosomes.[29–31] Thereafter, the viral life cycle goes on along with the differentiation program in keratinocytes and it is dependent on several cellular and viral factors and proteins.[29] Interactions of various viral and cellular pathways involving mainly proteins and other biomolecules like micro RNAs, E6 and E7, contribute towards progression of cervical epithelial cells to a cancerous state. Outgrowth of dysplastic lesions is said to be facilitated by increasing expression of viral oncogenes, E6 and E7, which themselves interact with various proteins that regulate the cell cycle.[31]

A number of factors, including HIV infection, age at sexual debut, Chlamydia trachomatis infection, oral contraceptives, smoking, gravidity, use of multiple sexual partners, and other forms of immunosuppression, are associated with cervical cancer.[21,32–36] There is limited information regarding the current magnitude of cervical cancer or its premalignant lesions and associated factors in resource-limited settings, which is normally attributed to poor data collection and reporting systems.[37,38] Available information[39] is too old to be relied on for decision making. Uganda is experiencing a very low cervical cancer screening rate,[40–42] and this might increase the burden due to cervical cancer or its premalignant lesions as a result of late detection and treatment.[43] The low screening rate for cervical cancer is said to be driven by scarcity of knowledge on cervical cancer and its screening services, religious barriers, community misconceptions, fear of embarrassment during screening procedures, fear of screening outcomes, and long distances travelled to reach screening centers among others.[44,45] There is still a lot of unknown information regarding prevalence and factors associated with cervical intraepithelial lesions, as well as barriers to screening. This study, therefore, aimed at determining the prevalence of cervical intraepithelial lesions and associated factors among women attending the Mbarara Regional Referral Hospital (MRRH) cervical cancer clinic in South Western Uganda.

Materials and methods

Study design

A cross-sectional study was conducted, including all women who presented at the MRRH cervical cancer clinic from 1 April to 30 June 2023, seeking cervical cancer screening, testing, and treatment services. Results of this study have been reported following the STROBE guidelines for cross-sectional studies.

Study setting

We conducted this study at the MRRH cervical cancer clinic from 1 April to 30 June 2023. MRRH is a public and free access tertiary health facility and is the primary teaching hospital for the medical school of Mbarara University of Science and Technology (MUST); and serves the entire

Southwestern Uganda. The distance between MRRH and Kampala, the Ugandan capital, is about 260 km. At this clinic, an average of 15 women are screened for cervical lesions per day, and the clinic runs 5 days a week. Being the only cervical cancer screening and treatment center in the region of Southwestern Uganda, it receives women from all districts of Southwestern Uganda plus the neighbouring countries, including Tanzania, Rwanda, Burundi, and the Democratic Republic of Congo. Staff at the clinic include nurses, midwives, medical officers, and residents, and all are supervised by a gynecologic oncologist. Screening for cervical cancer is voluntary and free of charge, although some women get referred to the clinic by lower health facilities. There is no role played in cervical cancer screening by any insurance service provider. Primary screening tests at the clinic are visual inspection with acetic acid (VIA) and Pap smear cytology, while diagnostic tests include colposcopy and histology. Patients who get diagnosed with cervical lesions either get treatment onsite or get referred to radiotherapy or chemotherapy at the Uganda Cancer Institute. At the clinic, cervical cancer screening services are available to all women aged 21 years and above, following national guidelines. Conventional Pap smear cytology and general histology are performed at the histopathology laboratory in the pathology department of MUST. This laboratory is the regional histopathology laboratory for the Ministry of Health, and it is run by qualified and certified histotechnologists together with pathologists and participates in external quality assurance.

Variables

The main outcome of this study was the prevalence of cervical lesions. The independent variables for this study included age, district, and village of residency, history of blood pressure, diabetes history, marital status, the highest educational level attained, HIV status, smoking status, contraceptive use, type of contraceptive being used, family history of cervical cancer, age at first sexual encounter, total number of lifetime partners, and number of full-term births. For those who reported to be HIV positive, we captured HIV viral load and CD4 count.

Sampling procedure

We used simple random sampling to recruit study participants among all women who sought cervical cancer screening and testing services at the cervical cancer clinic of MRRH. This was adopted to avoid selection bias.

Sample size determination

For this cross-sectional study, the sample size of 364 participants was estimated using the formula by Kish Leslie 1965. For this calculation, we considered $d=0.05$, the level of precision which is assumed to be ± 0.05, $Z_{\alpha/2}$ 1.96 (Score of normal standard variance curve corresponding to 95% level of confidence) for a two-sided test (1.96); and a prevalence of CIN of 38.6%.[17] Thirteen participants were excluded from data analysis because their Pap smears were non-diagnostic on examination.

Data collection

Upon arrival at the clinic, participants were assessed for eligibility to participate in the study and then after, written informed consent was sought. After the provision of written informed consent, participants were taken through a description of the study and its objectives. Pap smears were then collected, and VIA and other tests were performed thereafter.

Demographic data collection

The midwives from the cervical cancer clinic served as study assistants and administered a pretested questionnaire to gather demographic data. This questionnaire had been used previously[35,46] and was pretested at the Uganda Cancer Institute among women seeking cervical cancer care. After the provision of written informed consent, participants were assisted in filling out the questionnaire to capture their demographic data, including age, district, and village of residency, history of blood pressure, diabetes history, marital status, the highest educational level attained, HIV status, smoking status, contraceptive use, type of contraceptive being used, family history of cervical cancer, age at first sexual encounter, total number of lifetime partners, and number of full-term births. For those who were reported to be HIV positive, we captured HIV viral load and CD4 count results from clinic files.

Cytological evaluation

Pap smear collection was performed by midwives at the clinic following a routine standard procedure. The patient was placed on an examination table, and the midwife used a cytobrush or spatula to delicately remove cells from the surface of the cervix while a speculum was gently pushed into the vagina to allow a clear view of the cervix. Smears were created on glass slides, promptly fixed with 95% alcohol, and then stained using the Pap staining technique in accordance with standard operating procedures in laboratories. Smears were examined microscopically by the principal investigator, together with a qualified and certified pathologist. All the examinations and grading followed the Bethesda grading system (2014).[47] The results were categorised as follows: squamous cell carcinoma (SCC), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), atypical squamous cells of undetermined significance (ASCUS), and negative for intraepithelial lesion or malignancy (NILM).

Statistical analysis

Data was collected by the principal investigator with the help of research assistants. It was then entered into an Excel spreadsheet (Microsoft Office Professional Plus 2013, version 15.0.4675.1003, Microsoft Inc, USA) and then imported into STATA 17 (Stata Corp LLC, College Station, TX, USA) software. Descriptive statistics were employed to describe the population using frequencies, means ± standard deviations (SDs), and median values for continuous variables. We also employed Chi-square tests, frequencies, and proportions to describe categorical variables. The prevalence of cervical intraepithelial lesions was presented as proportions. We used bivariate and multivariate logistic regression analysis to arrive at factors associated with cervical lesions. Bivariate analysis was done first, and we purposively selected variables ($p < 0.2$) for multivariate analysis. We considered a p-value of <0.05 as being significant.

Ethical considerations

Prior to participating in the trial, each subject gave their written informed consent. On all instruments used for gathering data and on laboratory specimens, we used study numbers rather than names. During data analysis, we disconnected all participants' personal information. All interactions between research assistants and participants happened in a quiet, cosy side room of the clinic that was only available to one person at a time. This study received ethical approval from the Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) (MUST-2022-612) as well as the Uganda National Council for Science and Technology (UNCST) (HS2722ES). We also sought administrative clearance from the MRRH Director before commencing the study. All women whose results indicated a diagnosis of cervical intraepithelial lesions received the standard package of care available at the cervical cancer clinic, and all participants received their Pap smear results.

Eligibility criteria

This study included all women of 21 years and above who reported to the MRRH cervical cancer clinic during the time of the study and provided written informed consent to participate. All women were recruited regardless of their family or personal history of cervical cancer. We excluded all those women who were too ill, as well as those who were undergoing treatment for cervical lesions.

Results

The response rate for this study was 100%, and recruited a total of 364 participants following our inclusion criteria. Thirteen participants were excluded from analysis because they had nonsatisfactory Pap smears, and hence, they were considered to have missing information. We eventually included 351 in the analysis. The mean age of study participants was 41.9 (±12.2). The majority of our participants (63.8%, 224/351) were residing in districts other than Mbarara city, were married (65.2%, 229/351), and were nonsmokers (98.3%, 345/351). More than half (58.1%, 204/351) of the study participants were HIV positive, and their mean CD4 count as well as viral load were 493.392 cells per µl and 4719.833 copies per µl, respectively, as shown in Table 1.

We assessed our study participants for other reproductive characteristics. The most common gynaecological complaint was vaginal discharge, with a prevalence of 12.8% (45/351). More than a third (37.6%, 132/351) were contraceptive users, specifically hormonal contraceptives (87.1%, 115/132). A big proportion (80.3%, 282/351) of our participants reported to have had their sexual debut at an age between 12 and 20 years. Almost 88% (307/351) had an unknown HPV status, while 69.1% (242/351) reported to have had multiple sexual partners, as shown in Table 2.

Prevalence of cervical lesions

We observed a total number of 23 participants with positive cytology results out of 351 participants. These participants were LSIL+,[24] and one participant who was ASCUS but got treatment following an earlier positive VIA test. This makes a prevalence of 6.6%. More than half (73.9%, 17/23) of all positive cases were LSILs. All participants (23/23) with positive Pap smears reported no history of high blood pressure. A good number of participants (19/23) with cervical lesions were married: 100% for ASCUS, 80% for HSIL, and 82.4% for LSIL. More than half (52.9%, 9/17) of participants with LSIL had attained a maximum of primary school education. The same proportion of participants (52.9%, 9/17) were HIV positive and had suppressed viral load, as shown in Table 3.

Of all participants who had LSIL, 82.4% (14/17) reported to have had their sexual debut at 31 years and above. Still, for those with LSIL, more than half (58.8%, 10/17) were active contraceptive users, especially hormonal contraceptives (52.9%, 9/17). Additionally, whereas 60% (3/5) of people with HSIL reported having two or more lifetime sexual partners, more than half (70.69%, 12/17) of participants with LSIL did the same. A big proportion of participants (80%, 4/5) with HSIL reported to have had 4 or more full-term births, as shown in Table 4.

Factors associated with cervical lesions

Several variables were considered for multivariate analysis after their bivariate analysis revealed p values of less than 0.2, as well as being biologically plausible in cervical cancer. These variables included the number of full-term births, lifetime sexual partners, age at sexual debut, HIV status, type of contraceptive used, family history of cervical cancer, and highest educational level. After adjusting for number of

Table 1. Socio-demographic characteristics of study participants at Mbarara Regional Referral Hospital, 1 April to 30 June 2023.

Variables	Category	n/351	Percentage
Age		41.9 (12.2)	
Age group	21–29	59	16.8
	30–39	99	28.2
	40–49	92	26.2
	50 and above	101	28.8
Region of residence	Central Uganda	2	0.6
	Other parts of Western Uganda	224	63.8
	Mbarara city	125	35.6
History of high BP	No	312	88.9
	Yes	39	11.1
Diabetes history	No	333	94.9
	Yes	18	5.1
Marital status	Divorced	47	13.4
	Married	229	65.2
	Single	70	19.9
	Widowed	5	1.4
Highest educational level	Never studied	45	12.8
	Pre-primary	30	8.5
	Primary school	138	39.3
	Secondary school	87	24.8
	Tertiary institution	35	10
	University	16	4.6
HIV status	Unknown	1	0.3
	Negative	146	41.6
	Positive	204	58.1
HIV viral load category	N/A	146	41.6
	Not suppressed	18	5.1
	Suppressed	187	53.3
HIV viral load copies per µl		4719.8 (16606.9)	
CD4 count		493.4 (234.5)	
Smoking	No	345	98.3
	Yes	6	1.7

Continuous variables, age, HIV viral load, and CD4 count are presented as mean (standard deviation).

full-term births, type of contraceptive used, HIV status, age at sexual debut, CD4 counts, HIV viral load, highest educational level, and number of life-time sexual partners, the results of the multivariate analysis showed that the likelihood of cervical lesions was three times higher in those who reported using hormonal contraceptives than in those who had not used any form of birth control. This difference was statistically significant (OR: 3.032, p-value=0.025, 95% CI: 1.150–7.989). Also, participants who reported to have used IUDs as contraceptives were six times more likely to develop cervical lesions compared to those who had not used any contraceptive, and this observation was also statistically significant (OR: 6.284, p-value=0.039, 95% CI: 1.094–36.095). Participants who reported a family history of cervical cancer were also four times more likely to have cervical lesions than those who reported not to have such a family history of cervical cancer. This observation was statistically significant (OR: 4.144, p-value=0.049, 95% CI: 1.004–17.101), as shown in Table 5.

Discussion

In this study, we found that 6.6% of all participants recruited were positive for cervical lesions. This prevalence was lower than is reported in most studies. Studies in other low resource African countries have reported higher prevalence rates, such as 26.7-37% in Kenya.[48] There was a reported prevalence of 18.2% in Bangladesh[49] and 22.1% in Ethiopia.[50] Previous studies that have been conducted among HIV-positive and negative populations have reported prevalence rates of 22.9% and 5.79%, respectively, in Swaziland.[51] This difference could be attributed to the fact that most of these studies were conducted among HIV-positive people, bearing in mind that HIV is a risk factor for cervical lesions.[52–54] However, other studies in Cameroon have reported lower prevalence rates of 3.33%.[55] A case-control study in Nigeria documented a slightly higher but comparable prevalence of abnormal Pap cytology results (8.0%).[56] This observation could be attributed to the comparability of study populations

Table 2. Reproductive characteristics of study participants at Mbarara Regional Referral Hospital, 1 April to 30 June 2023.

Variables	Category	*n*/351	Percentage
Other gynaecological conditions	None	264	75.21
	Cervicitis	35	9.97
	Vaginal discharge	45	12.82
	Warts	1	0.28
	Candidiasis	2	0.57
	Trichomoniasis	2	0.57
	Syphilis	2	0.57
Contraceptive use	No	219	62.4
	Yes	132	37.6
Contraceptive type (*N* = 132)	BTL	2	1.5
	Condom	2	01.5
	Hormonal	115	87.1
	IUD	13	9.9
Cervical cancer family history	No	331	94.6
	Yes	19	5.4
Age at sexual debut	31 and above	2	0.57
	21–30	67	19.09
	12–20	282	80.34
Lifetime sexual partners (number)	0–1	108	30.86
	2 and above	242	69.14
Full-term births (number)	0	12	3.42
	1–3	163	46.44
	4 and above	176	50.14
VIA test results	Negative	312	88.9
	Positive	37	10.5
	Suspicious for cancer	2	0.6
HPV status (16 and 18)	Negative	14	4
	Positive	30	8.5
	Unknown	307	87.5

All data was categorical, and the total number of participants was 351, except for type of contraceptive (*N* = 132).

in terms of socioeconomic status and sexual behaviour. Increased prevalence of cervical neoplasia in our study was found among participants who were married and less educated (primary education). This pattern of prevalence was earlier reported in similar studies by Bayo et al.[57] in Mali and Palacio-Mejía et al.[58] in Mexico. These women may have developed cervical neoplasia as a result of their early sexual experience and multiple sexual partners, both of which are significant risk factors for HPV infection.

We found that all participants (23/23) with positive Pap smears reported no history of high blood pressure. This is in agreement with other studies, which have shown that cervical lesions are significantly associated with other metabolic syndrome components except high blood pressure.[59–64] It would be imperative to think of an indirect link between high blood pressure and cervical lesions. For instance, from our recent studies in Ugandan populations, we also observed significant associations between other metabolic syndrome components with cervical lesions.[36,46] Besides, high blood pressure being associated with inflammation likely impairs one's immune system,[65] which would eventually predispose one to reduced ability to clear infections, including HPV.

This study presents a higher prevalence of cervical lesions among married women. This contradicts the commonly perceived notion about marriage as a protective factor against cervical lesions[66] and that single women are at increased risk of cervical cancer.[67] This indicates that married women in this study population are likely exposed to HPV or other risk factors for cervical cancer. In fact, population dynamics and trends indicate that 8.3% of all women in Uganda are married or cohabiting in polygamous relationships.[68] Such observations in this population could explain the increased prevalence of sexually transmitted infections, including Chlamydia Trachomatis and HPV, which are associated with cervical lesions.[35] However, an equally high prevalence of cervical lesions among married women was reported in a hospital population in Cameroon.[69]

We found out that usage of IUDs and hormonal contraceptives, as well as having a family history of cervical cancer, were significantly associated with cervical lesions in this study population. Although it deviates from many of the risk

Table 3. Prevalence of cervical lesions across selected socio-demographic characteristics of study participants at Mbarara Regional Referral Hospital, 1 April to 30 June 2023.

Variable	ASCUS	HSIL	LSIL	NILM	Test	p-Value
	N = 1	N = 5	N = 17	N = 328		
Age group						
21–29	0 (0.0%)	1 (20.0%)	3 (17.6%)	55 (16.8%)	Fisher's exact	0.79
30–39	0 (0.0%)	2 (40.0%)	6 (35.3%)	91 (27.7%)		
40–49	1 (100.0%)	0 (0.0%)	5 (29.4%)	86 (26.2%)		
50 and above	0 (0.0%)	2 (40.0%)	3 (17.6%)	96 (29.3%)		
Region of residence						
Central Uganda	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)	Fisher's exact	0.71
Other parts of Western Uganda	1 (100.0%)	4 (80.0%)	9 (52.9%)	210 (64.0%)		
Mbarara city	0 (0.0%)	1 (20.0%)	8 (47.1%)	116 (35.4%)		
History of high BP						
No	1 (100.0%)	5 (100.0%)	17 (100.0%)	289 (88.1%)	Fisher's exact	0.44
Yes	0 (0.0%)	0 (0.0%)	0 0.0%)	39 (11.9%)		
History of diabetes						
No	1 (100.0%)	5 (100.0%)	16 (94.1%)	311 (94.8%)	Fisher's exact	0.71
Yes	0 (0.0%)	0 (0.0%)	1 (5.9%)	17 (5.2%)		
Marital status						
Divorced	0 (0.0%)	1 (20.0%)	2 (11.8%)	44 (13.4%)	Fisher's exact	0.70
Married	1 (100.0%)	4 (80.0%)	14 (82.4%)	210 (64.0%)		
Single	0 (0.0%)	0 (0.0%)	1 (5.9%)	69 (21.0%)		
Widowed	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (1.5%)		
Highest educational level						
Never studied	0 (0.0%)	2 (40.0%)	2 (11.8%)	41 (12.5%)	Fisher's exact	0.97
Pre-primary	0 (0.0%)	0 (0.0%)	1 (5.9%)	29 (8.8%)		
Primary school	1 (100.0%)	2 (40.0%)	9 (52.9%)	126 (38.4%)		
Secondary school	0 (0.0%)	1 (20.0%)	4 (23.5%)	82 (25.0%)		
Tertiary institution	0 (0%)	0 (0.0%)	1 (5.9%)	34 (10.4%)		
University	0 (0.0%)	0 (0.0%)	0 (0.0%)	16 (4.9%)		
HIV status						
Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.3%)	Fisher's exact	0.70
Negative	0 (0.0%)	1 (20.0%)	8 (47.1%)	137 (41.8%)		
Positive	1 (100.0%)	4 (80.0%)	9 (52.9%)	190 (57.9%)		
Smoking						
No	1 (100.0%)	5 (100.0%)	17 (100.0%)	322 (98.2%)	Fisher's exact	1.00
Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)	6 (1.8%)		
HIV viral load category						
N/A	0 (0.0%)	1 (20.0%)	8 (47.1%)	137 (41.8%)	Fisher's exact	0.52
Not suppressed	0 (0.0%)	1 (20.0%)	0 (0.0%)	17 (5.2%)		
Suppressed	1 (100.0%)	3 (60.0%)	9 (52.9%)	174 (53.0%)		
CD4 count						
200≤	0 (0.0%)	2 (11.8%)	0 (0.0%)	49 (14.9%)	Fisher's exact	0.54
<200	0 (0.0%)	1 (5.9%)	0 (0.0%)	4 (1.2%)		
Not indicated	5 (100.0%)	14 (82.4%)	1 (100.0%)	275 (83.8%)		

Data for categorical variables were presented as frequency (proportion).

factors that have been widely accepted, this provides new information regarding risk factors for cervical intraepithelial lesions. For instance, many authors from previous studies reported smoking, HIV positivity, contraceptive use, and a family history of cervical cancer as possible risk factors for cervical cancer.[7,52,53,70–78] Furthermore, in a study on the risk factors for cervical neoplasia, Kjellberg et al.[41] discovered a substantial correlation between higher grades of CIN and HPV, smoking, and prolonged contraceptive usage. In addition, our previous study showed that obesity is significantly associated with cervical intraepithelial lesions.[79] For family history of cervical cancer, it is crucial to acknowledge the

Table 4. Prevalence of cervical lesions across reproductive characteristics of study participants at Mbarara Regional Referral Hospital, 1 April to 30 June 2023.

Variable	ASCUS	HSIL	LSIL	NILM	Test	p-Value
	N = 1	N = 5	N = 17	N = 328		
Age at sexual debut						
31 and above	1 (100.0%)	5 (100.0%)	14 (82.4%)	262 (79.9%)	Fisher's exact	0.87
21–30	0 (0.0%)	0 (0.0%)	3 (17.6%)	64 (19.5%)		
12–20	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)		
Contraceptive use						
No	0 (0.0%)	3 (60.0%)	7 (41.2%)	209 (63.7%)	Fisher's exact	0.11
Yes	1 (100.0%)	2 (40.0%)	10 (58.8%)	119 (36.3%)		
Type of contraceptive						
BTL	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)	Fisher's exact	0.18
Condom	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)		
Hormonal	1 (100.0%)	1 (20.0%)	9 (52.9%)	105 (32.0%)		
IUD	0 (0.0%)	1 (20.0%)	1 (5.9%)	11 (3.4%)		
None	0 (0.0%)	3 (60.0%)	7 (41.2%)	208 (63.4%)		
Family history of cervical cancer						
No	1 (100.0%)	4 (100.0%)	14 (82.4%)	312 (95.1%)	Fisher's exact	0.15
Yes	0 (0.0%)	0 (0.0%)	3 (17.6%)	16 (4.9%)		
Number of lifetime sexual partners						
1	1 (100.0%)	2 (40.0%)	5 (29.4%)	100 (30.6%)	Fisher's exact	0.57
2 and above	0 (0.0%)	3 (60.0%)	12 (70.6%)	227 (69.4%)		
Number of full-term births						
0	0 (0.0%)	0 (0.0%)	0 (0.0%)	12 (3.7%)	Fisher's exact	0.60
1–3	0 (0.0%)	1 (20.0%)	10 (58.8%)	152 (46.3%)		
4 and above	1 (100.0%)	4 (80.0%)	7 (41.2%)	164 (50.0%)		
Other gynaecological conditions						
None	1 (100.0%)	4 (80.0%)	14 (82.4%)	245 (74.7%)	Fisher's exact	0.62
Cervicitis	0 (0.0%)	1 (20.0%)	3 (17.6%)	31 (9.5%)		
Vaginal discharge	0 (0.0%)	0 (0.0%)	0 (0.0%)	45 (13.7%)		
Warts	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.3%)		
Candidiasis	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)		
Trichomoniasis	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)		
Syphilis	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.6%)		

Data for categorical variables were presented as frequency (proportion).

ole of genetic and epigenetic factors in cervical carcinogen-sis. For instance, the apolipoprotein B mRNA editing enzyme catalytic polypeptide like DNA-editing protein is aid to play an important role in molecular pathogenesis of ervical cancer as well as other cancers.[80–82]

We observed no association between smoking and cervi-al lesions. This could be explained by the fact that there vere very few cases of smokers in the study population. In act, smoking has been found not to be associated with any grade of cervical lesions, as evidenced in previous studies by Castellsagué et al.[83] However, smoking has been shown to be ssociated with cervical precancerous lesions.[69,84] and other nalignancies.[85–87] In our population, age was not associated vith cervical lesions, though it was earlier proposed by Bhatla et al.[88] that the age group of 21–40 years could be a isk factor based on the high prevalence of HPV and other actors in the same age group.

We found that usage of IUDs is significantly associated with cervical lesions among the study population. Previous studies have reported contradicting results in regard to IUDs and cervical lesions.[89–92] A systematic review by Cortessis et al.[93] reported that cervical cancer is less frequent in women who have used IUDs, and hence, IUDs may be apparently protective against cervical lesions. This protective nature of IUDs against cervical lesions is based on observations that IUDs reduce HPV infection risk and also trigger immune system responses that tend to fight viral infections, including HPV.[94] From the case-control study, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) Study, non-significantly decreased risk of both CIN3/CIS and ICC was observed among IUD users.[93]

However, in agreement with our findings, recent evidence from a case-control study by Averbach et al.[95] showed that IUD usage is associated with CIN2+ but not with CIN3+ In

Table 5. Logistic regression analysis showing association between cervical lesions and selected variables of study participants at Mbarara Regional Referral Hospital, 1 April to 30 June 2023.

Variables	Category	COR	p-Value	95% CI	AOR	p-Value	95% CI
Number of full-term births	0	1			1		
	1–3	0.989	0.98	0.424–2.308	1.337	0.564	0.4982–3.591
	4 and above	1			1		
Number of life-time sexual partners	0–1	1			1		
	2 and above	0.826	0.674	0.339–2.011	0.849	0.748	0.312–2.313
Age at sexual debut	31 and above	1			1		
	21–30	0.614	0.442	0.177–2.130	0.980	0.976	0.257–3.731
	12–20	1			1		
HIV status	Negative	1			1		
	Positive	1.122	0.795	0.472–2.666	0.887	0.804	0.344–2.284
	Unknown	1			1		
Type of contraceptive	None	1			1		
	Hormonal	2.179	0.086	0.897–5.295	3.032	0.025*	1.150–7.989
	IUD	3.782	0.111	0.737–19.395	6.284	0.039*	1.094–36.095
	BTL	1			1		
	Condom	1			1		
Family history of cervical cancer	No	1			1		
	Yes	3.079	0.094	0.823–11.494	4.144	0.049*	1.004–17.101
CD4 count	200≤	1			1		
	<200	6.125	0.173	0.451–83.116	5.053	0.234	0.351–72.672
Highest educational level	None	1			1		
	Pre-primary	0.353	0.363	0.038–3.328	0.272	0.269	0.027–2.738
	Primary school	0.976	0.968	0.299–3.193	0.811	0.743	0.231–2.841
	Secondary school	0.625	0.5	0.159–2.453	0.493	0.36	0.108–2.244
	Tertiary institution	0.302	0.294	0.032–2.826	0.191	0.172	0.018–2.055
	University	1			1		

Data were adjusted for number of full-term births, type of contraceptive used, HIV status, age at sexual debut, CD4 counts, HIV viral load, educational level, and number of life-time sexual partners.
COR: crude odds ratio; AOR: adjusted odds ratio; 95% CI: 95% confidence interval.
*Statistically significant ($p \leq 0.05$).

some populations, including those in less developed countries, usage of IUDs is thought to increase the risk of sexually transmitted infections (STIs), including HPV, whose association with cancer of the cervix is well known.[96] A study among Kenyan sex workers found that usage of IUDs was significantly associated with increased incident C. trachomatis infection.[97] This is in agreement with our earlier study, which demonstrated that a co-infection of C. trachomatis and HPV is associated with cervical lesions.[35] Certain types of IUDs, including LNG-IUS, which may not be popular in our study population, have been shown to increase HPV acquisition and persistence.[98] Lekovich et al.'s[99] comparative study, which examined the differences in cervical cytology and high-risk human papillomavirus (HPV) infection clearance between levonorgestrel- and copper-containing IUD users, also supported the findings of our study by showing that levonorgestrel-containing IUDs may be linked to higher risk of high-risk HPV infection acquisition and lower clearance when compared to copper-containing IUDs.

We found a significant association between cervical lesions and usage of hormonal contraceptives. This is in agreement with results from a systematic review by Deese et al.,[100] who provided evidence that is supportive of an increased risk of many other STIs, including HSV-2 infection among users of other contraceptive methods, depot medroxyprogesterone acetate (DMPA). Iversen et al.'s[101] reported observations have also demonstrated that the risk of cervical cancer remains strongly correlated with the use of currently available contraceptives, particularly for women who have not received the HPV vaccine. We note that HPV vaccination among our study participants has a very low coverage [102,103] Moreover, with a low cervical cancer screening uptake [43] The usage duration of hormonal contraceptives could also have a significant correlation with cervical lesions. To further understand the potential impact of the usage duration of hormonal contraceptives on cervical lesions, a case-control study by Kusmiyati et al.[104] revealed that exposure to hormonal contraceptives for more than 5 years can increase one's risk of cervical cancer by 4.2 times.

It is well known that hormonal contraceptives change the way cells metabolise their substances, which can lead to long-term HPV infections and the eventual development of

cervical cancer. The expression of the oncogenes E6 and E7 of HrHPV genotypes may be encouraged by oestrogen and progestin. This process triggers the destruction of the p53 tumour suppressor gene and the incorporation of viral DNA, resulting in cellular transformation and the development of cancer.[105–107] Hormonal contraceptives are also known to bind to certain HPV-DNA sequences within transcriptional regulatory areas, which may control cellular death and promote HPV-DNA integration into the host cell genome.[108–114] With increased availability and usage of hormonal contraceptives in our population, there is an increased risk of cervical cancer as a result of hormonal contraception in a predominantly unvaccinated population.[102,103] This is based on recent observations that hormonal contraceptive use is also associated with a number of STIs, including HIV, HPV, and *Chlamydia Trachomatis*,[115] which are known risk factors for cervical lesions.

Recent evidence from a review by Anastasiou et al.[116] showed no evidence of an association between hormonal contraceptive use and cervical lesions. However, this study was limited by sample size that is, very few included studies. Additionally, case-control research conducted in Nigeria by Ajah et al.[56] had previously found no link between cervical neoplasia and hormonal contraception. This study did not reveal any significant association between oral contraceptive use and cervical cancer or any grade of cervical lesion. On the contrary, a recent systematic review has shown that longer duration of oral contraceptive pills use has an associated risk for developing cervical cancer, especially for Adenocarcinoma.[117]

Our study is limited by the inherent weaknesses of a cross-sectional study. This design is non-ideal for studying associations between population variables. Therefore, associations presented here may not be easily extrapolated to the wider population. We also acknowledge that classic Pap smear testing could have lowered the diagnostic power compared to liquid cytology. Another limitation of this study is that we only included women who visited the cervical cancer clinic. This could have caused a selection bias. However, the major strength of this study is its statistical power, which we considered while calculating the sample size. We also employed standard methods during collection, processing, examining, and reporting methods for Pap smear specimens. The results of our study could be generalised to the entire Ugandan population as well as other populations in resource-limited countries. This is so, considering the fact that screening uptake rates are said to be between 30.3%[41] and 44%,[42] with a low HPV vaccine completion rate of 43.3%.[102]

Conclusion

Based on Pap smear cytology, the prevalence of cervical intraepithelial lesions was 6.6%, which was lower than global estimates. Use of hormonal and IUD contraceptives, as well as family history of cervical cancer, showed significant associations with cervical intraepithelial lesions among our study population. Prospective studies are recommended to further understand associations between different types of IUDs, hormonal contraceptives, and cervical lesions.

Policy implications

The current burden of cervical lesions indicates the need to develop and maintain a national cervical cancer program and to put in place efforts that increase cervical cancer screening towards the World Health Organization targets.

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Author contribution

The corresponding author, FS, conceived the idea and developed the first draft of the manuscript. Co-authors TCR, DT, NN, and JN supervised the whole project, from data collection to analysis, and reviewed the manuscript. HL and CMC reviewed and provided overall guidance in the entire write-up and approved the final version prior to submission. JNN, RA, and AB participated in data analysis. All authors reviewed and approved the final version of the manuscript. All authors are accountable for all aspects of this manuscript.

Availability of data and materials

All the data from which this article was generated is available from the corresponding author upon meaningful request.

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Ethics approval

Ethical approval for this study was obtained from Mbarara University of Science and Technology Research Ethics Committee (MUST-2022-612) and Uganda National Council for Science and Technology (HS2722ES). We also sought administrative clearance from the Hospital Director, Mbarara Regional Referral Hospital, before commencement of the study.

Informed consent

Written informed consent was obtained from all subjects before the study.

Consent for publication

Not applicable.

Trial registration

Not applicable.

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Supplemental material

Supplemental material for this article is available online.

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