Addis Ababa University

College of Health Sciences

Department of Medical Microbiology, Immunology, & Parasitology

GENOTYPE DISTRIBUTION OF HUMAN PAPILLOMAVIRUSES & HPV E6/E7 mRNA TEST FOR THE DETECTION OF HIGH-GRADE CERVICAL INTRAEPITHELIAL NEOPLASIA (CIN2+) AMONG GYNECOLOGY COMPLAINTS IN NORTHWEST ETHIOPIA

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Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Philosophy (Ph.D.) in Medical Microbiology

July 2023

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Original Literary Work Declaration

I declared that the dissertation represents my own original work and not submitted to any other institution elsewhere to award any degree, diploma, or certificate. I duly acknowledged the various sources of information, views, and opinions that I consulted and used in the thesis.

Awoke Derbie ----------

Abstract

Background: In developing nations, cervical cancer (CC) is the main cause of cancer-related fatalities in women due to the absence of well-established vaccination and screening programs. Exploring the best triage test for women with cervical abnormalities is a timely area of research to advance cervical screening and management. Further, the distinct proportional impact of each HR-HPV on the distribution of cervical lesions varies geographically. There is a shortage of data regarding the clinical value of high-risk human papillomaviruses (HR-HPV) E6/E7 mRNA test and their molecular epidemiology in cervical samples from Ethiopia, particularly in the current study area. Moreover, despite the fact that HR-HPV infection is an essential biological cause of CC, other socio-demographic factors are not well studied in the nation. Therefore, this study aimed to fill these data gaps.

Objectives: The aim of the study was to determine the HPV genotype involved in cervical lesions, to evaluate the clinical use of HR-HPV E6/E7 mRNA for the early detection of CIN2+, and to explore factors associated with it among gynecology complaints in northwest Ethiopia.

Methods: Between March 2019 and October 2021, a cross-sectional study was carried out at Felege Hiwot Compressive Specialized Hospital (FHCSH). Among women who visited the hospital for gynecological examination, those who were eligible for visual inspection (VIA)-based screening were included. Cervical punch samples were obtained by a gynecologist for histological analysis. Cervical swabs collected and analyzed for HR-HPV DNA and HPV E6/E7 mRNA using the Abbott Alinity m system and real-time PCR, respectively at the Institute of Virology, Leipzig University, Germany. Demographic and gynecologic-related history were collected using a structured questionnaire. The distribution and frequency of HR-HPVs described using descriptive statistics. Histology was used as the reference test to determine how well the E6/E7 mRNA detected CIN2+.

Results: Of the 355 study participants (aged 30 to 80 years), more than half, 211 (59.4%), were unaware of CC, and their previous cervical screening practice was approximately 25%. Cervical biopsies from 41.8% (140/335; 95% CI: 36.6-47.1%) participants were diagnosed as cancer. The proportion of HR-HPV was 53%(188/355; 95%CI: 47.8-58.1%), with 13 different genotypes identified. HPV16 was predominant at 50.4% (95%CI: 29.4-39.2%), followed by HPV31 (9.7%), HPV33 (8.5%), HPV39 and HPV68 (5.8% each), and HPV18 at 4.7%. The

E6/E7 mRNA test was positive in 35.8% (127/355; 95%CI: 30.0-40.9) of cases for HPV16, 16 & 45. The proportion of positive HPV DNA test results for these three HR-HPVS was 42% (149/355). The total agreement of DNA and mRNA tests in the detection of these HPVs was at 92.7% (95%CI: 89.5-94.9) with a kappa value of 0.821. HPV16, at 108 (85%), was the most common genotype expressing E6/E7 mRNA. The mRNA assay had sensitivity, specificity, positive and negative predictive values (PPV and NPV) of 65.2% (95%CI: 57.5-72.2%), 90% (95%CI: 84.6-93.4%), 85.8% (95%CI: 78.5-91.0%), & 73.6% (95%CI: 67.2-79.1%), respectively for detecting histologically confirmed CIN2+. Specifically, the sensitivity and specificity of this assay in the detection of CIN2+ were 92.7% & 47%, respectively among HPV16, 18, & 45 DNA-positive cases. Likewise, the analytical sensitivity and specificity of the HPV-DNA test were 84.8% & 74.1%, respectively. CC increased steadily with participant age, with women older than 50 years about four times more likely to develop CIN2+ (AOR: 3.68 95%CI: 1.75-7.72, $p < 0.001$). Similarly, no cervical screening in the past five years (AOR: 2.04; 95%CI: 1.04-4004; $p = 0.038$), infection with HR-HPVs (AOR: 5.28; 95%CI: 2.66-10.47; $p < 0.001$) and tested positive for E6/E7 mRNA (AOR: 5.78; 95%CI: 2.73-12.24, $p < 0.001$) were statistically associated with CIN2+.

Conclusions: CC is still a significant issue for women's health in northwest Ethiopia that requires evidence-based interventions. The E6/E7 mRNA test and the HPV DNA test demonstrated good agreement and showed better diagnostic relevance in detecting CIN2+. Therefore, the test can be considered for colposcopy and biopsy triage. In particular, the mRNA test may be regarded as a potential triage for women who are HPV-positive , mainly in regions with a shortage of pathologists and colposcopy facilities. Vaccination and future HPV-based screening methods in Ethiopia should consider the important HR-HPV genotypes identified in such studies. To better assess the HPVs circulating in northwestern Ethiopia, community-based surveys should be conducted. Likewise, to optimize the E6/E7 mRNA analytical sensitivity and specificity , large-scale studies targeting major HR-HPVs should be considered. Finally, in accordance with the WHO recommendation women who are eligible for cervical screening need to be screened with a high-precision test, including HPV-based tests.

Keywords: Cervical cancer, CIN2+, HPV E6/E7 mRNA, HR-HPV DNA, northwest Ethiopia

Acknowledgments

First, I would like to convey my appreciation to the DMIP, CHS, Addis Ababa University for the opportunity it has given to me.

My heartfelt gratitude goes to Dr. Tamrat Abebe and Dr. Yimtubezinash Woldeamanuel for their unreserved professional support and guidance starting from the very beginning.

For their ongoing assistance, I want to convey my appreciation to the faculty of the DMIP, CHS, AAU.

I am very much grateful to the Virology Institute, Leipzig University Hospital, Germany for their substantial technical assistance during the HPV molecular analysis.

My special gratitude goes to Dr. Eva J. Kantelhardt for her kind assistance in the Department of Gynecology, Martin Luther University, Halle-Wittenberg, Germany. .

I would like to say thank you to the Center for Drug innovation and Therapeutics trial for Africa (CDT-Africa) at the CHS, AAU, which funded this research. I thank the Institute of Biotechnology at Bahir Dar University and the College of Health Sciences at Addis Ababa University for the partial funding provided for this research.

The study participants and data collectors are also acknowledged.

Finally yet importantly, I would want to express my gratitude to Bahir Dar University, College of Medicine and Health Sciences for supporting me financially.

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List of Symbols and Abbreviations

ASCUS	Atypical squamous cells of undetermined significance
CC	Cervical Cancer
CIN	Cervical intraepithelial neoplasia
CIN2+	High grade cervical lesions including cancer (CIN2+IN3+Cancer)
DNA	Deoxyribonucleic Acid
E6/7	Early protein 6/7
L1/2	Late Proteins 1 and/or 2
LCR	Long Control Region
FHCSH	Felege Hiwot Referral Hospital
HPV	Human papillomavirus
HIV	Human Immunodeficiency virus
IRB	Institutional Review Board
Kb	Kilo base pair
HR-HPV	High-risk Human papillomavirus
mRNA	messenger ribonucleic acid
NA	Nucleic Acid
NOS	Not Otherwise Classified
ORF	Open Reading Frame
Pap	Papanicylo test
PCR	Polymerase Chain Reaction
pHR	Probable High Risk
pRb	Retinoblastoma Protein
SCC	Squamous Cell Carcinoma
VIA	Visual Inspection with Acetic acid
WHO	World Health Organization

Glossary of Terms

The following working definitions were adapted from cited articles (L Bruni et al., 2017; Cuschieri & Wentzensen, 2008; Khieu & Butler, 2022; H. Y. Wang, Lee, et al., 2015).

- **HR-HPV**: the type of HPVs that are associated with cervical & other types of cancer, such as cancers of the anus, vagina, vulva, penis, and oropharynx.
- **HPV E6/E7 mRNA**: these the primary oncogenic transcriptions of HR-HPV oncogenes; that are associated with cell transformation. The E6 and E7 genes are transcribed polycistronically from a single promotor that is located at the 3' of the upstream regulatory region.
- **High-grade cervical lesions (CIN2+)**: Women with histologically verified CIN2, CIN3, and invasive carcinoma are said to have high-grade cervical lesions (CIN2+).

Chapter One: Introduction

1.1. Background

Cervical cancer (CC) is one of the most prevalent diseases in women worldwide, accounting for roughly 8% of all cancer-related deaths in women each year. It is the second most common type of cancer in women between the ages of 15 and 44 worldwide, ranking fourth among cancers that affect women. According to the 2021 world HPV information center report, approximately 604,127 women were diagnosed with CC and 341,831 die from the disease annually (Bruni, 2021; Fernandes et al, 2022). In 2030, the annual number of new cases and death are projected to upsurge to 700, 000 and 400, 000, respectively (Cancer, 2020; Hull et al., 2020). Close to 90% of the newly diagnosed cases and deaths were from countries with low and middle incomes (Bruni, 2021). Sub-Saharan Africa has been found to have the disease's highest burden. (Bray et al., 2018; De Vuyst et al., 2013; Hull et al., 2020; WHO, 2014a).

Ethiopia, with over 110 million people, is the second most populous nation in Africa after Nigeria (Federal Democratic Republic of Ethiopia, 2021). An estimated 33.7 million females aged $\geq$15 are at risk of CC. With an estimated 7445 new cases and 5338 fatalities each year, CC is Ethiopia's second-leading form of cancer in women behind breast cancer. Well-established data on the burden of CC in the general population of Ethiopia is missing (Bruni, 2021).

More than 99% of CC cases are associated with genital infection with certain types (called, HR,High-Risk) Human papillomavirus (HPVs). It is the most common viral infection of the reproductive tract on a global scale. (Kambouris et al, 2010; WHO, 2013a) that the majority of women experience shortly after becoming sexually active (Maine et al, 2011). The main cause of CC is persistent infection with HR-HPVs (Santos-Lopez et al, 2015; WHO, 2016). Along with CC, a significant number of other cancers (including anus, vulva, vaginal, and penis cancers) as well as an increasing number of head and neck malignancies have also been associated with HR-HPVs (Bruni et al., 2017; Centre, 2017; Chow et al, 2010; Doorbar et al., 2012; Wallace & Galloway, 2015).

More than 200 distinct HPVs identified and fully sequenced. About 40 of all types are transferred sexually and affect the genitalia (Doorbar et al., 2012; Kajitani et al, 2012; Münger et al., 2004; Narisawa-Saito & Kiyono, 2007; Williams et al, 2011). Significantly, HPV 16 &

18 account for >70% of all CCs worldwide. (Bouvard et al., 2009; Bruni et al., 2017; Kim, 2017). However, there are very little national statistics on Ethiopia's HPV genotype distribution. . Our present systematic review aimed at assessing the molecular profile of HPV from cervical samples in Ethiopia showed that full HPV genotype data is reported by very few studies in the country (Derbie et al., 2022). This implies that there is a need to have more data from different parts of the country for evidence-based vaccination and HPV-based screening approaches.

If caught early enough, CC is treatable. Early lesions develop slowly before cancer, often over a period of ten years, making premature discovery advantageous. (Leto et al, 2011). Hence, tests that predict the progression of cervical lesion, especially when the lesion is at its early stage are worth to discover.

Cervical precursor lesions, are detectable by a variety of methods (García et al., 2011). Globally CC screening and diagnosing strategies vary across countries. The most frequent method is cytology, and there are other approaches such as HPV-DNA tests & visual inspection of cervix with acetic acid (VIA) (L Bruni et al., 2017), the latter being practiced in resource-limited settings, including Ethiopia, where target women get the 'see and treat' services (Bruni et al., 2017). In the majority of developed countries, cytology and HPV-DNA based testing are widely accessible (Sahasrabuddhe et al, 2011). HPV-based tests are introduced as a supplement to cytology screening (referred to as "co-testing") or as the initial screening test to be followed by a secondary, more specific test, such as cytology or HR-HPV E6/E7 tests (Bruni et al., 2017; Cuschieri & Wentzensen, 2008; Wang et al., 2015).

On the contrary, in nations with poor resources, like Ethiopia, it has been extremely challenging to provide and maintain either cytology or HPV-based assays due to a number of factors; resource limitation and absence of professionals; for instance, in Ethiopia the number of pathologists are <200 [expert opinion]. Moreover, the CC screening program in Ethiopia is not well-established in most parts of the country (Sankaranarayanan et al, 2013; WHO, 2015). The VIA-based screening coverage for targeted women is much less than the African average (3.3%) (Federal Democratic Republic of Ethiopia, 2021) whereas screening coverage in Sub-Saharan Africa ranges from 2-20% in urban areas and 0.4-14% in rural regions (Louie et al, 2009).

Obtaining tools with adequate diagnostic performance for CC screening continues to be difficult in the global campaign against this disease. There has been a long-standing interest in the development and validation of new screening tools due to the shortcomings of existing methods as well as the limitations of cytology, VIA, & HPV-DNA including but not limited to the sensitivity/specificity issues and the ability to highlight the possibility of developing cancer. (Sahasrabuddhe et al., 2011). Currently, there are discoveries under went to develop CC screening biomarkers, of which, the HPV E6/E7 mRNA tests are promising noninvasive biomarkers for the detection of CIN2+ which enable to detect the HPV infection and to predict the change of progression to cancer (Cattani et al., 2009; Duvlis et al., 2015; Fontecha et al, 2016; Fontecha et al., 2017; Lie & Kristensen, 2008; Mariano et al., 2016; Castro et al., 2013; Varnai et al., 2008; Zhao et al., 2014).

As a result, it is claimed that detection of E6/E7 mRNA is more accurate and better able to predict CC risk than the HPV DNA test(Cattani et al., 2009; Duvlis et al., 2015; Fontecha et al., 2017; Lie & Kristensen, 2008; Mariano et al., 2016; Castro et al., 2013; Ratnam et al., 2010; Varnai et al., 2008; Zhao et al., 2014).

In the developed world, E6/E7 mRNA is well studies to clarify the oncogenic importance of HR-HPVs in CC and other types of malignancies. (Fontecha et al., 2016; Castro et al., 2013; Ren et al, 2018; Varnai et al., 2008; Wang, et al., 2015; Zhao et al., 2014). Studies also showed that E6/E7 mRNA test had greater specificity (Cattani et al., 2009; Duvlis et al., 2015; Fontecha et al., 2017; Lie & Kristensen, 2008; Mariano et al., 2016; Castro et al., 2013; Varnai et al., 2008; Wang, et al., 2015; Zhao et al., 2014) and compared to HPV DNA testing, it correlate better with the severity of the lesion, and may be a possible signal of women who are at risk of getting CC (Fontecha et al., 2016; Kristensen, 2008). Our recent systematic review on testing of E6/E7 mRNA in the identification of CIN2+ showed a very good result on this regard (Derbie et al., 2020a). However, researchers in the field recommended further studies in different settings to accumulate knowledge for additional steps to standardize the mRNA test (Cattani et al., 2009; Duvlis et al., 2015; Fontecha et al., 2016; Fontecha et al., 2017; Kristensen, 2008; Liu et al., 2018; Mariano et al., 2016; Castro et al., 2013; Varnai et al., 2008; Zhao et al., 2014).

HPV-based studies including the clinical role of mRNA in marking of CIN2+ are minimal in the developing world. Additionally, there is a dearth of information on the national genotype

distribution of HPV and the risk factors for CIN2+ in Ethiopia. Therefore, the objective of this study was to assess the analytical value of E6/E7 mRNA for CIN2+ detection, assess factors associated with CIN2+, and characterize the HR-HPVs. These are important entry points for treatment of HPV associated cervical lesions, helps to identify patients at higher risk for developing CC, and can be used for the development of vaccines and HPV-based screening.

1.2. Problem Statement

Most low- and middle-income nations lack access to cancer registry data, which is the foundation for designing and executing evidence-based cancer control initiatives (Bray et al., 2018), including Ethiopia. The Addis Ababa cancer registry, which was established in 2011, is Ethiopia's first institutional-based registry. According to this registry, CC (14.1%) was one of the leading prevalent malignancy in Ethiopia during the time of 2012 to 2015 (Memirie et al., 2018; Network, 2018; Timotewos et al., 2018).

CC becomes one of the challenges to public health in Ethiopia. Periodically, both the incidence and prevalence are rising (WHO, 2020a). In 2020, Ethiopian women's age-standardized incidence rate of the illness was 21.5/100,000 (Bruni, 2021). The international agency for research on cancer estimates that the predicted 7,500 new cases of CC in 2020 could increase to 15,300 cases in 2040. Similarly, the mortality from the disease could increase from ~5,340 in 2020 to 11,000 in 2040 yearly in Ethiopia (Cancer, 2020). Despite all these devastations, so far, the country does not introduce better CC screening practices and well-established CC vaccination programs although the Ministry of Health started vaccinating schoolgirls (using Gardasil 4) at the end of 2018. The VIA screening coverage is insignificant (3.3%) among women aged 18–69 years (Agency, 2011; Federal Democratic Republic of Ethiopia, 2021; WHO, 2010). On top of these, there is limited data on community-based HPV profile and important demographic factors associated with CIN2+ for integrated public health measures.

The suggested strategies to reduce the prevalence of CC are HPV vaccination, HPV-based screening and treatment of precancerous lesions. With the life-course approach, the WHO envisions a world in which CC is no longer a concern for public health. By 2030, each country should meet the 90-70-90 targets; i.e. 1) by the age of 15, 90% of girls must receive the full HPV vaccine; 2) by the ages of 35 and again by 45, 70% of women must have screening with high-performance screening tests, and 3) 90% of women identified with cervical disease shall receive treatment (WHO, 2020b). Though Ethiopia has issued a guideline to meet the global targets, the life-course approach for CC prevention is at an early stage (Federal Democratic Republic of Ethiopia, 2021). Most women are diagnosed at an advanced stage of the disease due to a lack of an efficient program and low uptake of the available strategy.

The global uptake of vaccination of young girls is low; 32 and 0.3% in both high- and low- and middle-income environments, respectively (Bruni et al., 2016). Although there are a variety of

tools for screening, it will take work to increase the service's effectiveness, accessibility, and uptake. The different tools available in the market have their pros and cons. Countries like Ethiopia that have no organized screening programs need to identify which of these different tools work best in their setting. So far, there is no one perfect method for CC screening; the early detection of CIN2+, however, can benefit from the integration of many strategies.

The WHO advises screening women between the ages of 30 and 49 using cytology or VIA every three to five years, or HPV DNA testing every five years, along with prompt treatment of precancerous lesions(WHO, 2020b). Cytology-based programs are, however, extremely challenging to implement in low- and middle-income countries, and where they are, the screening coverage is poor. Therefore, new strategies for screening and treatment had to be considered to increase coverage in all countries and ultimately decrease CC incidence and mortality. In the developed world, in addition to using HPV DNA and cytology for screening, countries are also studying the potential role of ThinPrep cytological test (TCT), mRNA based tests (e.g. Aptima and PreTect HPV-Proofer) and colposcopy for early detection of CIN2+, a stage where intervention is started (Zhang et al, 2018). Recent data also showed that Xpert HPV assay fulfills the HPV test criterion requirement to be used in the detection of CIN2+ (Akbari et al., 2018).

Women with abnormal cytology or HPV DNA test need to have a follow-up histopathological evaluation to look for the presence and stage of CINs. Abnormal cervical lesions could be missed or misdiagnosed due to the inherent limitations of VIA and cytology (WHO, 2007). Females at high risk must undergo routine retests to verify the veracity of the negative result due to the low sensitivity of the cytological test (Marth et al., 2017; WHO, 2007), which is expensive. Additionally, only a small percentage of individuals with negative or atypical squamous cells of unknown significance (ASCUS) but with positive HR-HPV DNA will ultimately develop CIN2+ (Apgar et al, 2009). To manage such women, it would be useful to use better biomarkers for triage (Zhang et al., 2018). Further, in developing countries like Ethiopia, where there are very few pathologists the problem becomes much worsen. Taking into account the prospective implementation of the HPV DNA test as a screening tool in Ethiopia, getting triage test for further steps to properly manage women with abnormal cytology is a timely area for research (Burger et al, 2011; Melnikow et al., 2018; Valenca et al., 2016).

Compared to similar testing (like VIA, Pap smear, and HPV DNA) that cannot distinguish between persistent and transitory HPV infections, the detection of E6/E7 oncogene products serve as a better predictor of the risk of developing CC (Burger et al., 2011; Cattani et al., 2009; Duvlis et al., 2015; Fontecha et al., 2017; Lie & Kristensen, 2008; Liu et al., 2018; Mariano et al., 2016; Castro et al., 2013; Ratnam et al., 2010; Varnai et al., 2008; Zhao et al., 2014). The HPV DNA is incorporated during the carcinogenesis process, which disrupts the E2 gene and causes an overexpression of E6 & E7. Therefore, testing these gene transcripts can be used as a potential marker for a greater risk of developing CC (Alaghehbandan et al., 2013; Ratnam et al., 2011). mRNA test helps lessen patients' worry and follow-up times while assisting in avoiding invasive procedures such as biopsies and over-referral for transit HPV infections (Duvlis et al., 2015).

Generally, it is not clear what to do with women who tested positive for HR-HPVs in Ethiopia. There are few colposcopy facilities and trained personnel to triage the high number of HPV DNA-positive women. Hence, there is a need to find a cost-effective triage-test for women who tested positive for HR-HPV DNA as it will not be feasible to consider colposcopy and histology for all HPV DNA-positive cases in Ethiopia.

On top of this, to draw up optimal vaccination and HPV-based screening approaches, it is crucial to identify the HPV genotypes circulating in the country, which is otherwise quite limited in Ethiopia (Bruni, 2021) and in the present study area in particular (Derbie et al., 2022). According to our recent review on HPV molecular epidemiology from cervical sample in Ethiopia (Derbie et al., 2022), we found only a few studies (Abate et al., 2013; Bekele et al, 2010; Leyh-Bannurah et al, 2014; Mihret et al., 2014) that provided data on this regard. Specifically, the only data in the present study area is almost none. Additionally, while HPV is not the only factor that leads to CC (Bray et al., 2018), exploring the possible associated epidemiological factors for CIN2+ will also play a great role in public health measures.

1.3. Research Questions

- Which HR-HPVs are linked with abnormal lesions of the cervical in northwest Ethiopia?
- What is the sensitivity/specificity of E6/E7 mRNA in the identification of CIN2+?
- Which epidemiological factors are associated with CIN2+ in the study area?

1.4. Hypothesis

- HO1: The types of HR-HPVs involved in CIN2+ in Northwest Ethiopia will be the same as other study findings in the country.
- HO2: E6/E7 mRNA expression correlates better with the severity of cervical lesions and could be considered as a potential biomarker for the detection of CIN2+.

1.5. Objectives

1.5.1. General Objective

To characterize the genotype epidemiology of HR-HPVs involved in abnormal cervical lesions, to study the analytical performance of HR-HPVs E6/E7 mRNA test for identifying CIN2+, & to explore epidemiological factors associated with CIN2+ among women with gynecology complaints in northwestern Ethiopian.

1.5.2. Specific Objectives

- To describe the cervical histopathological profile of the participants,
- To identify HR-HPVs associated with abnormal cervical lesions,
- To evaluate the analytical performance of the E6/E7 mRNA test in the identification of CIN2
- To investigate epidemiological elements connected to CIN2+ ,

1.6. Significance of the Study

Even though CC-related morbidity and mortality are extremely prevalent in Ethiopia, HPV-based research on screening/diagnostics, HPV genotype distribution from different grades of cervical lesions and their epidemiological predictors are at an infancy stage. Let alone HPV-based research, basic epidemiological data on CC prevalence, incidence and mortality with its predictors are scarce in resource-limited settings, including Ethiopia (Shrestha et al, 2018). Our study provided data on some of these topics.

About 80% of people will contract HPV at some point in their lifetime (Arbyn, et al., 2010; Muller, et al., 2010), consequently, more precise indicators, such as E6/E7 mRNA, are required for the early detection of aberrant cervical lesions (Shen et al., 2013). Women were more likely to be positive for mRNA if they had CIN2+ & persons who tested negative for the mRNA are at low risk of progression to CIN2+ (Rijkaart et al., 2012; Shen et al., 2013). As a result, based on previous study reports, a low sensitivity rate and high specificity of an mRNA test can result in a low referral rate (Shen et al., 2013) which indirectly reduce cost (Zappacosta et al., 2015). The mRNA results together with physician's assessment used to guide better management (Arbyn et al., 2012; HOLOGIC, 2017; Tezcan et al., 2014). The mRNA assay can potentially be utilized as a triage test to find women who are more likely to develop CIN2+, as mRNA tell us about the active replication of the virus in the cervix (Wang, et al., 2015; Zhao et al., 2014).

Furthermore, HPV profiling data from this study will be an input for vaccination and HPV-based screening strategies which facilitate the prevention and tracking of disease burden specific to each HPV type (Lagheden et al., 2018). CC screening might also contribute to the prevention of anal cancer in women (Lin et al., 2019). Because the carcinogenic potential of HPVs is reliant on particular genotypes, genotyping is crucial for clinical purposes (Chung et al., 2012). Hence, genotyping could resolve type-specific persistence questions correctly (Geraets et al., 2014). The triage of women with marginally abnormal cytology for colposcopy and as a test of cure following treatment, in particular, benefits from HR-HPV testing (Geraets et al., 2014; Meijer et al., 2009). Furthermore, because CC is caused by multiple factors, not just HPV (Bray et al., 2018), exploring the potential factors will be a key component of public health measures. Additionally, in order to evaluate the long-term effects of HPV vaccination and to determine the priority of public health measures, epidemiological information from women with CIN2+ will be essential.

Therefore, in this molecular, histopathological and epidemiological-based study, the finding will serve as input to policy makers and other stakeholders for vaccination programs, HPV-based CC screening and public health measures to decrease the burden of CC in the country. The finding will be also used as a potential source for further HPV-based large-scale studies in Ethiopia.

Chapter Two: Literature Review

2.1. HPV Virology

Human papillomavirus was discovered in 1956 (UK, 2014). In 1984 Harald zur Hausen discovered, cloned, and attributed CC to HPV16 and 18 (UK, 2014). The man was honored with the Nobel Prize in 2008 for his contribution to science. He made the early hypothesis that CC is caused by papillomaviruses. He was successful in identifying the two most prevalent HR-HPV kinds (HPV16 and HPV18) at CC, and subsequent actions improved our understanding of the mechanism behind HPV-associated cancer development and paved the way for the creation of a preventive vaccination. (Prize, 2008; UK, 2014).

HPVs refers to a group of >200 related viruses that belong taxonomically to Papillomaviridae family (Institute, 2022). They are ds-DNA viruses can infect the skin and mucous membranes because of their preference for epithelial cells. They are named because of the warts (*papillomas*) which some HPV types causes (Prevention, 2016). Based on the primary L1 capsid protein nucleotide sequences, HPVs grouped into five distinct genera. These are: *Alpha*, *Beta-*, *Gamma-*, *Mu-*, & *Nupapillomavirus.* The *Alphapapillomavirus* that contains the mucosal HR HPV types are linked to anogenital, and head & neck cancers (Institute, 2022). Each HPV virus in this large group is assigned with a number (HPV 6, 11, 16 etc.), in the order of their discovery, which is called its HPV type. The last HPV type recorded in the International HPV center database is HPV229 (Institute, 2022).

It is not possible to propagate HPV in vitro, and therefore, its identification and characterization is based on molecular analysis (Abreu et al, 2012). They are epitheliotropic, small (~55nm diameter), non-enveloped with an icosahedral capsid (Münger et al., 2004; Pinidis et al., 2016).

The DNA is linked to histone-like proteins and enclosed in 72 capsomeres made of L1 and L2. (McMurray et al, 2001). As depicted below in Figure 1, its genome is divided into 3 area: a ~4-kb Early (E), a ~3-kb Late (L) and a ~1-kb long control region (LCR). Only one of the two DNA strands of HPV (that is approximately 8,000 base pairs) is actively transcribed (Münger et al., 2004). The **E** region contains ORFs (open reading frames) encoding non-structural proteins that designated from E1 to E8. Two of the late genes encode the L1 and L2 coat proteins, necessary for virion formation, transmission and spread. Genes of the E&L region are

numbered based on their size; the higher the number, the smaller the corresponding ORF (Münger et al., 2004; Pinidis et al., 2016). The LCR region is not well conserved among the HPVs and is involved or regulates gene expression and viral replication (Leto et al., 2011; McMurray et al., 2001). HPV genome is static and changes in their sequence by mutation or recombination are very rare events (Leto et al., 2011).

Papillomaviridae contains about 16 genera (based on shared sequence homology within the L1 capsid gene), which are named with a Greek letter prefix (the alpha-, beta-, gamma-, delta-, mu-, nupapillomavirus and so on). Only the alpha- and beta-papillomavirus genera linked with cancer. HPVs infecting the genital tract (which are members of the α-papilomavirus) have been divided into two groups: HR or low risk based the tendency for malignant progression of the lesions that they cause (Chow et al., 2010; Santos-Lopez et al., 2015; Wallace & Galloway, 2015).

Most HPVs for example HPV6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81 are low risk that produces localized benign warts and do not undergo malignant progression (Sanjose et al., 2010). The high risk-HPVs include HPV16,-18,-31,-33,-35,-39,-45,-51,-52,-56,-58,-59,-66, & -68. These types associate with severe dysplasia and cervical carcinoma. Some other groups are classified as potential or probable high-risk (pHR) types including HPV53, 70, 73, and 82. HPV16 and 18 are the well-known oncogenic virus types, causing >70% of all CC burdens globally. Persistent infection with about eight types of HR-HPVs (HPV 16, 18, 45, 31, 33, 52, 58, and 35) are associated with approximately ~90% of all CC cases worldwide (Sanjose et al., 2010; FMoH, 2013; WHO, 2010, 2013a). It is believed that changes in HR-HPVs' E6 and E7 expression are associated with cancer. (Egawa et al, 2015; Fontecha et al., 2016; Hesselink et al., 2008; McBride, 2017; MicroBiotics, 2013; Münger et al., 2004; Santos-Lopez et al., 2015).

By a variety of ways, HR-HPVs reduce immune clearance; like, resulting in long-lasting infections without symptoms, long-term virion generation, and minimal viral gene expression (Egawa et al., 2015). The E6 & E7 onchoproteins could each hinder innate immunity by inhibiting interferon signaling and the E5 proteins can also down regulate MHC I expression, therefore avoiding immune surveillance (Patricio et al, 2016).

When HPVs infect squamous epithelial cells, several clinical consequences can result, from non-life-threatening infections to cancer that is fatal. Malignancy is associated with the E6 & E7 oncoproteins (which are supposed to produced following HPV DNA integration with the genome of the host) that eventually bind & damage host tumor-suppressor-proteins. E6 changes p53, which prevents the cell cycle from continuing in order to repair DNA damage. This results in the changed DNA continuing to replicate constantly, which raises the mutation rate. E7 changes retinoblastoma, which prevents a cell from growing quickly, and improves a cell's response to growth factors. Malignant transformation results from the loss of these two inhibitory systems (Prize, 2008; UK, 2014).

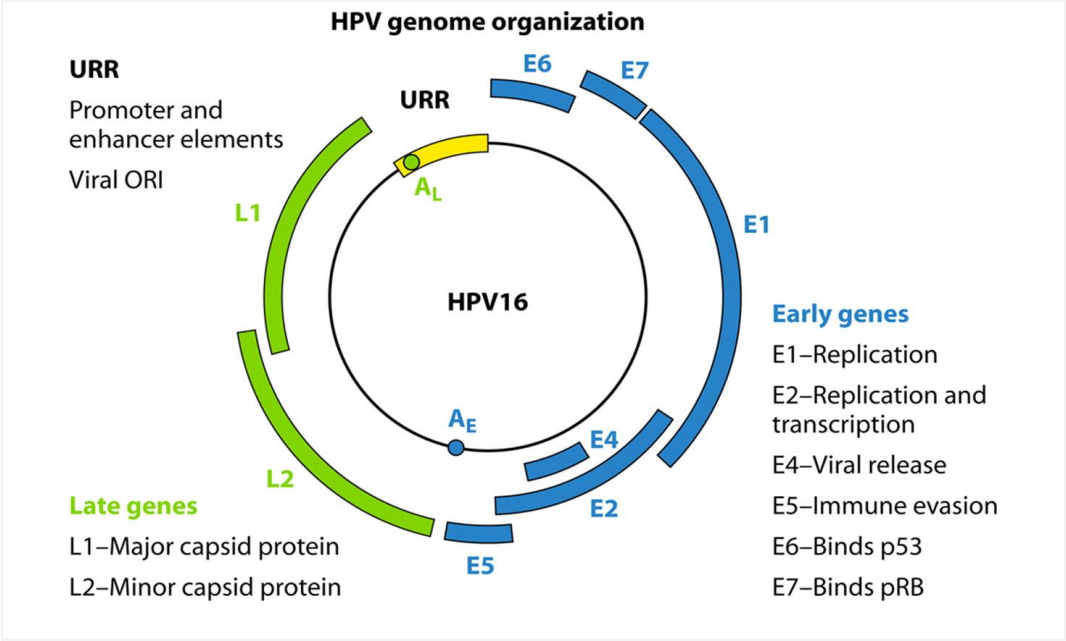

Figure 1: Diagrammatic representation of the HPV16 circular dsDNA.

The E, L genes, & LCR, are depicted here. The late genes (L1 and L2) bundle HPV virions, while the early gene products aid in genome proliferation. The genome is enclosed by a capsid composed of two structural proteins, the major protein L1 (which is highly conserved) and the L2 protein, which is internally located multifunctional protein with roles in genome encapsidation. The genome is approximately 8kbp. The early ORF (E1,E2,E4,E5,E6 & E7) expressed at the various stages of epithelial differentiation. Whereas the L1 / L2 are expressed in cells replicating viral DNA in the upper epithelial cells. The E3 & E8 genes are belived to be found only in a few forms of papillomaviruses (HPV1,-11,-16,-31,& HPV33). ***Source****: http://cmr.asm.org/content/25/2/215/F2.expansion.html and (Avci, 2012; Horvath et al, 2010; Narisawa-Saito & Kiyono, 2007).*

2.2. HPV Replication

HPV replication cycle is firmly associated with differentiation cycle of the infected epithelium (Sapp & Bienkowska-Haba, 2009; Wallace & Galloway, 2015). The life cycle is biphasic, consisting of both viral maintenance and amplification steps (Wallace & Galloway, 2015). Like other viruses, HPVs must introduce their genome and auxiliary proteins into host cells before using the cellular machinery for replication. HPV particles travel from the cell surface to the cytosol and then to the nucleus, where they replicate (Horvath et al., 2010; Howie et al, 2009).

HPVs initially infect basal epithelial cells, probably through abrasions & access cells via receptors (α-6 integrin of HPV-16) then the viral genome replicates slowly along with its host cell (Wallace & Galloway, 2015). The only cell layer in an epithelium that is actively dividing is the basal epithelial cells. The virus keeps minimal copies of its genomic DNA in the nuclei of infected basal cells, preventing the viral productive lifecycle from progressing. If the infected daughter cell moves laterally, remaining in the basal layer, HPV continues the maintenance portion of its lifecycle. It is clear that viral replication and host differentiation systems closely interact because the productive lifespan requires host cell differentiation. (Kajitani et al., 2012; Wallace & Galloway, 2015). In terminally differentiated cells, the HPVs replicates to high-copy, late-genes expressed, viral DNA packaged into capsids and progeny virus is produced and released to re-initiate infection (Kajitani et al., 2012; Münger et al., 2004; Narisawa-Saito & Kiyono, 2007). Summary of HPV replication cycle is illustrated in Figure 2.

Another important aspect of the infectious cycle of HPV is establishment of long-term persistence in squamous epithelia, where cells constantly undergo differentiation and differentiated cells shed. Their carcinogenic potential strongly influenced by the particular strategies that HR-HPVs have developed to overcome these obstacles. (Münger et al., 2004).

As the infected epithelial cell develops, the HR-HPVs activate the cell cycle to produce a replication-competent environment that permits viral genome amplification and packaging into infectious virion. The E6, E7, and E5 gene products arbitrate this. Due to their high affinity to bind with p53 & pRB, the HR-HPV E6 and E7 proteins to stimulate cell proliferation in the basal and parabasal layers as well as to enhance cell cycle entry in the higher epithelial layers sets them apart from low risk varieties. (Doorbar etal., 2015). Hence, targeting the gene products of E6 & E7 as potential markers to detect precancerous lesions in the management of

patients with abnormal cervical cytopathology is becoming a novel approach in the screening and diagnosis of CC research.

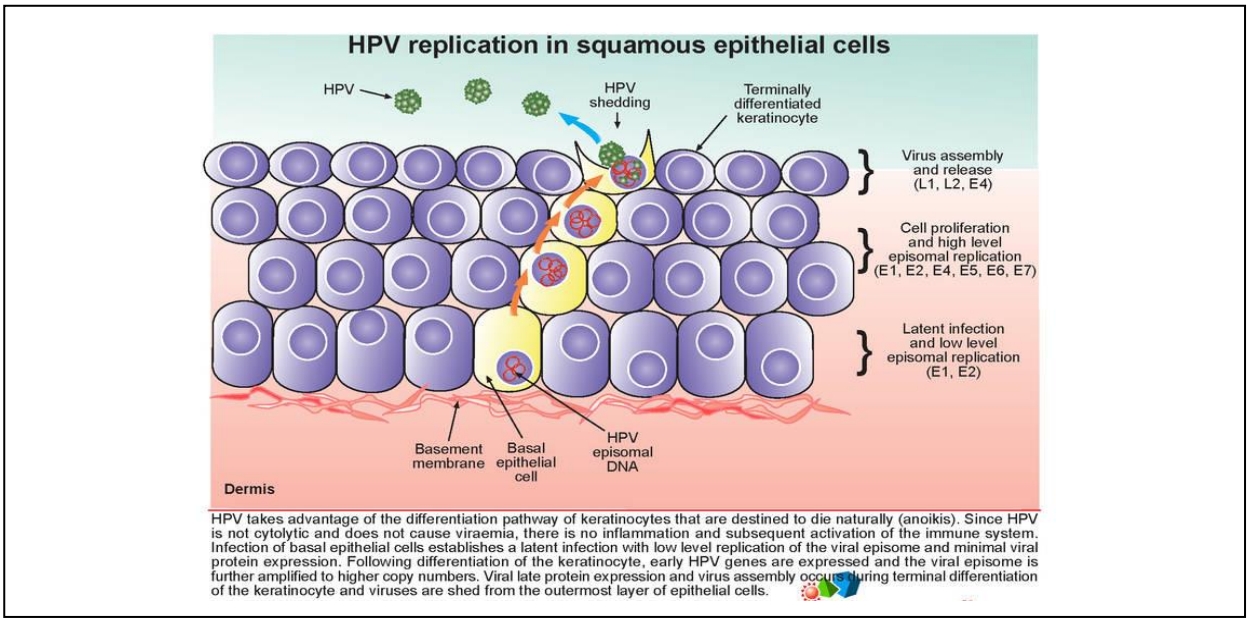

Figure 2: HPV replication in squamous epithelial cells of the cervix (Immunopaedia).

Through micro-abrasions or by infecting the transformation zone—an abrupt change from a columnar to a squamous epithelium—HPV can enter basal cells. Normal squamous epithelial cells divide as stem cells or transit-amplifying cells as they mature as stratified epithelium in the basal layers. One of the daughter cells migrates upward after dividing and starts to go through terminal differentiation, while the other one stays in the basal layer and continues to cycle slowly and self-renew. By attaching to and unwinding the replication origin, the E1 and E2 genes play a direct role in viral replication. In order for the infected stem cells to remain in the lesion for an extended amount of time, they support viral DNA replication and segregation. Viral late gene products (L1 and L2) are created to start the vegetative phase of the HPV life cycle as infected daughter cells move to the higher layers of the epithelium, leading to high-level amplification of the viral genome. The late HPV proteins form the viral capsid transcribed only during the latter portion of viral amplification, when infectious viral particles are produced. To uncouple differentiation from continued cellular propagation, HPVs express two oncoproteins, E6 and E7 that have cell-transforming properties (Howie et al., 2009; Narisawa-Saito & Kiyono, 2007; Wallace & Galloway, 2015).

2.3. The Role of HPV E6/E7 Oncogenes in the Development of CC

Viral oncoproteins are expressed during the integration of the viral genome into the host genome, but no contagious viral offspring is created. It is well known that during typical productive HPV infections, the expression of the E6 and E7 oncoproteins is tightly regulated, and infected cells hardly ever undergo malignant transformation. Cells are more susceptible to undergoing transformation when oncogene expression is dysregulated as a result of viral integration into the host genome or as a result of epigenetic changes to the viral gene. This results in an increase in cellular lifespan (including resistance to death), DNA synthesis, genomic instability, and disruption of the immune system's ability to respond to viruses and tumors(Patricio et al., 2016; Yim & Park, 2005).

The development of CC associated with HR-HPV is dependent on viral inactivation the p53 and retinoblastoma protein-pRb & the successive accumulation of DNA damages (Immunopaedia, 2018). Tumor suppressor proteins interact with the E6 and E7 oncoproteins of HR-HPV types, which inhibits their actions and results in unchecked cell proliferation and immortalization (Avci, 2012). Increased synthesis of E6 and E7 occurs following chromosomal integration of HPV genomes carrying a disrupted E2 gene. The HPV E2 gene encodes a transcription factor E2 that suppresses the transcription of E6 & E7 proteins. These transcription can only be increased if the E2 gene is disrupted (Immunopaedia, 2018).

p53 and pRb are essential in preventing cells with damaged DNA from dividing, thereby preventing transformation, and are known as tumour suppressor proteins. When the synthesis of E6 and E7 proteins is higher, E6 binds to p53 in the cytosol and recruits the E6AP (E6 activating protein) ubiquitin ligase that ubiquitinates (‘a process for protein inactivation’) p53 and targets it for proteosomal degradation. Similarly, HPV E7 binds to pRb in the cytosol and takes on the cullin-2 ubiquitin-ligase that destruct pRb & promotes proteosomal destruction. Damage of p53 and pRb proteins ultimately allows a cell with DNA damage to divide thus increasing the likelihood of developing cancer . (Immunopaedia, 2018; Hickman et al., 2002; Sherr & McCormick, 2002).

Lacking the function of both the p53 and pRB pathways contributes a significant part in cancer development. Deregulated cellular proliferation and apoptosis happen when there is a loss of pRB, whereas a loss of p53 desensitizes cells to checkpoint signals, including apoptosis (Hickman et al., 2002), eventually cancer will be evident.

2.4. Role of E6/E7 mRNA in Marking CIN2+

To reduce the impact associated with CC, early detection of cervical lesions should focus on the use of appropriate methods (Laudadio, 2013; WHO, 2013b). The various available tools to detect different grades of cervical lesions include cytology, HPV DNA test, and VIA (L Bruni et al., 2017; WHO, 2013b). Cytology is the most frequent method, the other are alternatives. VIA is an option to cytology-based screening in low-resource settings, including Ethiopia. All these tests suffer from poor specificity in detecting CIN2+.

Cytology-based screening (introduced in the 1940s) (Inc., 2018) has several limitations. It is dependent based on the individual's subjective assessment of the level of nuclear atypia (Sahasrabuddhe et al., 2011). It has a mild level of sensitivity (approximately 55%) for the detection of CIN2+ and suffers from subjectivity as well (Duvlis et al., 2015; Tian et al., 2014). Results from Pap tests that are unclear or only marginally abnormal necessitate costly retests or other diagnostic services like colposcopy and cervical biopsy-histology. In developed nations, Cytology screening is combined with HPV DNA testing ('co-testing') or as the primary screening test (Bruni et al., 2017; WHO, 2013b) to be followed by a secondary test that is with better specificity, such as HPV E6/E7 gene products (mRNA or its corresponding proteins) (Shi et al., 2017; Zhang et al., 2018).

For the triage of women with questionable cytology and for follow-up after treatment of high-grade cervical lesions, testing for carcinogenic HR-HPVs is becoming more popular (Grce & Davies, 2008). Here, the bottleneck is to find out a marker or a tool that demonstrate reasonable accuracy, reproducibility and clinical utility in developing countries (Lie & Kristensen, 2008). In low-resource nations, including Ethiopia, it is hard to launch and endure cytology and HPV-based testing tools, due to resource constraints (Sahasrabuddhe et al., 2011). In such settings, limited access to effective screening means that CC is often not detected early. In addition, prospects for the treatment of such late-stage disease may be poor.

Researchers in western countries are recommending testing HR-HPV E6/E7 mRNA assay that provides valuable clue as an alternative new tool to screen cervical abnormalities associated with HPV infection; it is important as a potential biomarker for early forecast and successive progression to cancer (Duvlis et al., 2015). Studies have demonstrated that the testing for HR-HPV DNA may not be as indicative of CC risk as mRNA (Kraus et al., 2006; Molden et al., 2005). HPV E6/E7 mRNA testing may be a useful biomarker for the diagnosis of CIN2+ in women infected with some strains of HPV genotypes, according to prior studies in this field (Fontecha et al., 2016; Castro et al., 2013; Ren et al., 2018; Varnai et al., 2008; Wang, et al., 2015; Zhao et al., 2014).

Additionally, compared to HPV DNA testing, HPV E6/E7 mRNA testing for HR-HPVs appears to correlate better with the severity of the lesion, making it a potential marker for the triage of women at risk of developing CC. Consequently, commercial tests for the majority of common high-risk HPV types' simultaneous genotyping and detection of E6/E7 mRNA are now becoming accessible (Lie & Kristensen, 2008). Additionally, it has been claimed that the

E6/E7 mRNA could be a trustworthy test for both primary CC screening and the triage of borderline cytological abnormalities (Ratnam et al., 2011). Furthermore, the E6/E7 mRNA-based test can compensate for the HPV-DNA test's limited specificity in the identification of CIN2+ (Munkhdelger et al., 2014). The risk of cervical lesions turning into cancer is higher in women who tested positive for HPV E6/E7 mRNA, necessitating additional care and sooner check-ups (Bruno et al., 2018)

A meta-analysis by Yang and his colleague showed that women with positive HPV E6/E7 mRNA test results are more likely than women with negative results to proceed to CIN2+ in two years (Yang et al, 2017). Additionally, this study discovered that a positive HPV E6/E7 mRNA result indicates that the women with atypical squamous cells of unclear significance (ASCUS) or low-grade squamous intra-epithelial lesions (LSIL) were in a strictly dangerous stage, which is a bad prognostic indicator. It was recommended that cervical lesions continue to grow and that these women should be referred for a colposcopy and immediate strengthening of follow-up. Conversely, women whose mRNA testing results were negative could extend their follow-up period, avoiding needless colposcopy and lowering the incidence of colposcopy and biopsy (Yang et al., 2017).

Other similar studies also revealed that, testing for HR-HPV E6/E7 has the same sensitivity as HPV DNA tests, but is more effective for detecting CIN 2+. In addition, for determining the likelihood of progression and offer a viable tool for triage during CC screening, it may serve as a more specific test (Cattani et al., 2009; Zhao et al., 2014). It may, however, overlook some CIN 2+ cases. This is a significant reported limitation (Zhao et al., 2014). Women with negative mRNA test results cannot be assumed to be free of CIN2+ due to the lesser sensitivity, and therefore need to be monitored more closely (Verdoodt et al., 2013). However, the mRNA-based test is unable to either detect all HR-HPVs or identify the specific types of identified HPVs. In order to avoid this, a study recommends utilising a real-time PCR assay to find type-specific E6/E7 mRNA. Additionally, funding is provided for more study to establish a gold standard for RNA expression detection so that it can be incorporated into the regular cervical screening programme (Fontecha et al., 2017).

There are currently a number of commercially available E6/E7 mRNA-based tests, two of which are frequently used in HPV research: the PreTectTM HPV-Proofer (which targets HR-HPVs; 16, 18, 31, 33, and 45) and the Aptima HPV assay (which targets 14 types; HPV16,18,31,33,35,39,45,51,52,56,58,59,66, & HPV68) (Shen et al., 2013).

2.5. Genotype Distribution of HPVs in Ethiopia

The prevalence and type distribution of HR-HPVs vary greatly in different geographical areas. For the introduction of vaccination and HPV-based diagnostics, nationwide data set on the molecular distribution of HPVs from CC is essential (Sanjose et al., 2010). The most frequently identified genotypes from CC were HPV16,18,31,33,35,45,52, &58, with a combined worldwide relative contribution of 91 percent, according to a study on the distribution of HPV genotypes that included 38 countries from Europe, north & south America, Asia, Oceania, & Africa. In this large investigation, CC patients with HPV types 16 & 18 were found in 71% of cases (Sanjose et al., 2010).

Another similar large-scale study conducted in Ghana, Nigeria, and South Africa found that the most common HPV types from CC cases were HPV16 (51.2%), HPV18 (17.2%), HPV35 (8.7%), HPV45 (7.4%), HPV33 (4.0%), and HPV52 (2.2%). The study also revealed that the distribution of HPV genotypes appeared to vary by tumour type (Denny et al., 2014). Further, a systematic review aimed to assess the frequency of HR-HPV and its genotype distribution in sub-Saharan Africa, the first ten predominant types identified from cervical samples were HPV16, 52, 18, 56, 31, 35, 45, 58, 51 and 68 (Seyoum et al., 2022).

Based on the IARC report, globally the prevalence of HPV16 & or HPV18 among women with CIN2 and CIN3 and cancer was at 51.9%, and 69.4%, respectively (Bruni et al., 2017). Precancerous lesion risk was 10% and 15% for persistent HPV types 16/18 infection, whereas it was <3% for all other HPVs in combination (Wright & Schiffman, 2003). As a result, HPV genotyping is crucial for identifying specific HPV strains that cause cancer as well as for demonstrating risk stratification (Abreu et al., 2012). Characterizing HPVs is important for both patient evaluation with HPV infection and routine surveillance investigations (Nilyanimit et al., 2018).

Although it is essential for pre-vaccine and HPV-based test research, currently there is limited data on a nationwide prevalence and genotype distribution of HPVs in Ethiopia, although the government started HPV vaccination to schoolgirls at the end of 2018. There are few studies conducted on HPV genotype distribution in Ethiopia from cervical samples (Abate et al., 2013; Bekele et al., 2010; Eshetu, 2015; Fanta, 2005; Leyh-Bannurah et al., 2014; Mihret et al., 2014; Ruland et al., 2006). These studies used different kinds of cervical specimens and molecular

techniques to characterize HPVs and all have reported HPV16 as the most common genotype followed by other different types as depicted in Table 1. The observed HPV genotype heterogeneity suggests the importance of periodic genotyping surveillance at the national level for vaccine and HPV-based screening effectiveness (Seyoum A, 2023).

Table 1: Summary of HR-HPV genotype distribution among women in Ethiopia, 2009-2023.

Article	Study area	Sample size	HR-HPV, %	Dominant HR-HPVs
Seyoum et al., 2023	East Ethiopia	886	12.4	HPV16, 31, 52, 58, 35
Teka et al., 2021	Butajira	764	20.5	HPV16, 35, 52, 45, 18
Gebremeskel et al., 2018	Multi-center	915	55.5	HPV16, 56, 59, 52, 35
Mihret et al., 2014	Addis Ababa	20	100	HPV16, 33, 38, 45, 58
Leyh-Bannurah et al., 2014	Atta	537	16.0	HPV16, 52, 56, 31, 35
Bekele et al., 2010	Jimma	132	100	HPV16, 18, 56, 45, 39
Wolday et al., 2018	Addis Ababa	233	83.2	HPV16, 35, 45, 31, 56

Compiling six of these articles, a recent systematic review of the molecular profile of HPV in Ethiopia showed that HPV 16(37.3%), HPV 52(6.8%), HPV 35(4.8%), HPV 18(4.4%) & HPV 56(3.9%) were the predominate genotypes identified in some areas of Ethiopia (Derbie et al., 2022). However, data on the HVP genotype profile in the North and Southern part of Ethiopia is missing.

Because of considerable geographical differences in the HPV genotype distribution globally, data are required on HPV genotyping for a specific nation not only for vaccination but also to support HPV-based CC screening programs (Kietpeerakool et al., 2015) as profiling the types of HPV may help to advance screening programs effectiveness & to reduce overtreatment. Hence, considering this knowledge gap, the present study will attempt to characterize genotype of HPVs from cervical samples in the northwestern parts of Ethiopia where data are limited in this field.

2.6. Epidemiology of Cervical Cancer in Ethiopia

The term "cancer" refers to a broad range of illnesses , >200 (Finn, 2012) it may impact any physical part & are characterized by uncontrolled growth and spread (Cancer.Net; 2012). It is a major public health problem worldwide. Cancer incidence and mortality are rapidly growing globally. The reasons are complex, several of which linked to socioeconomic development (Bray et al., 2018). In 2020, there will be 9.9 million deaths from cancer and 18.1 million new cases. In low- and middle-income nations, cancer deaths account for more than 70% of all fatalities. The estimated global burden of cancer in 2040 will be 28.0 million new cases and 16.2 million cancer-related deaths (Sung et al., 2021). HPVs are known as the etiological agent of CC, the 2nd most frequent cancer worldwide (among 15-44yrs), and infections generally are connected with around 20% of all cancers (Bruni et al., 2017; Lehoux et al, 2009; Patricio et al., 2016).

According to the 2021 world HPV information center report, approximately 604,127 women were diagnosed with CC and 341,831 die from the disease annually (Bruni L, 2021; Fernandes et al., 2022). In 2030, the annual number of new cases and death projected to upsurge to 700, 000 and 400, 000, respectively (Cancer, 2020; Hull et al., 2020). The majority of CC burden i.e., close to 90% of the newly diagnosed cases and deaths were from resource limited settings (Bruni, 2021). Sub-Saharan Africa is said to have the disease's highest recorded burden (Bray et al., 2018; Vuyst et al., 2013; Hull et al., 2020; WHO, 2014a). CC is the 2nd most common female cancer worldwide in the age range of 15 to 44 years, ranking 4th among cancers that affect women. (Bruni et al., 2017).

Based on the WHO estimates, the incidence of CC is rising steadily in Africa (WHO, 2017), with approximately 75,000 new cases and nearly 50,000 fatalities per year, a toll made worse by HIV infection (Mboumba et al., 2017). However, these figures might represent under estimation due to challenges in health information systems and cancer registries in Africa primarily in sub-Saharan area (WHO, 2017) where the prevalence and mortality of CC are among the highest in the world (Denny et al., 2014). The rise in CC cases in Africa could impede the gains gained by African women in lowering maternal mortality (Mboumba et al., 2017). On top of this, in the African continent, most women detected at an advanced stage of disease, which may leads to poor outcomes (WHO, 2017).

CC becomes one of the emerging public health challenges in Ethiopia. Knowledge about the disease among Ethiopian women is poor (Derbie et al., 2023). Due to population expansion and age, as well as an increase in the prevalence of known risk factors, the incidence and prevalence are occasionally rising (WHO, 2020a). The incidence rate of CC among women from Ethiopia was 21.5/100,100 in 2020 (Bruni, 2021). The worldwide institute for research on cancer estimates that the 7,500 new cases of CC expected in 2020 could increase to 15,300 cases by 2040. Similar to other countries, Ethiopia could see an increase in disease-related deaths from 5,340 in 2020 to 11,000 in 2040 (Cancer, 2020). It is evident that cervical cancer is growing to be a significant cause of morbidity and mortality among women in Ethiopia despite the lack of high-quality cancer registries and trustworthy data in the field and the fact that the nation is unable to maintain effective cervical cancer prevention strategies.

The majority of people on the planet have HPV at some point in their lives. However, most HPV infections do not result in symptoms or disease and clear up on their own; over 90% do so within two years (WHO, 2016). However, persistent HR-HPV infection results in precancerous lesions i.e Cervical intraepithelial neoplasia (CIN) that have the potential to develop into cancer if left untreated, however this progression often takes several years (WHO, 2016; UK, 2014).

A premalignant lesion called CIN which can be in one of three stages: CIN1, CIN2, or CIN3. Untreated CIN2 or CIN3 can develop into CC. (WHO, 2013b). Abnormal cells often become normal over time, but can sometimes turn into cancer. These cells usually be treated, depending on their severity and on the woman's age, past medical history, and other test results. CC associated with HPV infection has different stages. The stage of cancer describes the extent of cancer in the body. It helps determine how serious the cancer is and how best to treat it (American Cancer Society, 2017b). CC stage ranges from stages I through IV as described previously (Mayo Clini 2017; American Cancer Society, 2017b). Stage I: the cancer is the only in the cervix. Cancer in the cervix and upper vagina are found at stage II. Stage III: The disease has spread to the pelvic sidewall or the lower part of the vagina. Stage IV: The cancer has progressed to other parts of the body, such as the lungs, liver, or bones, or to neighboring organs like the bladder or rectum (Mayo Clini 2017; American Cancer Society, 2017b).

Despite the fact that cervix HPV infections do not always result in cancer, HR-HPVs have been linked to >95 percent of CC cases (Doorbar, 2006; Moody & Laimins, 2010; Yu et al., 2005). This was reported about four decades ago and these days there is overwhelming evidence that HR-HPVs are the causative agents of CC (Moody & Laimins, 2010; Prize, 2008; UK, 2014). Among the HPV types infecting the anogenital epithelia, a subset of 14 HPV types classified as HR; causing alterations of the cervical mucosa and ultimately cancer in women (Bruni et al., 2017; Chow et al., 2010; Doorbar et al., 2012; Wallace & Galloway, 2015). A central component of the association between HR-HPVs and cervical carcinogenesis is the ability of HPV to persist in the lower genital tract for long periods without being cleared because of its different immune evasion strategies (Pinidis et al., 2016).

2.7. Factors Associated with CIN2+

Skin-to-skin contact between intimate partners can spread HPV. By engaging in vaginal, anal, or oral sex with someone who has HPV, a person can become infected with the virus. Most frequently, it is spread through vaginal or anal sex (Prevention, 2016). Precancerous cervical lesions and CC are caused by HR-HPVs, but this is not a sufficient cause. According to reports from epidemiological studies, the continuation of HPV infection and development of cancer influenced by many factors. These include, age (first intercourse and first childbirth), smoking tobacco, high fertility, prolonged usage of hormonal contraception, HIV coinfection, *Chlamydia trachomatis* & HSV-2, immunosuppression, & certain dietary deficiencies (like folate), absence of regular screening (pap), a previous history of CIN (Barchitta et al., 2018; Bruni et al., 2017; Luhn et al., 2013; Tifaoui et al., 2018; Wang et al., 2017).

WHO has reported that early sexual intercourse, having many sexual associates and HIV coinfection & by different HPVs are significant factors for the development of CC and persistence of HPV infection (WHO, 2016).

Although they have not yet been fully defined, genetic and immunological host factors as well as viral parameters other than type, such as variations of HPV type, viral load, and viral integration, are expected to be significant (Bruni et al., 2017). Women with a sister or mother who had CC are also at increased risk (WHO, 2014a). Other reported viral related factors include; the loads of viral DNA, and the expression amount of mRNA of the E6/E7 gene from HRHPVs (Ren et al., 2018).

Vaccination status also influences the development of CIN2+ (Niccolai et al., 2017). The stage of different CINs at which the above factors would put their influences on carcinogenesis process is not clearly established.

There is limited data in Ethiopia on epidemiological factors associated with CIN2+ among women with cervical pathologies. However, few studies conducted in the country showed that women's knowledge, willingness and acceptance of CC screening were low (Belete et al., 2016; Tefera & Mitiku, 2017). The scarcity of information reveals a substantial need for further studies on CC prevalence, incidence and mortality with associated risk factors in resource-limited settings for strong public health interventions to minimize the impact of the disease in Ethiopia.

2.8. Prevention and Control of Cervical Cancer

By the age of 50 years, about 80% of women contract HPV infection. While most individuals are clear of the infection within two years, some types of HPV carry a high-risk of progressing to cancer (Broomall et al., 2010). Precancerous alterations are the hallmark of the most prevalent kind of CC, and there are ways to prevent the disease from progressing. First to prevent infection (through vaccination) and the second is to deal with pre-malignancies before they develop into actual cancers (screening) (American Cancer Society, 2017a).

CC may become a rare disease in the coming decades if HPV vaccination and HPV-based screening, including self-sampling, are scaled up globally. WHO has launched a Global Initiative to expand preventive, screening, and treatment programs in order to eradicate CC as a public health issue in the twenty-first century; a picture of the future in which CC is no longer a concern for public health. The Future Health Organization has established a goal for a world where the life-course approach is used to eradicate CC as a public health issue. Each nation should achieve the 90-70-90 targets by 2030, which call for: 1) 90 percent of girls to receive the HPV vaccine in its entirety by the age of 15; 2) 70 percent of women to undergo high-performance screening by the ages of 35 and 45; and 3) 90 percent of women with cervical disease to get therapy (WHO, 2020b).

Through the advent of preventive HPV vaccines, several countries are using Cervarix™ (that targets HPV16 and 18), Gardasil ™ (HPV6,11,16,18) & recent nonavalent i.e Gardasil-9 that covers close to 90% of all HR-HPVs (HPV6, 11, 16, 18, 31, 33, 45, 52, and 58) (Zhai & Tumban, 2016). Gardasil-9 prevents 80-95 percent of other HPV-associated anogenital malignancies in both men and women and the HPV varieties linked to almost 90% of incidences of CC in women worldwide. Nevertheless, because of the diversity in HPV-type specific distribution, the vaccine provides different proportions of defense in different areas; the nonavalent vaccine provided protection against HPVs linked to∼87.7% of CCs in Asia, 92% in N.America, 89.5% in S.America & the Caribbean, 91.7% in Africa, 90.9% in Europe, & 86.5% in Australia (Zhai & Tumban, 2016). A third-generation HPV vaccine is now necessary in order to provide total protection against all HPV strains that cause CC (Zhai & Tumban, 2016).

HPV-based vaccination in Ethiopia was started in October 2018 for schoolgirls only. The vaccine, Gardasil-4®, targets HPV6, 11, 16, and 18. According to the information from WHO African region report, so far about 2 million girls aged 9-14 were vaccinated in the country (WHO, 2021). However, based on the available HPV molecular data in some parts of the country and our review result (Ali et al., 2019; Derbie et al., 2022; Derbie et al., 2019; Wolday et al., 2018) the proportion of girls who could be protected from HR-HPV infections using this vaccine is <60%. Therefore, there is a need to upgrade the vaccine into Gardasil®9. A recent study revealed that compared to the Gardasil-4™, a nonavalent Gardasil®9 that targets close to 90% of all HR-HPVs is a profitable option for Ethiopia (Wondimu et al., 2022).

After a vaccination program is implemented, it will take several decades before the effects of HPV vaccine are fully apparent. In addition, ensuring enough coverage and ensuring that all girls of the proper age receive vaccinations remains difficult. At the same time, the HPV vaccine does not replace cervical screening; hence early detection of cervical intra-neoplasia is also very crucial.

Ethiopian national guideline for cervical cancer prevention and management stated screening should be offered with one or a combination of the three screening methods: These are VIA, HPV DNA, & cytological screening (Federal Democratic Republic of Ethiopia, 2021). However, the VIA is the most widely used screening modality in Ethiopia irrespective of the following pitfalls. (i) VIA is exclusively performed in a health facility, and it needs the woman

to undergo a genital examination with trained providers with the insertion of a speculum that is not culturally acceptable by women in Ethiopia. (ii) Both VIA and cytologic screening methods need more infrastructure and human resource training. (iii) VIA has low specificity (a limitation in identifying true negative), that may lead to unnecessary referrals, overtreatment, and adverse psychological consequences to the women. Therefore, it is critical to implement a screening method that is more specific, cost effective and women friendly. The economic burden to introduce and widely practice HPV-based study in Ethiopia must be also well studied.

The majority of Ethiopian women had little awareness and acceptability of cervical cancer screening, according to many studies conducted to measure its level of acceptance, uptake, and examine factors related to it. (Derbie et al., 2023). This also need a well-planned intervention.

Chapter Three: Methods and Materials

3.1. Study Setting

Between the first of March 2019 and the end October 2021, a hospital based cross-sectional study was carried out at Felege Hiwot Comprehensive Specialized Hospital (FHCSH). COVID-19 has influenced the data collection period of this study.

The FHCSH is located in Bahir Dar city (Figure 3) northwest Ethiopia, located about 565km away from the capital Addis Ababa. The FHCSH, with more than 500 beds, is a tertiary health care facility that provides several types of specialized referral services for about ten million people in northwest Ethiopia (Gojjam, Benishangul, Gondar, and Wollo). Hospital has been acting as a teaching facility for students at other private institutions as well as the Bahir Dar University College of Health Sciences. . Based on the information obtained from the hospital VIA clinic, before the time of data collection there were between 100 and 150 women per month coming to the clinic for CC screening service.

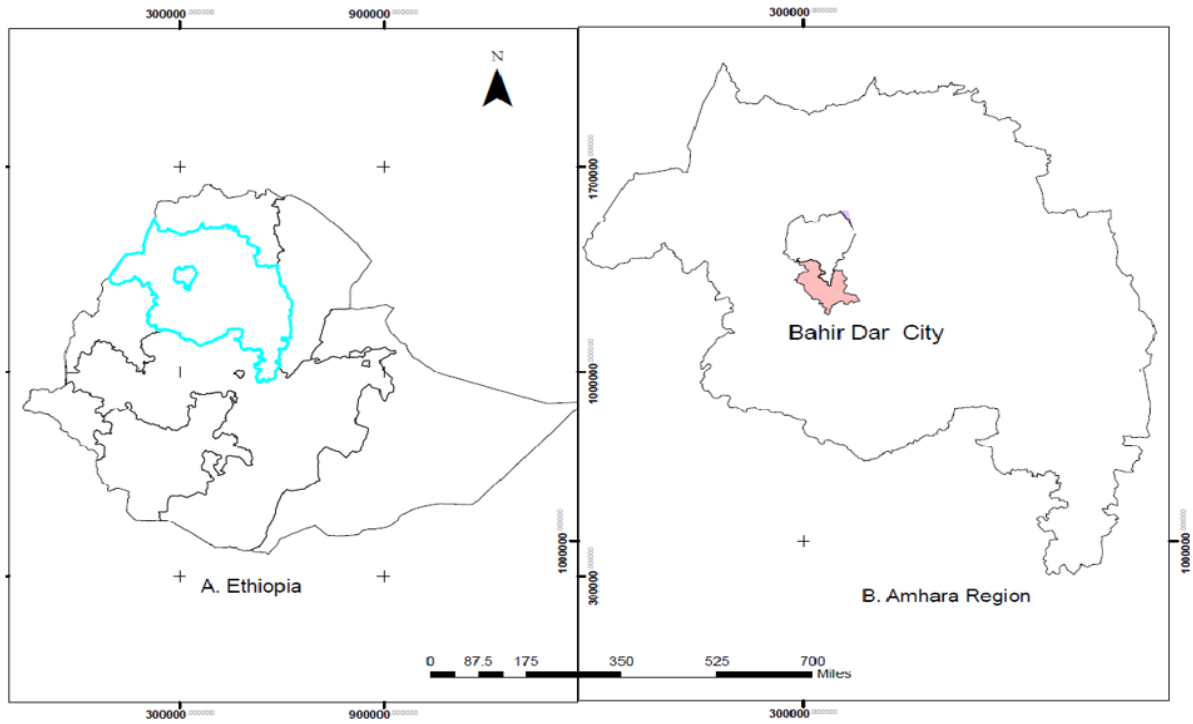

Figure 3: The study area.

3.2. Source Population

The source population was those women who were referred or directly came to FHCSH for gynecological examination because of any form of gynecological complaints in the hospital during the study period.

3.3. Study Population

The study population comprised of women who came for gynecological examination or who were admitted for investigation of any form of cervical abnormalities and were eligible for CC screening at the hospital fulfilling our inclusion criteria.

3.4. Sample Size Determination and Sampling Technique

The sample size was calculated using the percentage of high-grade squamous intraepithelial (HSIE)lesions, which was found to be 27% among women visiting the gynecology department's outpatient clinic at Black Lion Hospital, CHS, AAU (Wolday et al., 2018), with a 5% margin of error at a 95% confidence level. .

$$n = Z^2 p (1-p) / d^2$$

Where: Z = Z score for 95% confidence interval =1.96, p =prevalence (27%=0.27), d = tolerable error =5% (0.05)

The precision of the study and availability of resource was taken in to consideration during the determination of the sample size. Therefore, the minimum sample size was 340 (including 10% non-response rate). However, in the actual practice 355 women were included. For the specific objective related to determining the histopathological profile and factors associated with CIN2+, data was collected from 335 participants. For the rest HPV related objectives (i.e., HPV DNA and E6/E7 mRNA testing), data from 355 participants were analyzed. The period for sample collection was extended because of the COVID-19 epidemic. In order to back up the some of the firstly collected samples, we thought of using an additional 15 samples for HPV-based tests (thinking they might lose their quality owing to a power outage of the freezer).

The sampling technique was purposive in which all eligible women fulfilling the inclusion criteria and coming to the hospital throughout the time of data collection were included until the sample size was fulfilled.

3.5. Inclusion and Exclusion Criteria

- Women presenting to the hospital's gynecologic services with symptoms and signs that suggested an atypical cervix, such as abnormal vaginal discharge and vaginal hemorrhage (bleeding after sexual intercourse or any bleeding after menopause), complaining of painful sexual intercourse were considered for participation. These women should have also the following characteristics: ≥30 years of age, had a history of sexual activity, were not pregnant at the time of data collection, had a complete uterus and cervix, and weren't menstruating (Federal Democratic Republic of Ethiopia, 2021).
- On the other hand, women who were seriously ill and those who were under treatment for invasive CC were excluded.

3.6. Variables

- **Dependent Variables**: The proportion of HPV positive cases, the identified HPV genotypes, HPV E6/E7 mRNA test profile.
- **Independent Variables**: Demographic, reproductive and gynecologic related variables and the stage of cervical lesions.

3.7. Data Collection Process

After ensuring the informed and voluntary participation of the study participants by using a written information sheet and obtaining a signed consent form, data were generated from: 1) a questionnaire 2) molecular tests, and 3) histopathology.

3.7.1. Demographic Data

An interviewer-administered questionnaire was utilized to gather information on the demographics of the participants (like, participants' age, residence, and educational background), reproductive history (like, parity, # of sexual partners, and age @ first sex) and gynecologic related features (awareness about CC and previous cervical screening practice). Two trained nurses working in the hospital's gynecology department collected the questionnaire-based data. The tool was prepared following previous similar works (Barchitta et al., 2018; Bruni et al., 2017; P. Cattani et al., 2009; Luhn et al., 2013; Niccolai et al., 2017; Tifaoui et al., 2018; Wang et al., 2017; WHO, 2014a) (Appendix C).

3.7.2. Cervical Swab Collection and Handling

Study participants underwent a general and pelvic examination in a compassionate and respectful process after ensuring their informed consent. Then, for HPV DNA and mRNA testing a gynecologist collected cervical swabs with a single-use broom-type brush (Digene.HC2DNA.collection device:Qiagen,Hilden, Germany) following the manufacturer's instructions. Briefly, after removing excess mucus from the cervical os and the surrounding ectocervix using a cotton swab, the brush was inserted 1-1.5 cm into the os of the cervix till its largest outer bristles touched the ectocervix. The brush was then taken out of the canal and put into the bottom of the transport tube after being turned three full rotations. The shaft was finally broken off, and the tube was safely capped. Specimen Transport Medium (STM) that contains 1ml 0.05% sodium azide as a preservative accompanied the sample collection tube. Swabs were labeled with a special code and the patient card number, and then kept at -80^0C in the CMHS research laboratory at BDU. Finally, the frozen specimens were transported on dry ice packs to Leipzig University for molecular analysis. The STM was validated for HPV DNA test using the Alinity m system in the Institute of Virology for routine diagnostic service.

3.7.3. Cervical Biopsy Collection and Processing

During the time of data collection, the hospital didn't have a colposcopy facility hence traditional cervical punch biopsy was collected by a gynecologist using the four quadrant random biopsies collection (four punches each 0.5 cm, at positions of 3, 6, 9, and 12 o'clock) by the use of punch biopsy forceps following the recommended approach (Hu et al., 2017). Then, cervical biopsies were placed in screw-capped and labeled bottles that contained 20ml of 10% formol-saline fixative solution and transported to the hospital pathology laboratory for downstream processing by the senior pathologist as described previously (Ameya et al, 2017).

Briefly, using a fully automated tissue processing equipment, a senior pathologist processed the biopsy samples. While processing, water was removed by using ethanol. This was followed by adding a hydrophobic clearing agent, i.e xylene, to remove the alcohol and finally molten paraffin wax was used to infiltrate and replace the xylene. Subsequently, using LE/GA and EG1110 device the tissue was embedded in which tissue in paraffin wax remain attached to cassettes. About 3-5 mm tissue sections were cut with a rotary microtome and afterwards floated on a water bath that had been heated to between 60 and 65 °C. This makes picking sections on a microscopic slide easier and helps get rid of wrinkles that developed during sectioning. Then the slide was placed in an oven setted at >70°C for ~40 minutes so that it will

have time to adheres to the slide and the wax will melted. This is followed by staining of slides using hematoxylin and eosin (H&E). Finally, the slides were examined and reported based on the CIN reporting system: *normal histology, CIN1, 2, 3, cancer*, & other findings.

The CIN (also named cervical dysplasia) describes how far the irregular cells have gone into the layers cervix. CIN is classified into grades as CIN1, CIN2, and CIN3 based on cell changes. Treatment is usually given to remove abnormal cells in CIN2 and above(WHO, 2013b, 2014b). For this particular study, we used the acronym CIN2+ to represent high-grade precancerous lesions (CIN2 and CIN3) and CC together.

3.7.4. E6/E7 mRNA Extraction

Frozen cervical swabs were thawed from freezer and relabeled with a sequential barcode. Then each tube was vortexed (3-5 seconds) to homogenize the cells located on the brush with the preservative solution. In some samples with visible blood clots up to 400µl phosphate buffer solution (PBS) was added prior to vortexing. The brush was removed after release of the cells into the preservative solution. For the E6/E7-mRNA PCR, 200 µl of the sample was extracted using the Kit (which is DNA&Viral NA SmallVolume) using the MagNAPure96 device (Roche,Mannheim, Germany) as per the manufacturer instructions with elution volume set to 50 µl (Roche, 2021). The remaining vortexed sample in the original sample collection tube was directly used for HPV DNA detection and genotyping on the Alinity-m-System (the Abbott Molecular, DesPlaines, IL-United States of America).

3.7.5. HPV DNA Detection and Genotyping

Test principle: the molecular detection and characterization of HPVs involved in the sample from a cervical lesion are on the principle in which the HPV-DNA will be available in the epithelial layers of the affected tissue and can be detected easily with PCR-based technologies (Kambouris et al., 2010). HR-HPV detection and typing are becoming an another option for screening using cytology(Meijer et al., 2009) and become an vital alternate test in the dx & treatment of cervical pathologies associated with HR-HPVs (García et al., 2011). Since the ability to cause cancer differs among the HPVs, HPV detection followed by genotyping is valuable in the stratification of the risk of women who tested positive for HR-HPVs.

For this particular study, HR-HPV detection and characterization were made using Alinity-m HR-HPV AMP Kit on Alinity-m System (software version: 1.6.3) (Abbott Molecular, Des Plaines, IL, USA). The Alinity m System offers a fully automated random access platform to different molecular assays. It is a fully integrated and automated molecular analyzer introduced in 2019 (Dhillon etal., 2021). The assay fulfills the international consensus guideline criteria for primary CC screening (Garland et al., 2023; Oštrbenk et al., 2019). The time to the first result is less than two hours (Abbott, 2022).

Using real-time PCR and ReadiFlex® technology, the Alinity test is qualitative that targets the L1 conserved region for the detection of DNA from 14 HPVs; HPV16,-18,-31,-33,-35,-39,-45,-51,-52,-56,-58,-59,-66, & HPV68 from clinical specimens (Oštrbenk et al., 2019). HR-HPVs are detected with genotype-specific probes in five distinct channels: HPV16, HPV18, HPV45, and other high-risk genotypes group A (HPV31, 33, 52, and 58), and other high-risk genotypes group B (HPV35, 39, 51, 56, 59, 66, and 68) (Dhillon et al., 2021).

To ensure its quality performance, in addition to testing external positive and negative controls, the Alinity detects human endogenous β-globin as a Cellular Control signal to evaluate efficiency of sample extraction, amplification, and amount of cells (Abbott, 2022).

The steps of the Alinity assay consist of preparation of samples, RT-PCR assembly, amplification/detection, & calculation and reporting of results. All steps of the assay are performed automatically by the Alinity m system. In order to trap HPD DNA on magnetic microparticles, 400L of cervical swabs were pretreated and lysed with chaotropic chemicals during sample preparation. Following binding, the pure DNA was washed and eluted. Using the eluate and activation reagent, a lyophilized amplification master mix made up of DNA poly, primers, probes, and dNTPs was rehydrated. The finished PCR mixture was then put into a reaction vessel, covered, and moved to the amplification and detection element. All these steps including result generations are performed automatically (Abott, 2020; Jang et al., 2021).

Samples (n=49) which were positive for G- A and/or B with the Alinity m were further processed using the INNO LiPA HPV Genotyping Extra II assay (INNO-LiPA; Fujirebio Europe, Ghent, Belgium) for identification of the specific genotypes following the manufacturer instructions and as described previously (N.V., 2011; Tewari et al., 2021).

3.7.6. The E6/E7 mRNA test

Test principle: In comparison to HPV DNA-based assays, the measurement of viral gene expression both directly (through mRNA) and indirectly (via protein) may provide a more precise method for determining clinically relevant infection. The direct detection of viral mRNA transcripts allows for the monitoring of HPV E6/E7 gene expression and signs of its dysregulation (Cuschieri & Wentzensen, 2008). Progression to CC requires the overexpression of the E6/E7 mRNA in the integrated HPV genome (Cattani et al., 2009); hence, testing for this transcripts could be a useful tool for both primary screening and triage of women with border line cytology. It is also used in patient management, providing more accurate predictions of risk of abnormal lesion progression (Andersson et al., 2011; Paola et al., 2009).

The E6 & E7 area of DNA/genes are transcribed polycistronically (the genes transcribed into one full-length mRNA transcript coding for both proteins) from a single promotor that is located at the 3' of the upstream regulatory region (URR) (Andersson et al., 2011; Pal & Kundu, 2019).

Due to time and money constraints, the E6/E7 mRNA multiplex assay was reduced to detect the genotypes HPV16, 18 and 45. Primers for corresponding HPV genotypes were used according to the previously published primers and probes, Metabion, Germany (Pan et al., 2018). The following pair of E6/E7 region primers of these HPVs (Table 2) were used in a duplex format for the mRNA detection and the reaction and amplification conditions were performed based the in-house protocols of the Leipzig University Hospital Institute of microbiology and Virology. Similarly, corresponding probes from the publication were modified with for higher stability (TibMolbiol, Germany) (Table 2).

Table 2: Primers & probes used in the testing of HR-HPV E6/E7 mRNAs.

Primers		
HPV type	**Forward**	**Reverse**
HPV16	F 5'- GAGATACACCTACATTGCATGA-3	HPV16_R 5'- GCTGGACCATCTATTTCATCCTC-3
HPV18	F 5'- CATTGTATTGCATTTAGAGCCC-3	HPV18_R 5'- GCTACTACTAGCTCAATTCTGGC-3
HPV45	F 5'- GTAGGGAAACACAAGTATAGCA-3	HPV45_R 5'-CAACAGGTCAACAGGATCTAAT-3
Probes		
HPV16	HPV16P_LNAFAM 5'- 6FAM-TgCAACCAgAg+AC+A+ACT-BHQ1-3'	
HPV18	HPV18P_LNAFAM 5'- 6FAM-TCCggTTgACCTTCT+AT+gT -BHQ1-3'	
HPV45	HPV45P_LNAFAM 5'- 6FAM-TCCggTTgACCTTCT+AT+gTAAgTATgCATggA+C+CCC -BHQ1-3'	

The RT-PCR was performed using the SuperScript III Platinum One-Step qRT-PCR Kit based on the manufacturer's instruction.

Cell-Control was changed to target RNase P as described previously (Wozniak et al., 2020). Primers (RP2-F: 5'- AGA TTT GGA CCT GCG AGC G-3' and RP2-R: 5'-GAG CGG CTG TCT CCA CAA GT-3', Metabion, Germany) were used according to the publication, the probe (RP-P-YAK: 5'- YAK-TTCTgACCTgAAggCTCTgCgCg-BBQ-3') reporter dye was changed to YAK to allow differentiation of HPV and cell control.

For DNA-depletion, nine microliters of RNA mixed with 10 µl of 2fold DNase I buffer and 1u DNase I (Takara) the incubated @ 37°C for about 30 minutes. This is followed by inactivation of DNase by addition of 1µl 50 mM EDTA and incubation at 65°C for 10 minutes. The mRNA-RT-PCR reaction mix (40 µl) consisted of 20 µl 2fold reaction mix, 0.64 µl $MgS0_4$ (final concentration 4mM), 1 µl of each 10 mM-Primer, 0.5 µl of each 4 mM Probe, 1 µl BSA, 1 µl SSIII/TaqEnzymeMix and 4.36 µl H_2O and 10 µl of digested sample.

PCR cycling conditions on a LightCycler 96 (Roche, Mannheim, Germany) were as follows. Reverse-transcription at 42°C for 30 minutes, denaturation at 80°C for 2 min and 94°C for 2min which is followed by 40 cycles of 94°C 30s, 58°C 30s and 72°C for 30s with single signal detection at 72°C. Through determining the cycle threshold(CT), mRNA expression level was quantified. RNase P was used as cell-control system to avoid false negatives. Samples were re-tested without RT-Step to confirm sufficient DNA-depletion.

The overall procedural flow of the sample collection and analysis is summarized in Figure 4.

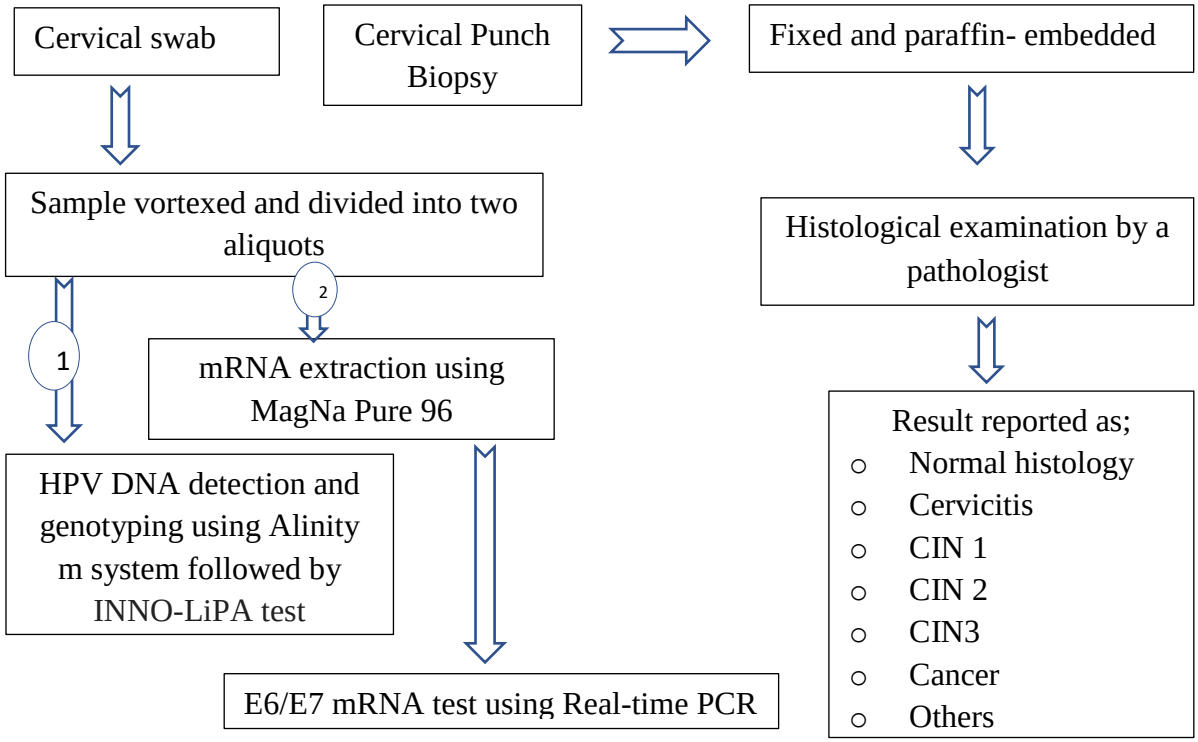

Figure 4: A chart representing the overall procedural workflow of the cervical sample collection and processing.

3.8. Data Quality Control

Standard operating procedures were strictly followed during the sample collection, storage, and transportation and during the time of sample processing steps. While working with the HPV molecular analysis, all steps were performed following manufacturers' instructions carefully. The questioner prepared to generate epidemiological data was translated into the local language (*Amharic)* and consistency of the items was ensured.

3.9. Data Analysis

SPSS version 25 employed to enter and analyze the collected data. To present the demographic, clinical characteristics, and histopathological results of the study participants descriptive statistics was employed. The type distribution of the identified HR-HPVs with a 95% confidence interval (CI) was calculated. The results are presented as simple counts, percentages, and mean with standard deviations.

The detection of the E6/E7 mRNA test was calculated from different histopathological grades. CIN2+ detection by E6/E7 mRNA test then calculated from all and specifically HPV16, 45 and 18 positive samples. Finally, the analytical performance of E6/E7 mRNA (Sensitivity, Specificity, PPV & NPV with (95% CI)) was calculated using histologically confirmed CIN2+ as the gold standard. Histopathological examination of cervical biopsies remains an important procedure that is most widely used as clinical endpoints by which the performance of new techniques for CC prevention is currently evaluated.

A stepwise multivariable logistic regression model run to find out factors associated with CIN2+. Variables with a *p*-value of $\leq$ 0.25 univariable analysis picked & subjected to the multivariable run (Bursac et al., 2008). A forward stepwise regression analysis was carried out. The model fitness was checked by the Hosmer-Lemeshow goodness of fit test value (>0.5). In multivariable analysis, significance was calculated at a *p*-value <0.05 to declare the risk factors considered in our study to be major explanatory factors associated with CIN2+ among studied women.

3.10. Ethical issues

The Institutional Review Board (IRB) of the College of Health Sciences at Addis Abeba University and the Research and Ethics Committee of the Department of Medical Microbiology, Immunology, and Parasitology (DMIP) both gave their approval to the study methodology (Protocol number: 087/19/DMIP) (Appendix E). As the sample was processed abroad, ethical clearance was additionally ensured by the Ethiopian NRERC (National Research Ethics Review Committee) at the Ministry of Education (Ref number: 7/2-149/m259/35) (Appendix F).

The procedure of specimen collection explained to all participants using their mother tongue. Then, informed consent (written) was ensured from the study subjects to take part voluntarily after they learn about the goal and aim of the research. The histology and molecular test results (including abnormal findings) were communicated to the hospital VIA clinic. All data gathered from respondents kept private and is only utilized for this study project.

Chapter Four: Results

4.1. Demographic and Other Features of the Participants

In this study, 355 women were included. At enrolment, the study participants were aged 30–80 years (mean 46.4 years). Most of the study subjects were married 272(76.6%), housewives 314(88.5%), were from rural settings 232(65.9%), and did not have formal education 272(76.6%) (Table 3).

Moreover, most of the study participants at 277(79.4%) started sex before 18 years of age (mean age at first sexual debut was at 15.7±2.6). Besides, 48(13.5%), 106(30.0%), and 180(51.6%) of the participants were HIV positive, had a history of vaginal discharge, and had multiple sexual partners, respectively.

A majority of the study subjects at 211(59.4%) knew nothing about CC. The participants' screening practice for CC and screening in the last five years were 97(27.3%) and 88(24.8%), respectively. Besides, their knowledge about the disease was very limited, and among those only 11(8.9%) of them know that the disease is caused by a sexually transmitted pathogen (Table 3).

Table 3: Distribution of the study participants by their demographic and clinical characteristics, northwest Ethiopia, 2021.

Characteristics		n (%)
Age groups (in years)	Mean, SD	46.4, 11.4
	30-40	142 (40.0)
	41-50	115 (32.4)
	>50	98 (27.6)
Permanent residence	Urban	121 (34.1)
	Rural	232 (65.9)
Marital status	Single	4 (1.1)
	Married	272 (76.6)
	Divorced	38 (10.7)
	Other*	40 (11.3)
Educational status	No formal education	272 (76.6)
	Primary	46 (13.0)
	Secondary	17 (4.8)
	Tertiary	20 (5.6)
Type of occupation	Housewife	314 (88.5)
	Private employee	17 (4.8)
	Government employee	22 (6.2)
	Other (prostitute)	2 (0.6)
Age at first sexual intercourse	Mean, SD	15.7, 2.6
	<18 years	277 (79.4)
	≥18 years	72 (20.6)
Life-time number of sexual partners	Mean	1.7
	1	169 (48.2)
	≥2	180 (51.6)
HIV sero-status	Positive	48 (13.5)
	Negative	301(84.8)
	Unknown	6 (1.7)
Previously treated for vaginal discharge	Yes	106 (30.0)
	No	247 (70.0)
Aware of CC	Yes	144 (40.6)
	No	211 (59.4)
How is the pathogen that causes CC transmitted?	Sexually	11 (8.9)
	Do not know	114 (91.1)
History of cervical screening	Yes	97 (27.3)
	No	258 (72.7)
History of cervical screening in the last five years	Yes	88 (24.8)
	No	267 (75.2)
HR-HPV	Detected	188 (53.0)
	Not detected	167 (47.0)
E6/E7 mRNA (HPV16,18,45)	Detected	127 (35.8)
	Not detected	228 (64.2)

***Other** types of marital status include: widowed, separated.

4.2. Histopathological Profile of the Participants

A cervical histology report was available for 335 participants. Of which, the majority of cervical histopathological findings were CC 41.8% (140/335; 95%CI: 36.6-47.1%). The proportion of high-grade precancerous lesions and CC together (CIN2+) was 49.0% (164/335: 95%CI: 43.6-54.2%). Seventy-four (22.1%; 95%CI: 18-26.8) of the participants had normal histology. The detailed histological profile of the study participants is presented in Figure 4.

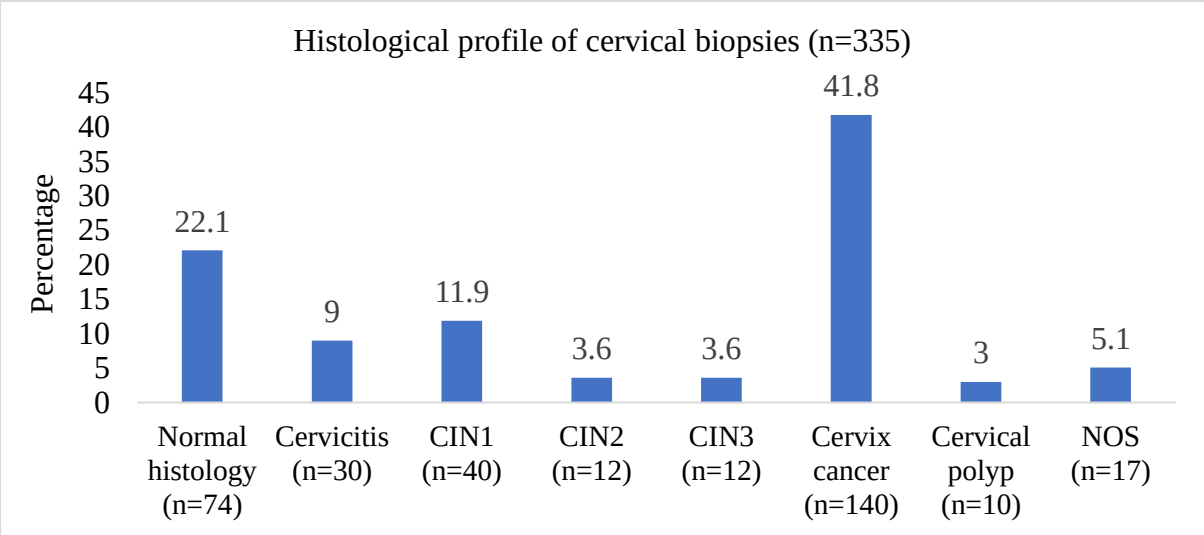

Figure 5: Percentage distribution of cervical histopathology findings, northwest Ethiopia, 2021.
CIN: Cervical intraepithelial neoplasia.
NOS: Not otherwise classified**;** these categories include atypical endocervical glandular proliferation (#1), focal of atypical columnar epithelium lining (1), unremarkable endocervical tissues (6), myoma (3), benign polypoid endocervical tissues (2), cervical wart (1), napotian cyst (1), bloody and non-diagnostic sample (2).

4.3. Type of the Identified HR-HPVs

For the detection and genotyping of HR-HPVs, data from 355 study participants were used. The prevalence of HR-HPV was 53.0% (188/355; 95%CI: 47.8-58.1%). From these samples (n=188), 13 different HR-HPVs were identified. The total frequency of the identified HR-HPVs including co-infections was 258. As participants ages grew, there was a noticeable increase in the detection of HR-HPV. Similarly, the detection HR-HPVs was found to be different based on screening history of the study participants (*p*-value <0.05) (Table 4).

HPV16 was the most often identified HR-HPV which accounted for 50.4% (130/258: 95%CI: 29.4-39.2%) proceeded by HPV31 9.7% (25/258: 95%CI: 6.7-13.9%), HPV33 8.5% (22/258: 95%CI: 5.7-12.6%), HPV39 and HPV68 each 5.8% (15/258: 95%CI: 3.6-9.4%) and HPV18 4.7% (12/258: 95%CI:2.7-8.0%). The least detected genotype was HPV58 (0.4%). The seven HR-HPVs (HPV16,-18,-31,-33,-45,-52, &-58) that are part of the HPV-nonavalent vaccine accounted for 79.1% (204/258:95%CI: 73.7-85.6%). Similarly, the combined prevalence of HPV16 and HPV18 was 55.1% (142/258: 95%CI: 48.9-61.0%) (Figure 5).

Among HR-HPV positive samples (n=188), mono-infection was documented in 143 (76.1%) whereas 27 (14.4%) and 18 (9.6%) samples were found to be positive for two and more ($\geq$3) HR-HPV types, respectively. Up to five different HR-HPVs were found infecting a single patient. The HR-HPV most prevalent coinfection was HPV31 with HPV33 then comes HPV39 with HPV59 & HPV68 (Table 5).

Table 4: The detection rate of HRHPVs genotypes across socio-demographic, sexual behavior, and clinical variables, northwest Ethiopia, 2021.

Variables		HR-HPV		*p* - value
		Detected, n(%)	Not detected, n(%)	
Age group	30-40	57(16.1)	85(23.9)	<0.001
	41-50	64(18.0)	51(14.4)	
	>50	67(18.9)	31(8.7)	
Marital status	Single	4(1.1)	0	0.01
	Married	131(36.9)	141(39.7)	
	Divorced	25(7.0)	13(3.7)	
	Other	27(7.6)	13(3.7)	
Educational status	No formal education	147(41.4)	125(35.2)	0.01
	Primary	17(4.8)	29(8.2)	
	Secondary	14(3.9)	3(0.8)	
	Tertiary	10(2.8)	10(2.8)	
Age at first sexual practice	<18	150(43)	127(36.4)	0.53
	>18	36(10.3)	36(10.3)	
Lifetime number of sexual partners	1	88(25.2)	81(23.2)	0.73
	>2	97(27.8)	83(23.8)	
HIV sero-status	Positive	26(7.3)	22(6.2)	0.78
	Negative	158(44.5)	142(40)	
	Unknown	4(1.1)	2(0.6)	
Cervical screening history	Yes	42(11.8)	55(15.5)	0.02
	No	146(41.1)	112(31.5)	
Screening in the last five years	Yes	37(10.4)	51(14.4)	0.01
	No	151(42.5)	116(32.7)	
Treated for vaginal discharge	Yes	54(15.2)	54(15.2)	0.52
	No	133(37.5)	114(32.1)	

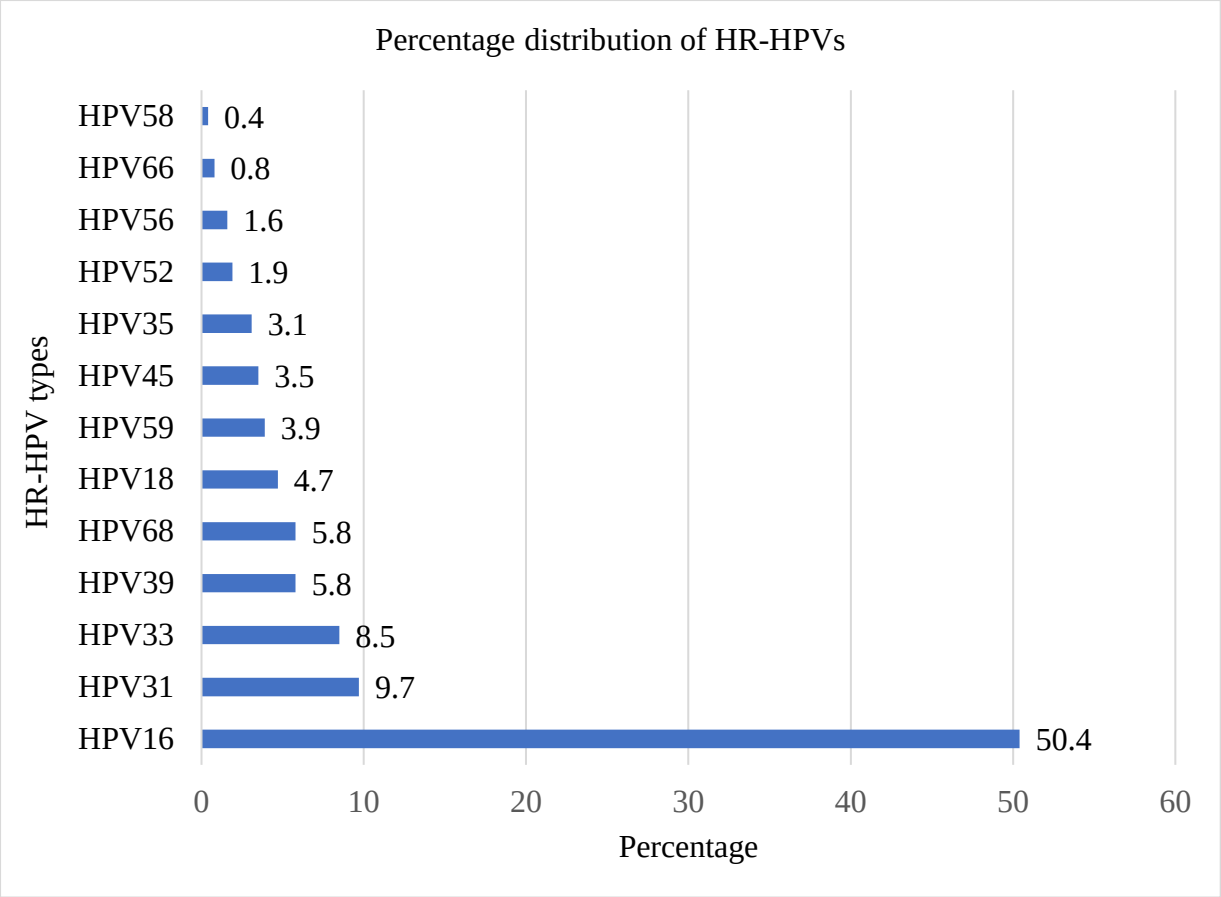

Figure 6: HR-HPV genotype distribution among the study participants, northwest Ethiopia, 2021.

4.5. HR-HPV mRNA Assay Result

The E6/E7 mRNA was tested targeting specifically HPV16, 18 and 45 and we detected 35.8% (127/355; 95%CI: 30.0-40.9) positive cases. About 42% (149/355) of these three HR-HPVS tested positive for HPV DNA. The total agreement of the DNA & the E6/E7-mRNA for these HPVs was at 92.7% (95%CI: 89.5-94.9) having a kappa (k value) score of 0.821 .

Among samples positive for mRNA (n=127), the most predominant type that expresses mRNA in different grades of cervical histology was HPV16 at 108 (85.0%) followed by HPV18 11(8.7%) and HPV45 (4.7%). In addition to this, co-infection of HPV16 and HPV45 E6/E7 mRNA transcripts was found in 2 (1.6%) cancer cases.

4.4. The Distribution of HR-HPVs in Different Histopathological Grades

Concerning the types of HR-HPVs involved in different histopathological grades, all HPV16, HPV18, HPV35, & HPV45 were discovered to be linked to either high-grade lesions (CIN2+) or CC, whether they were present singly or in coinfections. Likewise, 79(80.6%), 7(87.5%), 3(75%), and 5(100%) of HPV16, HPV18, HPV35, and HPV45 mono infections were identified in histopathologically confirmed cancer cases, respectively. Similarly, 11(78.6%) HPV31&33 coinfections were recovered from cancer cases.

Specifically, the detection of rate of HPV16 was increased with the higher degree of lesions in which the proportion was 12.5% (n=5), 16.7% (n=2), 41.7% (n=5), and 60% (n=79) in CIN1, CIN2, CIN3, and cancer cases, respectively. Seven of the eight (87.5%) HPV18 were detected in CC cases. In 9.7% (13/134) of CC cases, HR-HPVs were not detected (Table 6).

The proportion of mRNA noted with an increased level along with the higher degree of lesions of the cervix from 5%(2/40) in CIN1 to 25%(3/12), 50%(6/12), and 70.1% (94/134) in CIN2, CIN3 and CC, respectively (Table 5). Likewise, 78.8% (n=80) of HPV16, 88.9% of HPV18 (n=8) and 66.7% HPV 45 (n=4) E6/E7 mRNA were detected among cancer cases, respectively (Table 6).

Table 5: The detection of DNA and mRNA based on cervical histologies, northwest Ethiopia, 2021.

Tests		Cervical histology, n (%)				
		Normal	CIN1	CIN2	CIN3	Cancer
HR-HPV DNA	+	15 (20.5)	11 (27.5)	3 (25)	10 (83.3)	121 (90.3)
	-	58 (79.5)	29 (72.5)	9 (75)	2 (16.7)	13 (9.7)
E6/E7 mRNA	+	8 (11)	2 (5)	3 (25)	6 (50)	94 (70.1)
	-	65 (89)	38 (95)	9 (75)	6 (50)	40 (29.9)
	Total	73	40	12	12	134

Table 6: The distribution of HR-HPVs in different cervical histopathologic grades, northwest Ethiopia, 2021.

HPV type (s)	Histopathological classes					
	Normal	**CIN1**	**CIN2**	**CIN3**	**Cancer**	**Total**
HPV16	14	5	2	5	79	105
HPV18	0	0	0	0	7	8
HPV45	0	0	0	0	5	5
HPV35	0	0	0	1	3	4
HPV16,45	0	0	0	0	2	2
HPV16,56	0	0	0	0	1	1
HPV31,33	0	0	0	1	11	12
HPV39,59	0	0	0	0	1	1
HPV39,68	0	1	0	1	1	3
HPV56,66	0	1	0	0	0	1
HPV16,31,33	0	0	0	0	2	2
HPV18,31,33	0	0	1	0	0	1
HPV18,39,59	0	0	0	1	0	1
HPV31,52,66	0	1	0	0	0	1
HPV35,39,68	1	1	0	0	1	3
HPV35,56,68	0	0	0	0	1	1
HPV39,59,68	0	1	0	0	3	4
HPV16,31,33,52	0	0	0	1	0	1
HPV16,39,59,68	0	0	0	0	2	2
HPV18,31,33,52,58	0	0	0	0	1	1
HPV31,52,39,59,68	0	1	0	0	0	1
HPV not detected	58	29	9	2	13	111
Total	**73**	**40**	**12**	**12**	**134**	**271**
E6/E7 mRNA						
HPV16	8	2	2	6	80	98
HPV18	0	0	1	0	8	9
HPV45	0	0	0	0	4	4
HPV16&45	0	0	0	0	2	2
mRNA not detected	65	38	9	6	40	113
Total	**73**	**40**	**12**	**12**	**134**	**271**

- To make the data more precise, cervicitis, cervical polyp and other histological findings (NOS) are not included in this table. For example,
    - E6/E7 mRNA of HPV16 detected: (#1) from cervicitis, (2) from polyps, and (1) from other (NOS) histological types. Six of the HPV16 mRNA does not have histological result.
    - E6/E7 mRNA of HPV18 detected: (1) from the NOS group. One of the HPV18 mRNA does not have histological result.
    - E6/E7 mRNA of HPV45 detected: (1) from cervicitis and (1) from the NOS group.

4.6. Sensitivity, Specificity, PPV, and NPV E6/E7 mRNA Test

The sensitivity & the specificity of the mRNA assay in the identification of histologically confirmed CIN2+ were 65.2% (95%CI: 57.5-72.2%) and 90% (95%CI: 84.6-93.4%), respectively. Conversely, although the mRNA assay targets only three HPVs, the detection rate of the DNA was higher with an analytical sensitivity of 84.8% (95%CI: 78.4-89.6%) but with a lower specificity at 74.1% (95%CI: 67.1-80.1%) than the mRNA testing (Table 7). The sens & spec of the mRNA test in the identification of histologically confirmed CIN2+ was 92.7% & 47%, respectively among HPV16, 18, & 45 DNA positive cases (Table 8).

Table 7: The Sens, Spec, -PPV, & -NPV of the E6/E7 mRNA and HR-HPV-DNA tests for the identification of CIN2+ (n=328), northwest Ethiopia, 2021

Test		CIN2+		%(95% CI)			
		Yes	No	Sensitivity	Specificity	PPV	NPV
HR-HPV DNA	+	134	44	84.8	74.1	75.3	84
	-	24	126	(78.4-89.6)	(67.1-80.1)	(68.5-81.0)	(77.3-89.0)
E6/E7 mRNA	+	103	17	65.2	90.0	85.8	73.6
	-	55	153	(57.5-72.2)	(84.6-93.4)	(78.5-91.0)	(67.2-79.1)

PPV: Positive Predictive Value, **NPV**: Negative Predictive Value

Table 8: The Sens, Spec, -PPV, & -NPV of the E6/E7 mRNA test for the detection of CIN2+ among HPV16, 18 & 45 DNA positive cases (n=141), northwest Ethiopia, 2021.

Test		CIN2+		% (95% CI)			
		Yes	No	Sensitivity	Specificity	PPV	NPV
E6/E7 mRNA	+	101	17	92.7	46.9	85.6	65.2
	-	8	15	(86.2-96.2)	(30.9-63.6)	(78.1-90.8)	(44.9-81.2)

4.7. Factors Associated with CIN2+

In univariable logistic regression analysis, the participants' age group, number of deliveries, years of contraceptive use, having no information about CC, lifetime, and the last five years of screening history & HPV DNA & mRNA profile were found statically associated with CIN2+ (*p*-value <0.05) (Table 9).

However, in our forward model of multivariable logistic regression analysis, only three variables were found to be statistically significant (*p* value <0.05), with CIN2+. Elderly women >50 yrs (AOR: 3..68 95%CI: 1.75-7.72, 0.001), had no history of cervical screening in 5 years period (AOR: 2.04; 95%CI: 1.04-4004; *p*=0.038), tested positive for HR-HPV-DNA (AOR: 5.28; 95%CI: 2.66-10.47; p<0.001) and tested positive for E6/E7 mRNA of HPV16,18 & 45 (AOR: 5.78; 95%CI: 2.73-12.24, *p*<0.001) (Table 10). A marginal statistical association also observed for CIN2+ among those who had many sexual-mates (AOR: 1.69; 95%CI: 0.94-3.02, *p*-value 0.07).

Table 9: Univariable logistic regression analysis of factors associated with high-grade and cancerous lesions (CIN2+), northwest Ethiopia, 2021.

Variables		CIN2+		
		Yes, n (%)	No, n (%)	COR (95%CI)
Age groups (in years)	30-40	44(13.1)	88(26.3)	1
	41-50	53(15.8)	58(17.3)	1.82 (1.09-3.07)
	>50	67(20)	25(7.5)	5.36 (2.99-9.62)
Educational status	No formal education	138(41.2)	121(36.1)	1.57 (0.61-4.03)
	Primary	13(3.9)	29(8.7)	0.62 (0.20-1.89)
	Secondary	5(1.5)	10(3.0)	0.69 (0.17-2.81)
	Tertiary	8(2.4)	11(3.3)	1
Type of occupation	Housewife	151(45.1)	149(44.5)	1.88 (0.73-4.85)
	Private business	6(1.8)	9(2.7)	1.24 (0.31-4.93)
	Government employee	7(2.1)	13(3.9)	1
Body-mass index (BMI)	Underweight	11(3.6)	5(1.6)	1
	Normal	95(31.1)	100(32.8)	0.43(0.15-1.29)
	Overweight	47(15.4)	47(15.7)	0.46(0.15-1.41)
Age @ the first sexual contact	<18	137(41.3)	128(38.6)	0.59(0.34-1.02)
	≥18	26(7.8)	41(12.3)	1
Life-time number of sexual partners	1	74(22.5)	89(27.1)	1
	≥2	89(27.1)	77(23.4)	1.39(0.90-2.15)
Gave birth	Yes	159(47.5)	168(50.1)	0.57 (0.13-2.41)
	No	5(1.5)	3(0.9)	1
Number of children	1-2	20(6.2)	39(12.0)	1
	3-4	37(11.4)	49(15.1)	1.47(0.74-2.92)
	≥5	101(31.2)	78(24.1)	2.53(1.34-4.67)
Use of hormonal contraceptive	Yes	26(7.7)	44(13.1)	0.54 (0.32-0.94)
	No	138(41.2)	127(37.9)	1
Years of hormonal contraceptive use	1-3	9(15.8)	23(40.4)	1
	4-6	9(15.8)	6(10.5)	3.83 (1.06-13.91)
	>6	5(8.8)	5(8.8)	2.56 (0.59-11.00)
HIV sero-status	Positive	22(6.6)	22(6.6)	1.05 (0.56-1.98)
	Negative	142(42.4)	149(42.4)	1
Treated for vaginal discharge	Yes	47(14.0)	56(16.7)	0.83 (0.52-1.31)
	No	115(34.3)	117(34.9)	1
Heard about CC	Yes	59(17.6)	83(24.8)	1
	No	105(31.3)	88(26.3)	1.68(1.08-2.60)
Cervical screening history	Yes	32(9.6)	61(18.2)	1
	No	132(39.4)	110(32.8)	2.29(1.39-3.76)
History of cervical screening in the last five years	Yes	27(8.1)	57(17.0)	1
	No	137(40.9)	114(34.0)	2.54(1.51-4.27)
HR-HPV detected	Yes	134(40.9)	44(13.4)	14.52(8.44-25.00)
	No	26(7.9)	124(37.8)	1
E6/E7 mRNA detected (HPV16,18, & 45)	Yes	17 (5.2)	103 (31.5)	16.34(9.0-29.7)
	No	151 (46.2)	56 (17.1)	1

Table 10: Multivariable analysis of factors related to CIN2+, northwest Ethiopia, 2021.

Variables		CIN2+ Yes, n (%)	CIN2+ No, n (%)	COR(95%CI)	AOR(95%CI), *p*-value
Age groups (in years)	30-40	44(13.1)	88(26.3)	1	1
	41-50	53(15.8)	58(17.3)	1.82 (1.09-3.07)	1.09 (0.55-2.17), 0.794
	>50	67(20)	25(7.5)	5.36 (2.99-9.62)	3.68 (1.75-7.72), 0.001
History of cervical screening in the last five years	Yes	27(8.1)	57(17.0)	1	1
	No	137(40.9)	114(34.0)	2.54(1.51-4.27)	2.04 (1.04-4.04), 0.038
HR-HPV detected	Yes	134(40.9)	44(13.4)	14.52(8.44-25.00)	5.28(2.66-10.47), <0.001
	No	26(7.9)	124(37.8)	1	1
E6/E7 mRNA detected	Yes	17 (5.2)	103(31.5)	16.34(9.0-29.7)	5.78 (2.73-12.24), <0.001
	No	151 (46.2)	56(17.1)	1	1

HR-HPV; High-risk Human papillomaviruses; CI: ConfidenceInterval; **COR**: the crude odds ratio; **AOR:** the Adjusted odds ratio

Hosmer-Lemeshow model fitness test result; Chi-square; 3.47, df; 8, significance; 0.90

Chapter Five: Discussion

In the last few decades, a decreasing trend in the burden of CC was documented in the developed world. However, the disease remains a major public health problem in resource-limited settings (BM., 2020; X. Zhang et al., 2021) because of the rising trends in risk factors, late diagnosis most of the time, and inadequate awareness of screening and other preventative measures (Zeferino & Derchain, 2006). According to different studies the scale of precancerous abnormalities/lesions and CC were steadily increasing in Ethiopia because of very low screening coverage and management of precancerous lesions (Ameya et al., 2017; Kasa et al., 2021).

In this study, a health facility-based estimate of HR-HPV and common genotypes associated with it were described. Moreover, the analytical performance of the HPV16, 18 & 45 mRNA for the identification of CIN2+ was evaluated. We also provided data on the cervical histopathology profile of punch biopsies and important demographic factors linked to CIN2+ among the study participants in northwest Ethiopia. Therefore, the discussion part of the thesis is organized successively describing the distribution of HPV genotype, the mRNA assay result and finally the histological findings along with factors associated with high-grade cervical lesions.

The HR-HPVs are linked with CC and the distinct proportional impact of each genotype on the prevalence of the disease varies geographically (Kulkarni et al., 2023). Hence, a countrywide data on the prevalence and molecular distribution of HR-HPVs amongst Ethiopian women is crucial. This, however, is not adequately available even though there is high burden of CC-related disease and death in the country. Vaccination and screening-led elimination of CC is highly dependent on optimizing HPV data of a particular country. Such data are decisive to appraise the long-lasting effect of the vaccines against HPVs and HPV-based screening, inform policymakers on the best alternative options for cervical and other HPV-associated cancer prevention, and control activities (Ardhaoui et al., 2016).

In our study, the prevalence of HRHPV among women who presented to VIA clinic with cervical abnormalities was 53.0% (188/355). Previously conducted similar studies in Ethiopia reported wide-ranging findings on this regard. The proportion of HR-HPV among women who visited the gynecology clinics and who were recruited from the general population in different

parts of Ethiopia previously ranged from 16 to 100% (Bekele et al., 2010; Gebremeskel et al., 2018; Leyh-Bannurah et al., 2014; Mihret et al., 2014; Teka et al., 2021; Wolday et al., 2018). A study by Gebremeskel *et al.* (Gebremeskel et al., 2018) that specially incorporated women from northwest Ethiopia, described the proportion of HR-HPVs to be 55.5% that is a copy our finding. The prevalence of HR-HPV in different African countries was also varied; in Togo 53.3% (Kuassi-Kpede et al., 2021), and in Zimbabwe 96% (Kuguyo et al., 2021). Dissimilarity in the proportion of HR-HPV across different researches might be due to the variation in the methods of HPV testing, study participants, degree of lesions of the cervix, sociodemographic, and other issues (Bekele et al., 2010; Gebremeskel et al., 2018; Leyh-Bannurah et al., 2014; Mihret et al., 2014; Teka et al., 2021; Wolday et al., 2018)

In this study, HR-HPV was detected higher amongst women >50 years and HIV-infected once (*p*-value <0.005). This suggests that these groups of women need a regular follow up and screening by use of a high precision test including by the use of validated HPV-based & new similar methods and for different reasons persistence may be suspected if there is a positive HPV test at this age (Sweet et al., 2020).

In our study, HPV16 at (50.4%) was the leading HR-HPV. In other similar reports in Ethiopia, other parts of Africa, and in general, globally, HPV16 was the single most common genotype identified in cervical samples. The 2nd, 3rd, 4th, & the rest commonly reported types were typically varied. Specially, HR-HPVs from cervical samples with different degree of lesion in Ethiopia were reported variably (Bekele et al., 2010; Gebremeskel et al., 2018; Leyh-Bannurah et al., 2014; Mihret et al., 2014; Seyoum et al, 2023; Teka et al., 2021; Wolday et al., 2018). This implies that, in different places & spot of time even within the same population, the circulating HRHPVs differ (Jeudin et al., 2021). Therefore, the observed HPV type heterogeneity in Ethiopia suggests the importance of periodic genotyping surveillance at the national level for the effectiveness of vaccination (Seyoum et al., 2023).

Similarly, the distribution of HR-HPVs among women in other African countries was reported to be different (Abate et al., 2013; Ardhaoui et al., 2016; Kuguyo et al., 2021; Tagne et al., 2021). Consistently HPV16 was the most frequently identified genotype among women with various degrees of cervical abnormalities (Ciapponi et al., 2011; Clifford et al., 2005; Ogembo et al., 2015; Peng et al., 2012). The genotype distribution of other HPVs is heavily dependent on the type of cervical lesions. Most often, HPV18, HPV31, 33, 52, and 56 are found as confecting genotypes (Conesa-Zamora et al., 2009).

Generally, the difference in the proportion and type of HR-HPVs among different studies in Ethiopia and other parts of the world might vary partly due to study-specific characteristics, heterogeneity in geographical location, age, life style, & socioeconomic condition of the subjects and differences of the study population. In addition, most importantly methods used for HPV detection (Ardhaoui et al., 2016; Sweet et al., 2020). In other part of the world access to vaccine account for variations in genotypes circulating, age, disease stage and physiologic conditions (like, menopause) are also among the factors that contributed for the difference (Malentacchi et al., 2020)

Globally, HPV18 is the most frequently detected HR-HPV next to HPV16 from advanced cervical lesions including cancer (WHO, 2022b). However, it was not that dominant in our study and we reported a similar result in our recently published systematic review (Derbie et al., 2022). The reason behind the low prevalence of HPV18 (4.7%: 12/258) in Ethiopia requires further studies.

The understanding that infection with high-risk human papillomaviruses causes CC has resulted in the opening of novel prospects for screening & vaccination globally to prevent CC. human papillomaviruses-based testing is becoming a cost-effective approach in most countries around the world (Sehnal & Sláma, 2020). WHO, in the year 2020, launched a worldwide approach in speeding eradication of CC globally. Accordingly, priority is given to vaccinating schoolgirls, screening, and Rx of pre-cancer changes/lesions (WHO, 2020). The initiative/milestone is primarily dependent on the availability of HPV genotype data for countries, like Ethiopia. Our findings together with other previous HPV genotyping reports can be used to partly evaluate the impact of vaccination in the future and for the CC screening program in Ethiopia. Additionally, it will be crucial to keep track of any changes in the distribution of HPV types in the population that has received vaccinations and had their HPV checked to better learn cross-protection & find potential new HR-HPV types (Schmitt et al., 2013).

The current vaccine cocktail used in Ethiopia since 2018 for schoolgirls is Gardasil-4® which targets HPV6, 11, 16, and 18. The vaccine does not target other HR-HPVs circulating in the country. In the present study, for example, the seven HR-HPVs (HPV16, 18, 31, 33, 45, 52 and58) that are included in the nonavalent vaccine accounted for 79.1%. While the combined

prevalence of HPV16 and HPV18 was 55.1%, which implies that a significant proportion of girls might not be protected despite they are vaccinated with Gardasil-4®.

In Ethiopia HPV vaccination is not part of the country level vaccination program. One reason may be the cost of the vaccine. However, in a pilot program, the uptake was very low even if the vaccine was made available. A recently published studies in different parts of Ethiopia showed that the acceptance of the vaccine was very low, at 44.4% in Ambo (Beyen et al., 2022), 45.3% in Bahir Dar (Lakneh et al,. 2022), 50.4% in Arbaminch (Ukumo et al, 2022) and 66.5% in Minjar Shenkora (Kassa et al., 2021). Using Gardasil-4® coupled with its low acceptance will complicate the fight against CC in Ethiopia. A recent cost-effectiveness analysis revealed that, compared to the Gardasil-4™, a nonavalent vaccine, Gardasil®9 targets close to 90% of all HR-HPVs (16, 18, 31, 33, 45, 52, and 58) and is a profitable option for Ethiopia (Wondimu et al., 2022).

A large proportion of HPV infections are sustained by multiple genotypes (Bello et al., 2009). In the present study, 23.9% (45/188) of the participants were found infected with $\geq$2 (i.e up to 5) HR-HPVs, especially among women in 30-40 years of age. A similar study reported 27.3% (30/110) of multiple HR-HPV infections in Eastern Ethiopia (Seyoum et al., 2023). Another study in China similarly reported 29.8% HR-HPV multiple infections from abnormal cytology (Song et al., 2020). Multiple HPV infections are usually common at a younger age of sexual debut (Bello et al., 2009). However, the role of such multiple infections in cervical carcinogenesis has not been well explained in Ethiopia. Wentzensen *et al*. reported up to 14 HPV types from a single cervical specimen although they did not observe type interactions among multiple genotypes (Wentzensen et al., 2009). We also noted infections with many HR-HPVs without the development of high-grade lesions. For instance, infections with HPV31, 52, 66, and infections with HPV31, 52, 39, 59, and 68 were not found to be associated with any form of CIN2+.

Contradicting reports about the role of multiple HR-HPV infections in cervical carcinogenesis are coming out. For instance, Adcock and his colleague reported that the type of HPV & the load of the virus, but not a multiple co-infections of HPV are significant predictors of high-grade lesions including CC (Adcock et ak., 2019). In contrast, a study by Kim et al. (2021) showed multiple HPV infections were found significantly associated with CIN2+ compared to infections with single HPV types. Furthermore, according to Kim *et al*. report, individuals with two or more HPVs infecting cervix exhibited a persistent and extended period of the infection compared to women having one HPV colonization (Kim et al., 2021). The character of multiple HR-HPV infections in the association of an advanced form of cervical lesions including the potential efficacy of HPV vaccines on such infections warrants further research in Ethiopia (Teka et al., 2021).

Concerning the types of HR-HPVs involved and histopathologic grade, all HPV16, HPV18, HPV35, and HPV45 were associated with either CIN2+ in the present study. Specifically, 79(80.6%), 7(87.5%), 3(75%), and 5(100%) of HPV16, HPV18, HPV35, and HPV45 were respectively observed in histopathologically confirmed CC. Specially, the detection of HPV16 was increasing with the level of advanced cervical lesions in which the proportion was 5(12.5%), 2(16.7%), 5(41.7%), and 79(60%) in CIN1, CIN2, CIN3, and cancer, respectively. Similarly, (87.5%) HPV18 were detected in CC cases. This implies that it would be appropriate to consider targeted protocols for the close follow-ups/management of individuals who tested positive for HPV16 and HPV18 (Maria et al., 2018). Previous works also reported a statstical association amongst HPV16 & HPV18 and progression of cervical lesions (Joharinia et al, 2019).

Similarly, in our study 11(78.6%) HPV31&33 coinfections were recovered from cancer cases. A study showed that next to HPV16, the hierarchy of HPV types based on their carcinogenic potential was reported to be HPV33 followed by HPV31(Adcock et al., 2019). Song *et al*. in China also discovered that HPV16 &33 were increased significantly with the higher severity of cervical lesions (Song et al., 2020).

According to the latest WHO report (2022), a large proportion of CC cases (>95%) are due to infection with HR-HPV (WHO, 2022b). In our study, 9.7% of CC cases were without HR-HPVs. A study in Belgium showed that up to 15% of CCs were reported to be without HPV infection (Tjalma, 2018). Additional study is required in this regard in Ethiopia.

Regarding the test performance of the mRNA test, we evaluated the analytical piece of this transcript of HPV16, 18 & 45, which are important genotypes commonly identified in Ethiopia (Derbie et al., 2022) taking histology as an a standard procedure. Multiple studies in the field have shown that for the initial/primary screening, testing for DNA is in general more sensitive than mRNA-based tests. In our study, using the mRNA test that targets HPV16, 18, and 45, we detected 35.8% (127/355) positive cases while the DNA test was positive at 53% (188/355). The DNA test included 10 additional HPVs than the test we employed in mRNA testing. For fair comparison of these tests in our setting, our study strongly suggests large-scale similar studies aimed at evaluating HR-HPV mRNA and DNA detection capacities on important HR-HPVs circulating in Ethiopia.

HPV DNA test for HPV16, 18, & 45 was positive at 42% (149/355). The total agreement of the DNA & the mRNA tests for these three HPVs was at 92.7% with a kappa value of 0.821. Similar finding was reported in China with an agreement of these two tests at 90.7% (kappa =0.8) (S. K. Zhang et al., 2020). Other studies also reported an over agreement of HR-HPV mRNA and DNA based tests over 90% (Castle et al., 2015; Cook et al., 2017)

We observed that the E6/E7 mRNA positivity was increased with a higher grade of histology from 5% in CIN1 to 70.1% in CC. This implies that the occurrence the E6/E7-mRNA transcripts is crucial in cervical disease progression (Andersson et al., 2011). This is because, for the initiation and development of cervical tissue dysplastic characteristic, a continuous expression of these mRNA required (Johansson et al., 2015). Our study justified the hypothesis that E6/E7 mRNA is a possible biomarker for the marking of women who are in risk of developing CC as it associates with the severity of cervical lesions as compared with the DNA-based test (Fontecha et al., 2016; Lie & Kristensen, 2008).

DNA-based HPV tests determine if HPV DNA is present or not. But, most of HRHPV infections tend to be temporary & go away on their own after a couple of years. Only actively infected cells with HR-HPVs produce E6/E7 mRNA, and this expression rises as CIN develops and progresses (Zhang et al., 2020). Henceforth, commercial assays aimed at concurrent typing and detection of the mRNA (E6/E7) for common HR-HPVs are becoming available (Garland et al., 2023).

For both initial/primary CC screening & triage of marginal pap-smear abnormalities It is also reported that E6/E7 mRNA detection could serve as a reliable test (Ratnam et al., 2011).

Women who tested positive for high-risk human papillomavirus E6/E7-mRNA had quite greater risk for CIN2+ & might warrant more attention and earlier health facility visits (Bruno et al., 2018; Dabeski et al., 2019). Besides, it was stated that the mRNA test demosntraated advanced diagnostic correlation in the estimate of Rx failure among surgically treated CC cases in Greek compared with the HPV DNA test (Carcea et al., 2021). Several similar reports support our finding in which HR-HPVs that are frequently associated with high-grade cervical lesions express E6/E7 mRNA more often than other HPV types (Bruno et al., 2018; Dabeski et al., 2019; Khieu & Butler, 2022).

Like the detection of HPV DNA, we observed higher E6/E7 mRNA positive rates in older women than the younger one (*p value* <0.001). The positivity rate increased from 26% among 30-40 years to 37% in those women who were above 40 years of age. A similar finding was reported previously (Zhang et al., 2020). There quick removal of for HPV infection and regression of CIN in younger women is common hence, conservative management of such women is important (Zhang et al., 2020).

Among mRNA-positive cases, HPV16 at 85% (108/127) was the most predominant type that expressed mRNA in different grades of cervical histology followed by HPV18 (8.7%) and HPV45 (4.7%). In one way, the higher proportion of HPV16 In the genotyping might correlate with the observed higher E6/E7 mRNA expression. Further, the link between these HR-HPVs with an increased cervical lesion pattern could be due to their potential to express cell-transforming E6/E7 mRNAs (Andersson et al., 2011). Specifically, 78.8% of the E6/E7 mRNA of HPV16, 88.9% of HPV18 and 66.7% HPV45 were detected among cancer cases. As expected, these HPV types, especially HPV18 & 16, are important in cervical disease and were previously reported in Ethiopia as well (Derbie et al., 2022).

The sen & spec of the mRNA test for the identification of histologically confirmed CIN2+ was 65.2% and 90%, correspondingly. While our DNA assay targets ten more HPVs other than HPV16, 18 & 45, the detection rate of the test was higher with an analytical sensitivity of 84.8% and a specificity of 74.1%. The high specificity of the mRNA test partly explained by the fact that the test targets only the three HPV genotypes (that constitute about 57.8% (149/258) of the identified HR-HPVs. However, studies in the field assert that the mRNA test has a very good specificity as far as CIN2+ detection is concerned (Dabeski et al., 2019; Derbie, et al, 2020). The reported higher specificity by mRNA test could be also illustrated by our

finding in that among 103 women with cervical histology of normal and cervicitis; mRNA for the three HPV types was detected in only 17(9.7%) while HPV DNA was detected in 25 (24.3%) cases.

Our finding implies that the mRNA test can used as a secondary triage test following a first test to decrease colposcopy/biopsy referral, & reduce overtreatment and adverse psychological consequences to the women because of its better specificity. Likewise, the PPV of the E6/E7-mRNA test was greater than DNA testing (85.8%, 75.3%) in the detection of CIN2+, which intern implies that E6/E7 mRNA of HR-HPVs could be an alternative valuable marker both in the triage of women and primary screening (Andersson et al., 2011). An improvement in test specificity is good for proper risk stratification and follow-up of women in general. Specifically, the larg spec and a low sens of the mRNA test in the primary screening suggests a low referral for colposcopy and biopsy (Cook et al., 2017; Shen et al., 2013) which is usually not easily available in most health facilities in Ethiopia. Further, the reported specificity, especially during triage could minimize women who supposed to undergo unrequired conization & costly repeated patient visit to health facilities (Burger et al., 2011; Sørbye et al., 2013) and patients psychologic burden associated with repeated testing(Mockel et al., 2011).

Compared to HPV-DNA assays that indicate merely the qualitative positive or negative results (presence/absence) of the virus, testing for the mRNA gives more insight into the activity of the virus and thus, it demonstrated better clinical relevance as it relate great with advanced nature of lesions. Hence, the mRNA test could be also taken into consideration as a possible bioagent/marker in the marking of individuals that gets at risk of developing advanced-stage lesions (Basu et al., 2016; Ca et al., 2012; Frega et al., 2011; Frega et al., 2016; Gustinucci et al., 2016; Mockel et al., 2011; Pan et al., 2018; Sørbye et al., 2013; Zhao et al., 2014).

In line with this, specifically, the sen and spe of the E6/E7 mRNA assay in the identification of histologically confirmed CIN 2+ were 92.7% and 47%, respectively among HPV16, 18, & 45 DNA positive cases. A similar study by Yao and his colleague reported comparable finding with our study in that the mRNA test demonstrated a sen of 89.5% and spe of 49% among DNA +ve patients (Yao et al., 2017). Likewise, Pruski *et al* also reported the test sensitivity, specificity of the mRNA was at 86%, and 54%, respectively in the identification of histology declared CIN 2+ (Pruski et al., 2019). This implies that after having more similar data and investigating its cost implications, the mRNA test could be considered as a secondary

test/triage/ alternative of women tested +ve for HR-HPV in Ethiopia. Especially this is vital in regions where there is lack of well-trained pathologist and colposcopy facilities (Yao et al., 2017). For both primary CC screening and triage of marginal lesions (like HPV positive cases), the mRNA might be used as a trustworthy marker to be considered (Ratnam et al., 2011).

Generally, the diagnostic relevance, most importantly the better specificity and PPV of the mRNA test to detect advanced cervical lesions and its high sensitivity among HPV-DNA positive cases makes it a potential tool to be considered in areas where there are limited cytology/histology testing facilities. However, our study encourages further large-scale studies including its cost implication in Ethiopia. As HPV-based tests are becoming evident in Ethiopia it might be useful to consider the most important HPVs circulating in the country for further studies to optimize the analytical sensitivity and specificity of this novel test.

As far as the histological profile of cervical biopsies is concerned, having data on this topic from institution-based studies, like ours, is important in reflecting the overall picture of cervical lesions including cancer of the cervix to help guide preventive works. The histopathological

profile of cervical biopsies is also providing the basis for a tailored management of patients (Ameya etal., 2017).

In our study, at the time of hospital visit, 41.8% of study subjects were diagnosed with CC and the proportion of CIN2+ generally was 49.0% that implies most women come to the hospitals at higher stage of the disease a long time later they experience the first symptoms of cervical lesions. A previous study also showed that most Ethiopia women present to hospitals at higher stage of the disease/CC (Begoihn et al., 2019). CIN-1 regress highly (>85%) in two years and it occasionally advances to cancerous lesions. However, if it left untreated, a large proportion of CIN2 and CIN3 in particular could progress to CC (Loopik et al., 2019). The good thing is that, as the disease progresses slowly it gives enough time for early detection and management. The higher rate of premalignant lesions and CC in our finding suggests the need to upsurge awareness of the community and strengthen early cervical lesion detection and treatment.

Few similar studies conducted in Ethiopia reported comparable findings with our result. For instance, a retrospective study by Ameya *et al*. in Hawasa, reported the proportion of cancerous lesions to be 49.3% among symptomatic women plus the level of precancerous lesions were 9.9% (Ameya et al., 2017). Another histopathological-based study in Addis Ababa by Ergete *et al.* on the objective of assessing the causes of postmenopausal bleeding reported that the proportion of CC was 84.8% amongst all cancers of genital tract (Ergete et al., 2001). The relatively higher proportion of CC in the Ergete *et al*. study might be explained by the difference in the type of studied population in which the study typically involved women with postmenopausal bleeding in contrast to our study which involved women aged $\geq$30 years.

A nine-year retrospective data analysis of 1,049 cervical histology reports in Ghana revealed that the majority of cervical samples at 99.4% were diagnosed as CC (BM., 2020). Another retrospective-based study aimed at analyzing 500 histological reports in Malawi showed that the proportion of cervicitis, endocervical polyp, and CIN to be 46.0%, 20.5%, and 24.4%, respectively (Kaseka et al., 2021). Further, a study in Nepal on the histological profile of cervical biopsy indicated that benign, inflammatory, borderline, and malignant lesions were the commonest findings. Proportions of CIN1, CIN2, and CIN3 were 5.8%, 2.0%, and 0.4%, respectively among women with different cervical pathologies who underwent cervical histological examination (Vaiday et al, 2017). Similarly, in India, among 200 women with

cervical histology examination, 35% had cervicitis, 12.5% had cervical polyps, 20% had CIN1, 3% had CIN2, 0.5% had CIN3, and 3.5% had CC (Jain, 2018).

The differences in the proportion of the histopathological findings across these studies might be explained by factors like variations in the severity of the lesions that women presented with, level of awareness of the participants for cervical screening practice, women's health policy of nations, time and setting difference among the studies and socio-demographic related aspects.

At large, the relatively higher proportion of high-grade and cancerous lesions in our report as compared to other countries could be due to disparities of study population, the dissimilarity in accessibility of better women health care practices including inadequate vaccination and cervical screening coverage and inadequate facilities for the management of precancerous lesions in Ethiopia. Further, as the majority of our study participants were from rural settings (66%) and had no formal education (77.3%), these might have also a link with poor awareness and knowledge among studied women about CC including its means of transmission, prevention, and the stage when they should seek medical attention. Most of participants in our research also reported that they have no idea about CC at all. Previous studies also stated that most Ethiopian women had poor knowledge about CC (Derbie et al., 2021; Kassie et al., 2020).

On top of this, the relatively higher prevalence of HIV in our setting might also be an important contributor to the high rate of CC in the present study. Despite majority of our study participants were from rural setting, the prevalence of HIV in our study was relatively higher (13.1%) than the national average. A study in Debre Tabor by Kiros *et al.,* reported that among women participants who came to hospital for cervical screening with VIA, the proportion of HIV positive once were 23.6% (129/546) which is much higher than our report. According to Kiros *et al.,* study, the majority at 60% were from rural setting. This is an interesting report that calls an action to reduce the burden of HIV among rural women in Ethiopia (Kiros et al., 2021).

The proportion of at least one time cervical screening practice was quite low (27.8%) in our study which was much lower than the WHO recommendations but relatively larger than the

national coverage (3.3%). A meta-analysis by Desta *et al*. revealed that CC screening among eligible women in Ethiopia was about 14.8%. The lowest national screening coverage was documented in Amhara Regional State (13.6%) where the present study was carried out. According to this study, that absence of women's formal education reduced screening utilization by 67% (Desta et al., 2021). Another study in Addis Ababa, Ethiopia reported that inadequate awareness about screening are the main perceived reasons amongst individuals who don't have previous CC screening practice (Getachew et al., 2019).

Concerning factors associated with CIN2+, studies in different parts of the world identified important predictors for CIN2+ although there was inconsistency among reports. Some of the commonly identified factors include younger age at first sex and pregnancy, having multiple sexual partners, increasing the period of contraceptive use, cigarette smoking, high parity, low socioeconomic status, and immunosuppression. Indeed, these factors either increased the risk of exposure to HR-HPV infection or might predispose to a compromised immune response to the infection (Anchalee, 2021; Bakari et al, 2017; Baldur-Felskov et al., 2019; Girianelli, & Thuler, 2009; Jean, 2015; Wudtisan et al, 2019; Zhu et al., 2015).

In our study, the multivariable logistic regression analysis showed that women >50 years of age were approximately 4x likely in developing CIN2+ than women aged 30-40 years. Similarly, those who were not screened, those with HR-HPVs infection and those tested positive for E6/E7 mRNA were about two, five, and six times more likely to develop CIN2+, respectively. However, most of the variables tested in the model did not show an independent statistical association with CIN2+. This might be because of a sample size matter, the role of chance and confounding variables influencing each other.

The proportion of CC steadily increased with the age of the study participants. From 21.2% at the age of 30-40 years, the proportion of CC increased to almost 71.3% among postmenopausal women above the age of 50 years (*p*-value <0.0001). Ameya and his colleague from Hawassa, Ethiopia also reported the same pattern matching our report (Ameya et al., 2017). A study in Jimma, Ethiopia also revealed that women above 50 years were probable to had advanced cervical patholohgies (Tesfaw et al., 2020). A study in Ghana also revealed that 71% of the patients with CC were ≥50 years old (BM., 2020), which is precisely in line with our report. Similarly, a study in Nepal reported that CC was primarily documented among women aged 51-60 years (Vaiday et al., 2017).

Studies in Ethiopia showed that patients with CC most often visit health facilities after a long time leading to advanced stages of the lesion during the time of diagnosis (Begoihn et al., 2019; Tesfaw et al., 2020). This might be due to deprived information about CC & the nature of the screening program in the country, which is mainly by using VIA technique. In poor nations like Ethiopia, where no available established cervical screening and vaccination coverage, such kind of disease pattern across age categories is highly predictable. The best way to avert the continued increase of the disease with age would be through comprehensive cervical screening of eligible women with a convenient method, like using HPV based tests. For instance, a trial-based study by Gizaw *et al*. demonstrated significantly higher levels of population-based cervical screening uptake and adherence for self-collection HPV testing than the VIA-based screening in Ethiopia (Gizaw et al., 2019).

In our project, the other important predictor towards CIN2+ was infection with HR-HPV. We noted higher proportion of HR-HPV with advanced degree of cervical lesion (*p*-value <0.0001). Studies in South Africa and Brazil also reported findings in line with our report (Girianelli et al., 2009; Johnson et al., 2020). In the present study, 265 (79.8%) of the participants started having sex before the age of 18 years. Mean age during the participants firsttime intercourse was 15.7$\pm$ 2.6 years. Moreover, 30.7% & 50.9% of the participants had a history of vaginal discharge and sex with multiple partners, respectively. These findings indirectly suggest the participants' risk of exposure to different kinds of sexual transmitted infections including HR-HPVs that proportionally increase the risk of advanced stage of cervical lesions and eventually CC development. It is a well-established knowledge that women who have persistent infection with HR-HPVs ultimately develop high-grade lesions including cancer in later years (Mittal et al., 2017).

We also noted that participants who were positive for E6/E7 mRNA were about six times more likely to develop CIN2+. This supports the fact that the existence of these mRNA is important in cervical lesion advancement (Andersson et al., 2011). This is because for the initiation and and keeping of the tumor phenotype, continuous expression of these RNAs in the integrated HPV genome is necessary (Johansson et al., 2015).

Lastly, we noted that women who had no CC screening practice in the last five years were about two times more likely to develop CIN2+. Some women not screened regularly could

potentially develop cancerous cervical lesions at the late stage of their life. In contrast, a study revealed that women who are sufficiently screened had less chance to be diagnosed with high-grade and cancerous lesions (Hammer, et al., 2019). Since the majority of our study participants were from rural settings, had no formal education, and without information about CC, they might miss important CC prevention approaches, including screening at regular intervals. Illiteracy among women results in poor health-seeking behavior (Gyenwali et al., 2013), especially for gynecologic-related symptoms, which most people in Ethiopia considered as a taboo. In the present study, participants' previous history of cervical screening practice was reportedly very low, at 27.8%.

Screening encompasses testing for HPV infection to detect precancerous lesions the earliest possible, followed by treatment when apposite. When screening detects HR-HPV infection or precancerous lesions, these can easily be treated and therefore advanced stages of the lesion could be prevented. The WHO recommends that screening should begin at the age of 30 in the general population, with a regular screening every 5-10 years, and from 25 years of age for HIV-positive women who should be screened frequently, every 3-5 years. Vaccination, cervix-screening, & Rx of pre-cancer changes are profitable ways in the prevention and control of CC (WHO, 2022a).

As far as associated factors for CIN2+ are concerned, the clinical implication of our findings implies that women above the age of 50, those who tested positive for HR-HPVs/ E6/E7 mRNA, and those who had no cervical screening history should get top priority for close follow-up.

Limitations of the study

Our findings should be interpreted in line with the following limitations. 1) We used a cross-sectional study that has inherit problem in predicting cause and effect association, including the chance of cervical advancement in line with E6/E7 mRNA expression of high-risk HPVs. 2) We have used biopsy taken blindly in four quadrants i.e. it is not colposcopy guided hence there is a chance for inadequate sampling. 3) The histopathological report was generated by a single pathologist; slides were not double-checked. 4) The mRNA analysis did not incorporate all the relevant HR-HPVs identified by our HPV DNA test due to time and financial constraints. 5) The use of CIN2+ endpoint using histology results when assessing the mRNA assay has its own drawbacks as lesions might regress (false +ve) or progress (false -ve) from histologically asserted cervical changes (Burger et al., 2011). 6) Finally, since our study is hospital-based, the conclusion is rather limited. As a result, our conclusion might not be inferred to the general patients.

Chapter Six: Conclusions and Recommendations

6.1. Conclusions

Key findings: *Among the 355 participants, the overall proportion of HR-HPV was 53.0% (188/355) from which 13 different genotypes were identified. Of which, HPV16 at 50.4% was predominant. The HPVs that are part of the nonavalent (HPV16,-18,-31,-33,-45,-52 &-58) vaccine accounted for 79.1%. Specifically, the combined prevalence of HPV16 & 18 was 55.1%. The E6/E7 mRNA test that targets three HR-HPVs (HPV16, 18 & 45) was positive at 35.8% (127/355). The positivity rate of the HPV DNA for these HPVs was 42% (149/355). The test agreement of HPV-DNA and -mRNA was at 92.7% with k score of 82.1%. The detection of both HR-HPV (DNA) & E6E7 mRNA enhanced with the age of the participants and degree of cervical lesions (p-value <0.001). For instance, the detection of mRNA was 5% in CIN1 but increased to 70.1% in CC. Among all samples, the mRNA test meaningfully demonstrated a clinical specificity of 90% and PPV of 85.8% for the detection of histologically confirmed CIN2+ while the HPV DNA test had better sensitivity at 84.8%. However, the Sen and Spe of mRNA assay was 92.7% and 47%, among HPV16, 18, & 45 DNA-positive cases. Majority of the study participants were unaware of CC (59.4%), and did not know that HR-HPVs are transmitted sexually (91.1%). The participants' cervical screening history was about 25%. Most of biopsies at 41.8% were diagnosed as CC. High-grade lesions (CIN2+CIN3+CC) in general accounted for 49.0% (164/335). Women being above the age of 50 years, with no screening history, those infected with HR-HPVs and tested positive for E6/E7 mRNA were significantly associated with CIN2+ (p value less than 0.05).*

⇒ We observed that the majority of the study subjects were unaware of CC and did not have a screening history despite the high burden of CIN2+ and HR-HPV infection in the study area. The vaccination and HPV-based screening practices in Ethiopia will be directly influenced by such studies. Access to cervical screening together with HPV vaccination is considered a key pillar in CC prevention, yet access to screening in the study area is far from where it needs to be. The E6/E7 mRNA test showed better specificity and PPV (with a better sensitivity among HPV-DNA positive cases). Hence, the test could be considered for colposcopy triage after the HPV DNA test so that it would minimize cost and patient anxiety. The test has also diagnostic relevance to detect CIN2+ and could be considered in areas where there is no histological test facility. The mRNA-based test could be a suitable alternative for primary CC screening and triage in Ethiopia once generating additional data and assessing its cost implications.

6.2. Recommendations

Based on our important findings, the following recommendations are forwarded to relevant stakeholders and researchers for future consideration.

- Action to expand and strengthen the ongoing activities in increasing the awareness of the public about CC in the study area shall be in place.
- As per the WHO recommendation, women who are eligible for screening needs to be screened with high-precision test, including with HPV-based tests.
- All forms of CC prevention strategies including the consideration of multivalent HPV vaccination shall be in place in the study area.
- Vaccinations and HPV-based screening tests should take into consideration the major HR-HPVs circulating in the country.
- Community-based similar studies with a better HPV detection method should be considered for improved appreciation of the HPVs circulating in northwest Ethiopia.
- The role of HPV18 and multiple HPV infections in high-grade cervical lesions entails further study in Ethiopia.
- A longitudinal study should be considered in our setting to set a period within which women with a positive HR-HPV DNA test, but a negative E6/E7 mRNA test would be safely followed.
- Lastly, to optimize the analytical sensitivity and specificity of the HR-HPV E6/E7 mRNA assay, large-scale studies that target important HR-HPVs circulating in Ethiopia should be considered.

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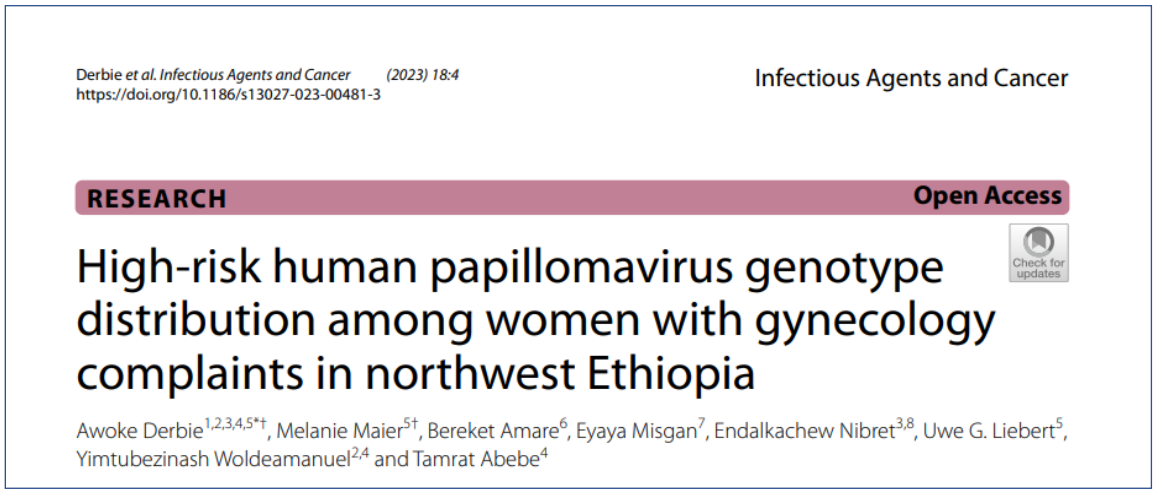
List of Publications and Papers Presented

Original Articles

1. **Awoke Derbie** *et al*. High-risk human papillomavirus genotype distribution among women with gynecology complaints in northwest Ethiopia. *Infectious Agents and Cancer* 2023; 18(1):4. DOI: 10.1186/s13027-023-00481-3


Derbie *et al. Infectious Agents and Cancer* (2023) 18:4
https://doi.org/10.1186/s13027-023-00481-3

Infectious Agents and Cancer

RESEARCH Open Access

High-risk human papillomavirus genotype distribution among women with gynecology complaints in northwest Ethiopia

Awoke Derbie[1,2,3,4,5*†], Melanie Maier[5†], Bereket Amare[6], Eyaya Misgan[7], Endalkachew Nibret[3,8], Uwe G. Liebert[5], Yimtubezinash Woldeamanuel[2,4] and Tamrat Abebe[4]

2. **Awoke Derbie** *et al*. Histopathological profile of cervical punch biopsies and risk factors associated with high-grade cervical precancerous lesions and cancer in northwest Ethiopia. *PLoS ONE* 2022; 17(9):e0274466. DOI: 10.1371/journal.pone.0274466.


PLOS ONE

OPEN ACCESS PEER-REVIEWED

RESEARCH ARTICLE

Histopathological profile of cervical punch biopsies and risk factors associated with high-grade cervical precancerous lesions and cancer in northwest Ethiopia

Awoke Derbie, Bereket Amare, Eyaya Misgan, Endalkachew Nibret, Melanie Maier, Yimtubezinash Woldeamanuel, Tamrat Abebe

Published: September 12, 2022 • https://doi.org/10.1371/journal.pone.0274466

Review Articles

1. **Awoke Derbie** *et al*. Cervical cancer in Ethiopia: a review of the literature. *Cancer Causes & Control* 2022; 34(3):1-11. DOI:10.1007/s10552-022-01638-y
2. **Awoke Derbie** *et al*. Advances in cancer immunotherapy: a review of the literature. *Ethiop Med J*, 2022, Vol. 60, No. 2
3. **Awoke Derbie** *et al*. Human papillomavirus genotype distribution in Ethiopia: an updated systematic review. *Virol J*. 2022 Jan 15; 19(1):13. DOI: 10.1186/s12985-022-01741
4. **Awoke Derbie** *et al*. Low level of knowledge about cervical cancer among Ethiopian women: a systematic review and meta-analysis. *Infect Agents Cancer* 16, 11 (2021). https://doi.org/10.1186/s13027-021-00350-x.
5. **Awoke Derbie** *et al*. HPV E6/E7 mRNA test for the detection of high grade cervical intraepithelial neoplasia (CIN2+): a systematic review. *Infectious Agent and Cancer*, DOI **:** 10.1186/s13027-020-0278-x>
6. **Awoke Drbie** *et al*. Human papillomavirus in Ethiopia: A systematic review. *VirusDis* 2019; 30(2), 171-179.

Manuscripts ready for publication

1. HR-HPV E6/E7 mRNA test for the detection of high-grade cervical lesions among women with gynecology complaints in northwest Ethiopia (Original MS)
2. Acceptance of Human papillomavirus vaccination and parents' willingness to vaccinate their daughters in Ethiopia: a systematic review and meta-analysis

Papers Presented

I have presented the following papers on different conferences (some of the certificates of recognition are attached with).

1. **High-risk Human papillomavirus genotype distribution among gynecology complaints in** northwest Ethiopia.
    - Annual conference of Ethiopian Public Health Association (EPHA), 2023
2. **Histopathological profile of cervical punch biopsies and risk factors associated with high**-grade cervical precancerous lesions and cancer in northwest Ethiopia.
    - Annual conference of Ethiopian Society of Obstetrics and Gynecology (ESOG), 2023.
    - Annual Consortium of Reproductive Health Association (COHRA), 2023
    - Annual conference of Ethiopian Public Health Association (EPHA), 2023
3. **Human papillomavirus genotype distribution in Ethiopia: an updated systematic review.**
    - Annual conference of Ethiopian Society of Obstetrics and Gynecology (ESOG), 2022.
    - Annual conference of Ethiopian Public Health Association (EPHA), 2022
    - Annual conference of Ethiopian Society of Hematology and Oncology, 2022.
4. **Low level of knowledge about cervical cancer among Ethiopian women: a systematic** review and meta-analysis.
    - Annual conference of Ethiopian Society of Obstetrics and Gynecology (ESOG), 2021.
    - Annual conference of Ethiopian Public Health Association (EPHA), 2021

Ethiopian Public Health Association
የኢትዮጵያ ጤና አጠባበቅ ማህበር

CERTIFICATE OF PARTICIPATION

THIS CERTIFICATE IS AWARDED TO

AWOKE DERBIE

For participation as Scientific Paper Presenter at the 34th Ethiopian Public Health Association Annual Conference.

Conference date: from 20/03/2023 to 21/03/2023G.C

Venue: Inter-luxury Hotel, Addis Ababa, Ethiopia

Allocated CEU:10CEU Unique ID: 406453747

Ato Tesfa Demelew
PDT Director, EPHA

Dr. Alemayehu Mekonen
Executive Director, EPHA

Certificate of Participation

This certificate is proudly awarded to

Awoke Derbie

For his presentation of an abstract entitled "Histopathological profile of cervical punch biopsies and risk factors associated with high-grade cervical precancerous lesions and cancer northwest Ethiopia." at the Consortium of Reproductive Health Association 3rd Annual RH conference. CORHA appreciates his excellent participation and presentation at the conference.

MARCH 22, 2023

Acc No: 02EMwA-CPDP1420

Issued Date: April 28, 2023

CERTIFICATE OF PARTICIPATION

The Ethiopian Society of Obstetricians and Gynecologists (ESOG) certifies that

Awoke Derbie

has presented a research entitled ***Histopathological profile of cervical punch biopsies and risk factors associated with high-grade cervical precancerous lesions and cancer in northwest Ethiopia*** at the scientific session of ESOG's 31st Annual Conference which was conducted from February 10-15, 2023. He/She is awarded **10 CEUs.**

DR. FERID ABBAS
Chair, CME Committee

DR. ABDULFETAH ABDULKADIR
President of ESOG

Certificate of Recognition

This is presented to

AWOKE DERBIE

for being a distinguished presenter of '**Human papillomavirus genotype distribution in Ethiopia; an updated systematic review**' at the 30th annual conference of the Ethiopian Society of Obstetricians and Gynecologists (ESOG) post-conference scientific session held from February 21-22, 2021

Dr. Abdulfetah Abdulkadir
Chair, CME Committee, ESOG

Appendix

Appendix A: Information Sheet

(English Version)

Name of Investigator: Awoke Deribie (PhD candidate)

Introduction

Dear Madam, you are invited to participate in a research study conducted by a PhD candidate, from Addis Ababa University. Your participation is absolutely based on a voluntarily basis. The research team includes a principal investigator, data/specimen collectors including a senior gynecologists and pathologists and supervisors from Addis Ababa University. Please, kindly take as much time as you need to read/listen the information sheet.

Aim of the Research Project

We request your voluntary participation in our study because we are planning discover more about new cervical screening tool, the type of viruses involved in cervical lesions and the epidemiological issues linked to CIN2+ among women to devise a solution in the prevention of CC.

Procedure

If you are willing to participate in this project, you need to understand and give your consent. The required clinical sample (cervical swab and biopsy) will be collected by an experienced gynecologist working at FHCSH following the recommended approach. No anesthesia is required to collect cervical biopsy. We will also ask you some questions about yourself by using a set of questions.

Anticipated risks and discomforts

Although we do not anticipate major risks associated with your participation in this project, however as all types of clinical studies, there might be a minimal risk. During the collection of cervical biopsies there might be some level of pain and slight bleeding but this does not produce serious pain and other anticipated complications.

Benefits to study participants and/or to the society

Based on the diagnostic result of histology and NA analysis, you will be linked to the gynecology clinic for better management and at the same time, you are indirectly benefiting through the generation of this data for better management of patients like you attending this hospital and other hospitals in the country associated with cervical pathology.

Payment for study participation

We do not offer any kind of fee being you are part of this study.

Confidentiality issues

All data generated from this study will be confidential and only be shared with your physician when necessary. Data collected about you will be utilized using codes, no name identified will be indicated for the public.

Study participation/withdrawal

It is your right to be part of this particular study. You will not be forced to remain in the study: withdraw at any time is possible. You can also deny giving clinical samples.

Person to contact:

If you have any question, you can contact any of the following at any time:

1. Awoke Deribie (PhD student): Cell: +251- 09 13 05 98 87 E-mail: awoke.derbie@bdu.edu.et
2. Dr. Tamrat Abebe (Advisor): Cell: +251-911447227, E-mail: tabebezeleke@gmail.com, AAU, CHS-DMIP.
3. Dr. Yimtubeznash Woldeamanuel (Co-advisor): Cell: +251-911225832, Email: yimtuwa@gmail.com, AAU, CHS-DMIP
4. Institutional Review Board, CHS, AAU. Telephone: 0118961396; E-mail: Chs.irb@aau.edu.et

Appendix B: Consent Form

Participant ID _____

Name of the participant _____

I get told about the research, which is planned to be done on a new cervical screening tool, the type of viruses involved in cervical lesions and the epidemiological factors associated with CIN2+ amongst women in NW ETH. The aim & application of the research project were narrated to me. I am also informed that all information collected from me will be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care and hospital access.

It is therefore with full understanding of the situations that I agreed to give the written consent to the researcher to use the specimen taken from my cervix for investigation. I also agreed that the cervical brush, histological sample and the HPV viruses identified might be stored and investigated further on similar grounds. In addition I have had the opportunity to ask a question about the project and I have got the clarification to my satisfaction.

Moreover, I was also told that results will be reported timely to physician in charge for appropriate treatment and management of my case. Hence, I agree to participate on this study with full voluntarism.

I _____ the undersigned hereby give my consent for giving the requested information and specimen for the aforementioned study.

- Signature of the participant _____
- Witness (Illiterate) _____
- Physician/principal investigator/data collector _____

Date ------/-------/----

የጥናቱ ማብራሪያ

መግቢያ

ይህ ምርምር አቶ አወቀ ደርቤ በተባለ የአዲስ አበባ ዩኒቨርሲቲ ህክምናና ጤና ሳይንስ ኮሌጅ የሶስተኛ ዲግሪ ተማሪ እና በአማካሪዎቹ የሚሰራ ሲሆን በዚህ ጥናት ለመሳተፍ የእራዎ ሙሉ ፈቃደኝነት ያፈልገናል። በቂ ጊዜ ወስደወ መጀመሪያ ይህን መጠየቅ እንዲያነቡ/እንዲያዳመጡ በአክብሮት ተጋበዘዋል።

የጥናቱ ዓላማ

የጥናቱ ዋና ዓላማ በኢትዮጵያ በአሳሳቢ ሁኔታ እየጨመረ በመጣዉ የማህጸን በር ጫፍ ካንሰር ቅድመ-ምርመራ፤አመጭ ቫይረሶች ልየታ እና ተያያዥ አጋላጭ ሁኔታዎችን ለማጥናትና ለቀጣይ የመፈተሄ ግበዓት የሚሆን መረጃ ለማመንጨት ነዉ።

መረጃ አሰባሰብን በተመለከተ

በዚህ ጥናት ለመሳተፍ ሙሉ ፈቃደኝነትን የሚጠይቅ ሲሆን እርሰዎም በጥናቱ ለመሳተፍ ፈቃደኛ ከሆኑ ለምርምሩ የሚሆን 1ኛ) በነርስ የሚሙላ መጠይቅ እንዲሁም 2)ከማህጸንዎ በር ጫፍ ለምድ ባለዉ ማህጸን እና ጽንስ ህኪም አማካኝነት ማደንዘዣ መረፌ ሳያስፈልግዎ የተለመደ አሰራሩን በመከተል ናሙና ይወሰዳል።

ሊፈጠሩ የሚችሉ ስጋቶች

በዚህ ጥናቱ በመሳተፈዎ የሚፈጠር የጎላ የጎንዮሽ ችግር ባይኖርም የማህጸን እና ጽንስ ህኪሙ ናሙና በሚወስድበት ጊዜ የተለመደ የአለመመቸት ስሜት ሊሰማዎት ይችላል፤ ከዚህ በተጨማሪ ናሙና ከተወሰደበተ ቦታ ላይ ቀላል የሚባል መድማት ሊኖር ይችላል። ይሁን እንጂ ስሜቱ ከተወሰኑ ደቂቃዎች በኋላ ስለሚጠፋ ቀጣይነተ ያለዉ ህመምም ሆነ ሌላ ለጤናዎት ስጋተ የሚሆን ችግር አይኖረዉም።

በጥናቱ በመሳተፈዎ የሚገኝ ጥቅም እና ክፍያን በተመለከተ

የናሙናዉ የምርመራ ዉጤት እንዳለቀ ለሚከታተሉት ህኪም ዉጤቱ ለተሻለ ህክምና የሚሰጥሎት መሆኑን እየገለጽን በዚህ ጥናት በመሳተፈዎ ግን የሚከፈል ምንም አይነት ክፍያ የለም። ይሁን እንጂ የጥናቱ የመጨረሻ አጠቃላይ ዉጤት በሀገራችን የማህጸን በር ጫፍ ካንሰርን ለመከላከል በሚደረግዉ ርብርብ የእርስዎ በምርምሩ መሳተፍ እንደ ግብዓት የሚያገለግል መሆኑን ልናሳዉቀዎት እንወዳለን።

የመረጃ አያያዘን በተመለከተ

በጥናቱ ዉስጥ የተሰበሰቡ ማናቸዉም ግላዊ መረጃዎች ሚስ□ራዊነት □ዙ□በቀ □ሆናል። የናሙና ምርመራ ዬ□ቶች- ን ለሚከተተሉ- ት ህኪም ከማ□ራት ዬ ቸ ከማንነት- □ር በቀዓ ተታ ተጸጸ□ነት ያላቸዬ መረጽ- ች በሙሉ በ□ና ተመራማሪዬ ሚስ□ራዊ በሆነ □መረጽ ጥንቅር □□ ከተቀ□ሩ በኋላ ብቻ ለምርምር ሂ□ቱ □ዎሚዬ ሉ ይሆናሉ።

ከጥናቱ ስለመዉጣት/ማቋረጥን በተመለከተ

ይህ ጥናት በፍቃደኝነት ላይ የተመሰረተ አንደመሆኑ መጠን በማንኛውም ወቅት በፈቃድዎ ከጥናቱ መዉጣት ወይም ናሙና አለመስጠት ይችላሉ::ከጥናቱ ቢዎ□ም □ተለመደዉን □ሕ□ምና አገለገሎት በጤና ተቋሙ ዉስጥ በማንኛዬ ም ዯ□ □ማ□ኜት መብትዎ የተጠበቀ ነዉ::

በመጨረሻ፤ ከጥናቱ ጋር በተያያዘ ማናቸዉም ጥያቄ ወይም አስተያየት ቢኖርዎ በሚከተለዬ አድራሻ ዓ ጸቄ- ን ማቅረብ ይችላሉ፤

1. **አወቀ ደርቤ፡ አድራሻ፡ አዲስ አባበ ዩኒቨርሲቲ፤ ስልክ ቁጥር 0913-059887/0963801526**
2. **□/ር ታምራት አበበ፡ አቴስ አባበ □ኒቨርሲቲ፤ ሞባ□ል ስል□ ቁዓ ር 0911-447227**
3. **□/ር ይምጡበዘናሽ ወለደአማኑኤል: አቴስ አባበ □ኒቨርሲቲ፤ ሞባ□ል ስል□ ቁዓ ር 0911-225832**
4. **አዲስ አባበ ዩኒቨርሲቲ ጤና ሳይንስ ኮሌጅ የምርምር ስነ-ም□ባር መቆ□□ሪጸ □አ ል፤ ስል□ ቁዓ ር 0118961396? ኢሜል**: chs.irb@aau.edu.et

የስምምነት ቅጽ

- □ተሳተኝዬ ምስ□ር ቁዓር ______________
- □ተሳተኝዬ ሙሉ ስም ______________

እኔ ስሜ ከ□ህ በታች □ተቶለ□ዬ በ□ህ ዓናት ተሳታኝ ለመሆን ስወስን □ዓናቱ □ላማ አሰራሮችና ቅድመ ሁኔታ- ች በግልጽ በመረዳትና እንዲሁም ከጥናቱ ተሳታኝነት ፈቃደኝቴን በማንኛዉ ደረጃ የማስወገድ መብቴን በማረጋገጥ ነዉ።

እኔ ______________________________ በ□ህ ዓናት በፈቃደኝነት ተሳተፊ መሆኔን በኝርማ□ እያረጋገጥኩ ይህንን ስወስን በዓናቱ ሳቢያ ሊከሰቱ የሚችሉ ስ□ቶችን በሚቶ □ተረቴሁና ከጥናቱ በማንኛዉም ደረጃ ራሴን ለማግለል ብወስን ተገቢ የሆኑ ህክምናዎችና እቶ□ ች ሁሉ እን□ማ□ነፈጉኝ በማመን ነዉ። ከ□ህ በተፊ ማሪ በመርምሩ ወቀት ከእኔ □ተወሰ□ □ናሙና ምርምራ ዉጤቶች ለሚከታተሉኝ ሀኪሞች እንደሚሰጡልኝ የተገለጸልኝ ሲሆን ናሙናዬ እንዲሁም ከ□ሁ □ተለ□ ቫ□ረሶች ለወደፊት መሰል ጥናት ቢቀመጡ □አና ዓቀም ላ□ ቢዬ ሉ ፈቃደኝነቴን እያሳወኩ እነ□ህ ሁሉ መረጃ- ች በምረቴዬ ቋንቋ በሚገባ የተገለጹልኝ መሆኑን በፊርማዬ አረጋግጣለሁ።

- □ተሳታኝዬ ሙሉ ስም____________________ ኝርማ________
- □መረጽ □ሚሰበስበዬ /ተመራማሪዬ ሙሉ ስም__________
  ኝርማ________
- □ምስ□ር ሙሉ ስም______________________________ ኝርማ________

Appendix C: Demographic Data Collection Tool
(Questionnaire)

I. Demographic characteristic

1. Participant ID/Card# _____
2. Age/in full years/ _____
3. Setting a. Urban b. Rural
4. Education a. unable to read & write b. < grade 8 c. Grade 8-12 d. College study
5. Occupation a. Housewife b. Private business/employ c. Gov't employs d. Other specify_____
6. Marital status a. Single b. Married c. Widowed d. Other, specify_____

II. Reproductive/Gynecologic data/risk related items (some from chart review)

7. Body Mass Index, (BMI--Kg/M^2) _____
8. Your age during the sex? _____
9. # of sex partner(s)? _____
10. Did you give birth? a. Yes b. No
11. If yes, how many children do you have? _____
12. Do you use hormonal contraceptives? a. Yes b. No
13. If yes, for how many years? _____
14. HIV Sero-status? a. Positive b. Negative c. Unknown
15. If HIV positive, current CD4 count and stage of AIDS _____, _____
16. Did you have previous history of sexually transmitted disease? a. Yes b. No c. Unknown
17. History of treatment for vaginal discharge? a. Yes b. No c. Unknown
18. Have you vaccinated for HPV? a. Yes b. No c. Unknown
19. Do you have sister or mother with history of CC? a. Yes b. No
20. Do you smoke tobacco a. Yes, (average smoke per day____) b. No
21. Do your husband/any other family membrane living with, smokes? a. Yes b. No
22. Have you ever had any type of cancer? a. Yes b. No c. Unknown
23. Have you screened for CC before? A. Yes (how many times___)b. No
24. Have you screened for CC in the last five years? a. Yes b. No
25. If yes for either, by which method? a. VIA b. Pap c. HPV based test d. other, specify____
26. Current presumptive gynecologic diagnosis? _____
27. Gynecologic image finding (like, ultrasound) _____
28. VIA screening result _____
29. OnchoE6 test result a. Positive b. Negative c. Invalid
30. If OnchoE6 test is positive, type of HPV identified? a. HPV 16 b. HPV 18 c. Both
31. Type of treatment given? _____

መጠይቅ እና ለበሽተኛዉ ክቻርት የሚሞላ

1. **የተሳታፊ መለያ ቁጥር** _____
2. **ዕድሜ** (በዓመት) _____
3. ***መኖሪያ*** ***ሀ. ገጠር*** ***ለ. ከተማ***
4. **የትምህርት ደረጃ** ሀ. ፊደል መቁጠር የማትችል ለ. ከ8ኛ ክፍል በታችቸ ሐ. ከ8-12ኛ መ. የኮሌጅ ት/ት
5. **የስራ አየነት** ሀ. የቤት እመቤት ለ. የግል ስራ/ተቀጣሪ ሐ. የመንግስተ ሰራተኛ መ. ሌላ _____
6. **የጋብቻ ሁኔታ** ሀ. ያላገባ ለ. ያገባ ሐ. የፈታ መ. ሌላ _____
7. ቦዲማስ ኢንዴክስ (BMI) (ኪ.ግ/ሜ^2)? _____
8. **በመጀመሪያዎ የግብረስጋ ግንኙነት ወቅት ዕድሜዎ ስንት ነበር?** _____
9. **እስካሁን ከስንት ሰዎች ጋር የግብረስጋ ግንኙነት ፈጽመዋል?** _____
10. **ልጅ አለዎት? ሀ. አዎ ለ. የለኝም**
11. **መልስዎ 'አወ' ከሆነ ምን ያህል ልጆች አሉዎት?** _____
12. **የሆረሞን ወሊድ መቆጣጠሪ ይጠቀማሉ? ሀ. አዎ ለ. አልጠቀምም**
13. **መለሰዎ አወ ከሆነ ለምን ያክግ ጊዜ?** _____
14. **በደምዎ የኤችአይቪ ሁኔታ ሀ. አለብኝ ለ. የለበኝም ሐ. አላቅም**
15. ኤችአይቪ በደም ዉስጥ ካለ የቅረብ ጌዜ CD4 ቁጥርና የኤዲስ ደረጃዉ? (ክቻርት) _____, _____
16. **የአባላዘር በሽታ ይዞዎት ያቃል? ሀ. አዎ ለ. የለም ሐ. አላቅም**
17. **ከብልት በሚዎጣ ፈሳሽ ሳቢያ ታክመዉ ያቃሉ? ሀ. አዎ ለ. የለም ሐ. አላቅም**
18. **የማህጸን በር ካንሰርን ለመከላከል የሚያገዝ ክትባት ወስደዋል? ሀ. አዎ ለ. የለም ሐ. አላቅም**
19. **አናትዎ ወይም እህት በማህጸን በር ካንሰር ታመዉ ያቃሉ? ሀ. አዎ ለ. የለም**
20. **ትንባሆ አጭሰው ያቃሉ? ሀ. አወ (በአማካኝ በቀን ስንት_____) ለ. አላቅም**
21. ባለቤትዎ ወይም ሌላ የቤተሰብ አባል ትንባሆ የሚያጨስ አብሮ ከቤተሰብ ጋር የሚኖር አለ? ሀ. አዎ ለ. የለም
22. **ለትኛውም አይነት ካንሰር ታመው ያቃሉ? ሀ. አዎ ለ. የለም**
23. **ለማህጸን በር ቅድመ ካንሰር ስክሪን (ምርመራ) አድርገው ያቃሉ?ሀ. አዎ ለ. የለም**
24. ለማህጸን በር ቅድመ ካንሰር ባለፍት አምስት አመታት ተመርምረው ያቃሉ? ሀ. አዎ ለ. የለም
25. **መልስዎ 'አወ' ከሆነ በየትኛው የምርመራ መንገድ?** _____
26. **አሁን ላይ የተጠረጠረው ችግር? (ክቻርት)** _____
27. **የቪአይኤ** (VIA) **ምርመራ ውጤት? (ክቻርት)** _____

28. **የአልትራሳዉንድ ወይም ሌላ የምስል ምርመራ ዉጤት(ከቻርት)**

_____

29. **ኦንኮ6 (OnchoE6) የምርመራ ውጤት? (ከቻርት)** _____

30. **ኦንኮ6 ፖዝቲቭ ከሆነ የተለየው የቫይረስ አይነት?(ከቻርት)**

_____

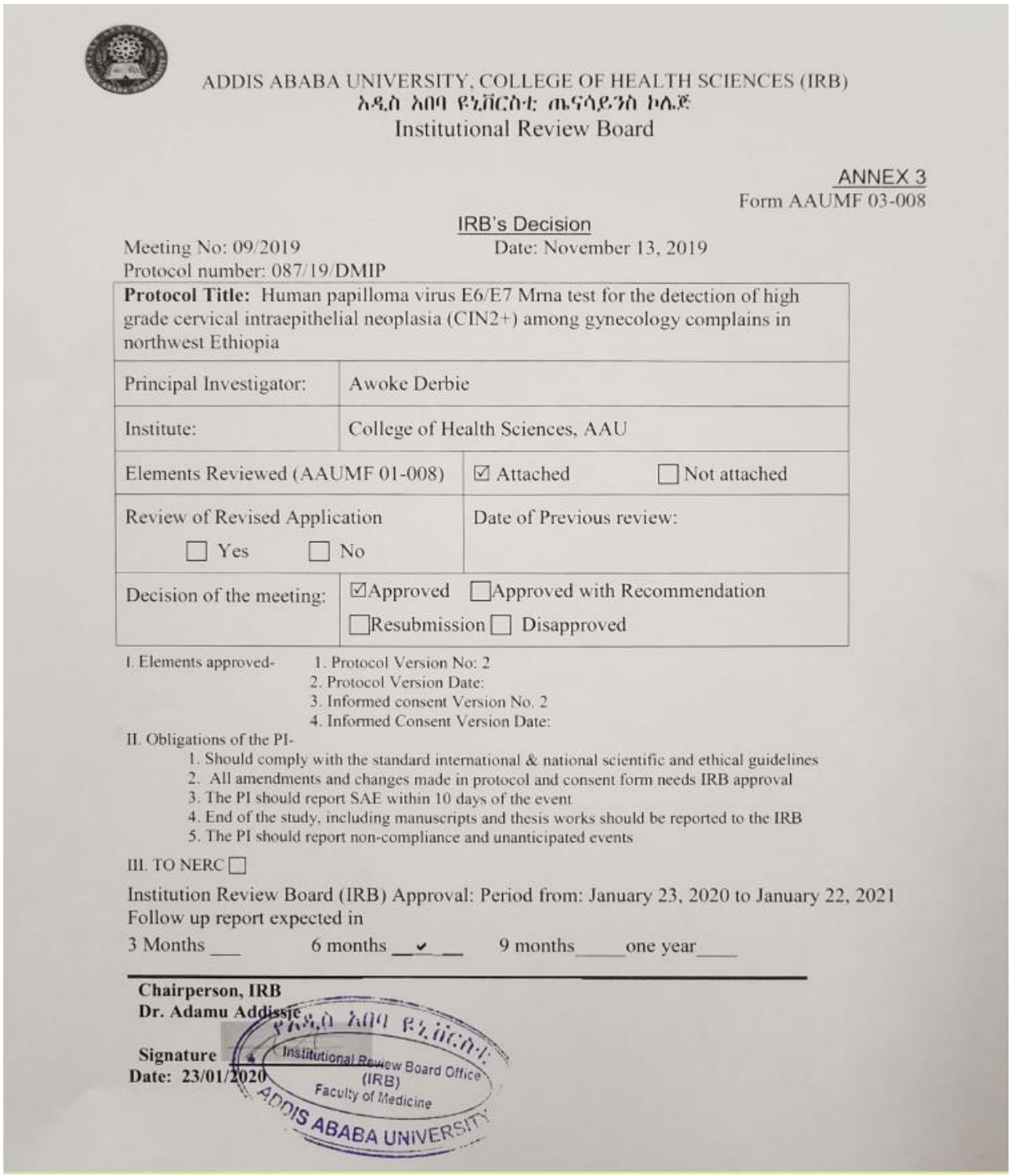
31. **የተሰጠው የህክምና አይነት? (ከቻርት)** _____

Appendix D: Project Administration and Staffing

This PhD thesis was administered by department of DMIP,CHS,AAU. The funding issues was handled by CDT-Africa, AAU-CHS, and BRI, Bahir Dar University (BDU). Supervisors were assigned from the department and collaborators were invited from FHCSH, BDU, CMHS, and from Germany at the Leipzig University Hospital.

The faculty that were involved in this project includes; the principal investigator, supervisors, collaborators (gynecologist and pathologist), and questioner administrator (nurses).

Appendix E: AAU, CHS, IRB-ethical clearance



ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)
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Institutional Review Board

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 09/2019 Date: November 13, 2019
Protocol number: 087/19/DMIP

Protocol Title: Human papilloma virus E6/E7 Mrna test for the detection of high grade cervical intraepithelial neoplasia (CIN2+) among gynecology complains in northwest Ethiopia		
Principal Investigator:	Awoke Derbie	
Institute:	College of Health Sciences, AAU	
Elements Reviewed (AAUMF 01-008)	☑ Attached	☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:	
Decision of the meeting:	☑Approved ☐Approved with Recommendation ☐Resubmission ☐ Disapproved	

I. Elements approved-
1. Protocol Version No: 2
2. Protocol Version Date:
3. Informed consent Version No. 2
4. Informed Consent Version Date:

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB
5. The PI should report non-compliance and unanticipated events

III. TO NERC ☐

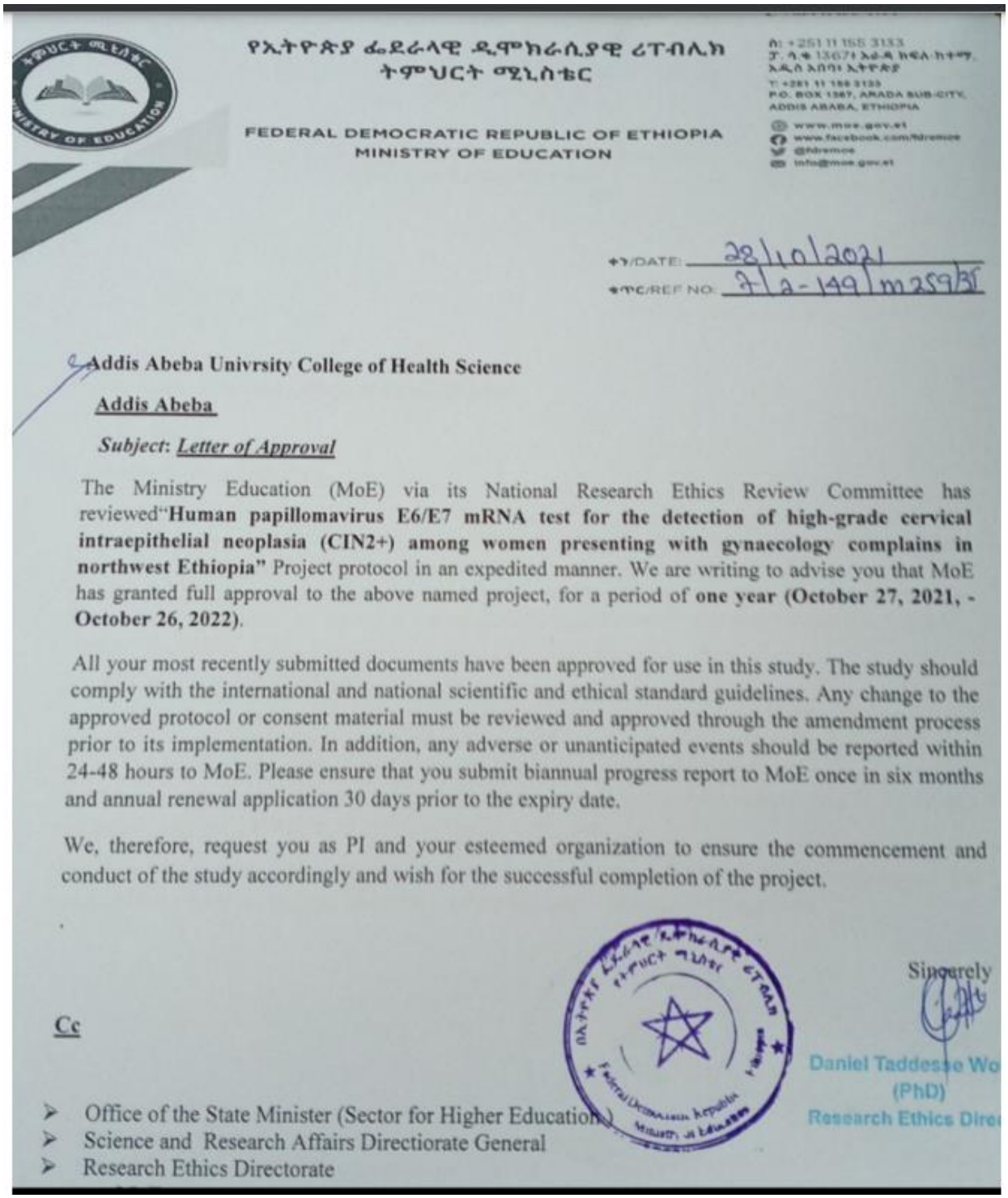
Institution Review Board (IRB) Approval: Period from: January 23, 2020 to January 22, 2021
Follow up report expected in
3 Months ___ 6 months __✔__ 9 months_____ one year____

Chairperson, IRB
Dr. Adamu Addissie

Signature
Date: 23/01/2020

የአዲስ አበባ ዩኒቨርስቲ Institutional Review Board Office (IRB) Faculty of Medicine ADDIS ABABA UNIVERSITY

Appendix F: National Ethical Clearance



የኢትዮጵያ ፌደራላዊ ዲሞክራሲያዊ ሪፐብሊክ
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ቀን/DATE: 28/10/2021
ማጣ/REF NO: ፖ/ሳ-149/m259/35

Addis Abeba Univrsity College of Health Science

Addis Abeba

Subject: *Letter of Approval*

The Ministry Education (MoE) via its National Research Ethics Review Committee has reviewed"**Human papillomavirus E6/E7 mRNA test for the detection of high-grade cervical intraepithelial neoplasia (CIN2+) among women presenting with gynaecology complains in northwest Ethiopia"** Project protocol in an expedited manner. We are writing to advise you that MoE has granted full approval to the above named project, for a period of **one year (October 27, 2021, - October 26, 2022)**.

All your most recently submitted documents have been approved for use in this study. The study should comply with the international and national scientific and ethical standard guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to MoE. Please ensure that you submit biannual progress report to MoE once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

Sincerely

Daniel Taddesse Wo
(PhD)
Research Ethics Dire

Cc

- Office of the State Minister (Sector for Higher Education)
- Science and Research Affairs Directiorate General
- Research Ethics Directorate

Appendix G: Material Transfer Agreement (MTA)

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Addis Ababa University, Department of Microbiology, Immunology and Parasitology, Ethiopia and Institute of Virology, Medical Faculty, Leipzig University in all transfer of research material (samples, derivatives, and specimens) related to the protocol ***"Human papillomavirus E6/E7 mRNA test for the detection of high grade cervical intraepithelial neoplasia (CIN2+) among gynecology complaints in northwest Ethiopia".***

Provider: Mr. Awoke Derbie, Addis Ababa University, Department of Microbiology, Immunology and Parasitology, Ethiopia

Recipient: Prof Uwe G Liebert, Institute of Virology, Medical Faculty, Leipzig University, Leipzig, Germany. Provider agrees to transfer to recipient's designated (provider) the following research materials /specimen; ***Cervical brushes*** for HPV genotyping and E6/E7 mRNA test.

The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

a) Are the research materials of human origin?

Yes [√] No [ ]

b) If yes, are they collected according to the details in the protocol and in adherence to National Research Ethics Review Committee (NRERC) and Addis Ababa University College of Health Sciences (AAU-CHS) Ethics Review Committee recommendations and their approval?

Yes [√] No [ ]

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows ***"Human papillomavirus E6/E7 mRNA test for the detection of high grade cervical intraepithelial neoplasia (CIN2+) among gynecology complaints in northwest Ethiopia".***
3. In all presentations or written publications concerning the research project, recipient will acknowledge provider's contribution of this research material unless requested otherwise.

4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research material and further agrees not to transfer the research material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project.
5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the recipient under conditions agreed to in the protocol on shipment of the samples. This research material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.
6. The recipient shall notify the provider in witting of any intention, improvement, modification discovery or development to the material or the information made by recipient or parties, collaborating with recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, and other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under- signed provider and recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.
8. Any additional terms (use an attached page if necessary):
9. The provider maintains, ownership right of the research material and its unmodified derivatives unless stated otherwise.

The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement

Signature page

For Recipient:

Read and Acknowledged by:

Recipient's Investigator

Prof. Uwe G Liebert

Signature

UNIVERSITÄTSKLINIKUM LEIPZIG AöR
Institut für Virologie
Direktor: Prof. Dr. U. G. Liebert
Johannisallee 30 · 04103 Leipzig
Tel. 0341 / 97-1 43 00 · Fax 97-1 43 09

Date: 02/09/20

Mailing Address for Materials:

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04103 Leipzig

Telefon:0341 - 97 14300

Fax: 0341 - 97 14309

Duly Authorized

Prof. Uwe G Liebert

Signature/ Stamp

UNIVERSITÄTSKLINIKUM LEIPZIG AöR
Institut für Virologie
Direktor: Prof. Dr. U. G. Liebert
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Date: 02/09/20

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For Provider:

Provider's Investigator

Mr. Awoke Derbie

Signature

Date: 02 Oct 2020

Mailing Address:

Addis Ababa University, Department of Microbiology, Immunology and Parasitology, College of Health Sciences, School of Medicine, Tikur Anbessa Specialized Hospital, P.o.Box 9086, Addis Ababa, Ethiopia

Tel: +251913059887

Duly Authorized

Prof. Daniel Asrat

Signature/ Stamp

Date: 02 Oct 2020

Mailing Address for Notices:

Addis Ababa University, Department of Microbiology, Immunology and Parasitology, Tikur Anbessa Specialized Hospital, 6th floor room number 8, P.o.Box 9086, Addis Ababa, Ethiopia

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