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Human papillomavirus genotype distribution, persistence, clearance, and characterizing cervicovaginal microbiota: A population- based follow up study

By

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Work Declaration

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I, Brhanu Teka Endallew, declare that this dissertation is my original work. I have written and submitted to AAU-CHS, Department of Microbiology, Immunology and Parasitology never to any other institution in any form for evaluation. All the information here is dully acknowledged and I have never used any other source except those cited ones.

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I hereby declare that I have advised this Ph.D. work, the thesis has been prepared under my supervision and has been submitted with my approval.

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Human papillomavirus genotype distribution, persistence, clearance, and characterizing cervicovaginal microbiota: A population- based follow up study

Abstract

Background: Human papillomaviruses (HPVs) are a group of small, non-enveloped, naked icosahedral (55nm) viruses that can cause cervical cancer (CC) and other cancers. Cervical cancer is by far the most common HPV-related disease, and it is the second leading cause of morbidity and mortality from all cancers in Ethiopian women. Persistent infection with hr-HPVs and progression to precancerous lesions are the most important steps in the carcinogenesis process. However, most infections are transient and rarely persist implying development of CC is a multifactorial and step by step process and may require other co-factors like cervicovaginal microbiome within the local microenvironment for its development.

Objective: To determine the prevalence of HPV infection, genotype distribution, the persistence and clearance rates within two years and compare the performance of different HPV tests. Furthermore, it aimed to characterize the cervicovaginal microbiota in women with premalignant dysplasia or invasive cervical cancer compared with that of healthy women.

Methods: The study was conducted in two cohorts; a population-based cohort from rural women in Butajira, south-central Ethiopia and women attending gynaecological clinic at Tikur Anbessa Specialized Hospital from October 2017 to February 2020. From Butajira, a total of 893 samples were tested at baseline. A self-sampling brush (Evalyn Brush®, Rovers, Oss, The Netherlands) was used for cervical specimen collection and HPV testing was performed using multiplexed genotyping (MPG) by BSGP5+/6+ PCR with Luminex read out. Follow-up testing was done at 6 and 24 months for baseline hr-HPV positive women. Moreover, three HPV DNA testing assays (MPG-Luminex Assay, Anyplex II HPV HR Detection, and EUROArray HPV) were compared and the analytical sensitivity and specificity of the assays in detecting hr-HPV infections was computed. At Tikur Anbessa Specialized Hospital, cervicovaginal microbiota of 120 women was characterised using the 16S rRNA cervical microbiome sequencing. Shannon and Simpson diversity indexes were used to evaluate alpha diversity. Beta diversity was examined using principal coordinate analysis (PCoA) of unweighted Unifrac distances.

Results: At baseline screening**,** the population-based HPV positivity rate was 23.2% (95% CI: 23.54-22.86%), of these 20.5% (95% CI=20.79-20.21), and 10.3% (95% CI=10.52-10.08) women were hr- and lr- HPV positives, respectively. Age-specific hr-HPV infection peaked in the age-group 30-34 years old (58.6%) and decreased in 35-39, 40-44, and 45-49 years to 20.4%, 4.5% and 3.8% respectively. The top five prevalent hr-HPV genotypes were HPV16 (57.1%), 35 (20.3%), 52 (15.8%), 31 (14.1%), and 45 (9.6%) in the Butajira district. hr-HPV infection clearance was observed in 70 women (73.7%) within 6 months and among 77 women (84.6%) within 2 years. In the control women (negatives at baseline), the hr-HPV incidence was 4.1%.

HPV68, 82, 53, 52, 56 were the most persisted genotypes with 100%, 75%, 42.9%, 31%, and 25% persistence rates respectively while after 24 months, HPV59, 68, 66, 52 and 16 were found to have persistence with 50%, 50%, 20%, 15.8% and 3.5% respectively. Twenty-nine (29.9%) of the 6 month follow up attended women were with abnormal cytology including ASCUS and HSIL constituted 10.3% of the tested women. Of the three HPV testing assays compared in this study, MPG-Luminex Assay found 18.2% positive for the 14 hr-HPV and 7.3% for the probable hr-HPV genotypes. Anyplex™ II HPV HR Detection assay and EUROArray HPV Assay identified 21.82% and 12.7% samples, respectively, for the 14 hr-HPVs and both 7.3% for the probable hr-HPV genotypes (κ=0.734). Among the 14 hr-HPV genotypes, the genotype-specific agreement of the three HPV genotyping assays was moderate or better for HPV16, 31, 35, 39, 52, 56, 66 and 68. The aggregated sensitivity in detecting the 14 hr-HPV infections of Anyplex™ II HPV HR Detection and EUROArray HPV assays was high, 100% and 70%, respectively. The specificities of Anyplex™ II HPV HR Detection and EUROArray HPV were 95.6% and 100%, respectively. In this study, alpha diversity was significantly higher in patients with cervical cancer than in patients with dysplasia and in healthy women ($p < 0.01$). Beta diversity was also significantly different in cervical cancer patients compared with the other groups (weighted UniFrac Bray-Curtis, $p < 0.01$). Microbiota composition differed between the dysplasia and cervical cancer groups. *Lactobacillus iners* was particularly enriched in patients with cancer, and a high relative abundance of *Lactobacillus species* was identified in the dysplasia and healthy groups, whereas *Porphyromonas, Prevotella, Bacteroides,* and *Anaerococcus species* predominated in the cervical cancer group.

Conclusion: This study provided new data on the overall prevalence of HPV infection and distribution of specific HPV types in rural Ethiopia. As a first population-based study in the country, our results can serve as valuable reference to guide nationwide cervical cancer screening and HPV vaccination programs in Ethiopia. HPV16, HPV35, HPV52, HPV31 and HPV45 were the most prevalent genotypes. Most of the hr-HPV infections among rural Ethiopian women were cleared within 2 years. This study has found differences in cervicovaginal microbiota diversity, composition, and relative abundance between women with cervical cancer, women with dysplasia, and healthy women. Additional studies need to be carried out in Ethiopia or in any other regions to further validate the role of cervical microbiome in development of cervical cancer. From this study, the three evaluated assays showed similar analytical performance in the detection of hr-HPV infections and moderate or better concordance in HPV genotyping.

Key words: Ethiopia, Butajira, high-risk HPV, HPV testing, HPV persistence, analytical performance, cervical dysplasia, cervical microbiota

DEDICATION

This PhD dissertation is dedicated to my family:

My Dad and Mom, you taught me values and beliefs, being a strong and to persevere no matter what, even in the toughest circumstances. The personality built in me hold true in both my professional occupation and personal life. Dad, you won't be with me as usual during this academic achievement, but you are always on my mind and in my heart every step of the way. Thank you and Rest in Peace.

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Glossary of Terms/Words

ASCUS:	Atypical Squamous Cells of Undetermined Significance
BV:	Bacterial vaginosis
CC:	Cervical Cancer
CDK:	Cycline dependent kinase
CIN:	Cervical intraepithelial neoplasia
CST:	Community state type
DNA:	Deocyribonucleic Acid
E gene:	Early gene
E6AP:	Early protein 6-associated protein
FMOH:	Federal Ministry of Health
GM-CSF:	Granulocyte- macrophage colony stimulating factor
HDSS:	Health and demographic Surveillance Site
HGD:	High grade cervical dysplasia
HPV:	Human Papilloma Virus
HR:	High Risk
HrHPV:	High risk Human Papilloma Virus
HSIL:	High- grade Squamous Intraepithelial Lesion
ICC:	Invasive Cervical Cancer
IL:	Interleukin
L gene:	Late gene
LCR:	Long Control Region
LD:	Lactobacillus dominant
LEEP	Loop Electrosurgical Excision Procedure
LGD:	Low grade cervical dysplasia
LSIL:	Low-grade Squamous Intraepithelial Lesion
NCDs:	Non-Communicable Disease
NF-I:	Nuclear factor 1
NGS:	Next generation sequencing
NLD:	Non-Lactobacillus dominant

NO:	Nitric Oxide
NOS:	Nitric Oxide Syntheses
NRERC:	National Research ethics review committee
ORF:	Open Reading Frame
p21:	21-kDa protein
PBS:	Phosphate buffered bovine serum
PCR:	Polymerase Chain Reaction
pRb:	Retinoblastoma protein
PV:	Papillomavirus
RNA:	Ribonucleic Acid
rRNA:	ribosomal RNA
SCC:	Squamous Cell Carcinoma
SOP:	Standard operating procedure
SPSS:	Statistical Package for Social Sciences
STI:	Sexually transmitted infection
TNF:	Tumor necrosis factor
URR:	Upstream Regulatory Region
VIA:	Visual Inspection with Acetic acid
VILI:	Visual Inspection with Lugol’s Iodine
VLP:	Virus like particles
VM:	Vaginal Microbiome
WHO:	World Health Organization

1. Introduction

1.1. Background and Rationale

Cervical cancer leads in prevalence among reproductive organ cancers causing morbidity and mortality among women. It is the second most common cancer in women worldwide but the commonest in developing countries (Ferlay *et al.*, 2010). In 2020, an estimated 604,237 women were diagnosed with cervical cancer globally, representing 6.5% of all female cancers (Sung *et al.*, 2021). In Ethiopia, cervical cancer is second the leading cause of morbidity and mortality from all cancers (FMoH, 2015). According to the Ethiopian ministry of health, about 21 million women were at risk of developing cervical cancer in 2010 (FMoH, 2015).

It has been well-established that human papilloma virus (HPV) is a necessary cause for cervical cancer (CC) (Racey, Withrow and Gesink, 2013). There are more than 40 HPV types identified to have high tropism specifically for anogenital mucosal epithelia. Depending on their oncogenic potential, HPVs are classified as either high-risk (hr) or low-risk (lr) HPVs. Those HPV types associated with cervical cancer and precancerous lesions are grouped as hr-HPV types while the others causing genital warts and benign lesions are labelled as lr-HPVs (Bosch *et al.*, 2002; Muñoz, 2003). Among the hr-HPV types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are the most clinically significant high-risk types and cumulatively responsible for 94.5% of all squamous cell carcinomas of the cervix worldwide (Clifford *et al.*, 2006). It was also established that HPV16 and 18 genotypes contribute for more than 70% of cervical cancer cases worldwide (Abreu *et al.*, 2012; Crow, 2012). In addition to HPV 16 and 18 genotypes, HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are also considered as carcinogenic or high-risk types while types 26, 53, and 66 are considered as probably carcinogenic (Muñoz, 2003).

Human papillomaviruses are a group of small, non-enveloped, naked icosahedral (55nm) viruses that belong to Papillomaviridae family (Tang *et al.*, 2007) and have 8000 bps in their double stranded circular DNA (Bleotu and Anton, 2010). They are widely distributed throughout the animal kingdom, specifically infect squamous epithelia, and cause the generation of warts (Munger *et al.*, 2004). They are grouped under five genera: *Alpha (α), Beta (β), Gamma (γ), Mu (μ),* and *Nu*

(ν)- papillomaviruses (De Villiers *et al.*, 2004), where viruses that belong to different genera have <60% similarity within the *L1* gene. Within a genus, HPVs are classified in species that share between 60 and 70% of similarity (Bzhalava, Eklund and Dillner, 2015). The Alpha and Beta HPVs are responsible for most of mucosal and cutaneous infections (De Villiers *et al.*, 2004) and each has its own tissues tropism (Stanley, 2012). According to the International HPV Reference Center (Karolinska Institute), 226 HPV genotypes have been described to date, and new types are regularly added to this list. It is also important to note that the number of HPVs belonging to the β- and γ- *PV* genera has rapidly increased in the last years (Mühr, Eklund and Dillner, 2018).

Long-lasting infections with hr-HPVs can cause cancer in parts of the body where HPV infects cells, such as in the cervix and oropharynx. HPV associated cancers include cervical cancers, oropharyngeal cancer, anal cancer, penile cancer, vaginal cancer, and vulvar cancer. However, cervical cancer is almost exclusively caused due to persistent infection of hr-HPV types; where 99.7% of all cases contain HPV DNA (Walboomers *et al.*, 1999). Infection with HPV is one of the most common sexually transmitted infections among sexually active persons (Ho *et al.*, 1998; Garland and Tabrizi, 2006; Satterwhite *et al.*, 2013) and the indicated risk factors for infection are early first intercourse and number of sexual partners, immunosuppression, HIV infection, cigarette smoking, Chlamydia infection, multiparity, and long-term use of oral contraceptives (Adam *et al.*, 2000; Munoz *et al.*, 2002; Castellsagué and Muñoz, 2003). However, the most important factor associated with cervical cancer prevalence is lack of screening (WHO, 2013).Since the screening programs have been implemented in different populations of developed countries, the incidence and mortality associated with cervical cancer have declined (Gustafsson *et al.*, 1997) but in developing countries it still remains an important cause of death in women (Bleotu and Anton, 2010).

HPV infection alone is not sufficient as a cause for cancer. In addition, most genital HPV infections are transient and asymptomatic (i.e., natural immune surveillance usually clears infectious HPV). Although about 80 percent of sexually active people are infected with HPV at some point in their lives, only 10% of these HPV infections will become persistent (Ho *et al.*, 1998). It is only in a small number of women; with chronic carriage of oncogenic or high-risk genotypes that severe dysplasia (CIN2/3) eventuates, over several decades, to cancer. The time needed for progression

from initial HPV infection to cervical cancer is 10 to 20 years (Snijders *et al.*, 2006). This is the unique advantage in cervical cancer control which enables early detection of neoplastic changes by screening and allows early treatment. In addition, carcinogenesis requires additional genetic changes like in some cases HPV integration and possibly other cofactors in complex pathways not totally understood (Garland and Tabrizi, 2006). Persistent HPV infection is the central factor in the development of cervical cancer and is a prerequisite for progression to high-grade cervical lesions. However, few HPV infections progress to cervical cancer, and most HPV infections are eventually cleared (Castellsagué, 2008; Koshiol *et al.*, 2008). Therefore, detection of persistent hr-HPV infection in a women gives us a clue for a high risk of cervical cancer precursor (Castle, 2008). Despite luck of consensus in the definition of persistent infection, "persistence" is often defined in terms of duration of type specific infection i.e two or more HPV positive time points and the median time of testing interval is six months (Rositch *et al.*, 2013).

Mechanisms associated with clearance or persistence of HPV infection is not well understood (Akaaboune *et al.*, 2018). Currently, different factors have been found to be associated with the development and persistence of HPV infection (Veldhuijzen *et al.*, 2010). Among these, other sexually transmitted infections (STIs) are found to be associated with HPV infections, but the association has not been fully elucidated. Several epidemiological studies have suggested that other STIs are frequently present in HPV infected women and play a role in the HPV infection and persistence (Bellaminutti *et al.*, 2014; Camporiondo *et al.*, 2016).

Among the STIs, genital chlamydia and HIV infection have been the most commonly evaluated in conjunction with HPV (Liu *et al.*, 2016); moreover, *Mycoplasma hominis* and *Ureaplasma urealyticum* have also been found to be co-prevalent with HPV infection (Zhang *et al.*, 2017). In this thesis, we determined STIs to see whether hr-HPV infections are commonly associated with other STI infections. The other factor associated with HPV persistence or clearance is Bacterial Vaginosis (BV). Along with higher rates of HPV infection, Bacterial Vaginosis (BV) has been associated with delayed clearance of the virus and with cervical intraepithelial neoplasia, suggesting that a diverse, *Lactobacillus*-depleted microbiome may play a mechanistic role (King *et al.*, 2011; Guo *et al.*, 2012). Studies indicated that advancing CIN disease severity is associated with increasing vaginal microbiota diversity and may be involved in regulating viral persistence

and disease progression (Mitra *et al.*, 2015). From studies conducted so far, there are no good direct data that show how altered vaginal microbiome (VM) influences local immune function. However, it is plausible that the stability and composition of the vaginal microbiome may play an important role in determining host innate immune response and susceptibility to infection as well as playing a role further downstream regarding the development of cervical disease. Several studies have shown that BV and BV-associated bacteria effect immune parameters within the vagina including cytokines/chemokines, antimicrobial proteins and immune cell populations (Onderdonk, Delaney and Fichorova, 2016). The other objective of this thesis project was, therefore, to characterize the cervicovaginal microbiome structure and diversity in women with different CIN grades, cervical cancer in comparison with women without cervical cancer.

In developing countries, including Ethiopia, almost all women with cancers present to health care facilities at late stages with advanced disease and poor prognosis (Pathfinder, 2010). Unlike other reproductive health cancers, cervical cancer can be prevented and even possible to be cured if identified in its early stages (Anorlu, 2008). Several studies are indicating that under-screened women (called hard-to-reach) experience a higher incidence of cervical cancer and elevated mortality rates compared to regularly screened women (Perehudoff *et al.*, 2020). As a result, due to effective screening and treatment of precancerous lesions and cancer, high-income countries have seen marked decreases in cervical cancer incidence and mortality in recent decade (Canfell, Sitas and Beral, 2006). Therefore, screening is acknowledged as the most effective approach for cervical cancer control efforts (Anorlu, 2008; Pathfinder-org, 2010; World Health Organization, 2013a; FMoH, 2015; Shin *et al.*, 2021).

Detection of hr-HPV in the cervix is recommended as primary screening for cervical cancer when possible (World Health Organization, 2013b). World Health Organization (WHO) primarily recommends the HPV test which is very sensitive and a convenient molecular test for cervical cancer screening (World Health Organization, 2013b). This approach is found to be less examiner-dependent, reduce the burden in health system, enhance the accuracy, efficiency and reduce cultural barriers (Cuzick *et al.*, 2006; Adefuye *et al.*, 2013; Moses *et al.*, 2015). Therefore, HPV testing is a future option in low and middle-income countries. Since several new HPV screening assays have been developed for hr-HPV testing, the main challenge especially in resource limited

countries is to select which HPV assay to be used. In this regard, we compared the performance and adequacy of three HPV genotyping assays and established an HPV DNA testing molecular biology laboratory for cervical cancer screening in Ethiopia.

Therefore, we conducted this first population-based study in Ethiopia to fill the above-mentioned gaps dealing with the prevalence of HPV and circulating genotypes in rural women believing that the knowledge of the molecular epidemiology of HPV diversity among women is crucial for nationwide cervical cancer screening and HPV vaccination programs in the country.

1.2. Statement of the problem

As mentioned above, HPVs are the most common sexually transmitted infections worldwide (Sanjosé *et al.*, 2007). More than 80% of sexually active women and men will acquire at least one HPV infection by the age of 45 years (Chesson *et al.*, 2014). However, most HPV infections are transient after detection of the first infection. In women, more than half of the HPV infections clear within 6 months and up to 90% of incident HPV genital infections clear within two years (Rodríguez *et al.*, 2008) .

Cervical cancer is a significant public health threat to women especially on the African continent. All of the top 20 countries worldwide with the highest burden of cervical cancer in 2018 were in Africa (Carter, 2018) and it is steadily increasing in sub-Saharan Africa with more than 75,000 new cases and 50,000 deaths yearly (Mboumba Bouassa *et al.*, 2017) . The tragedy is that while this type of cancer is one of the most preventable, poor access to prevention, screening and treatment contributes to 90% of deaths (Carter, 2018). Ethiopia is among these high burden countries (Leyh-Bannurah *et al.*, 2014).

Worldwide, the most common hr-HPVs are 16 and 18, and approximately 70% of cervical cancers are due to infection by these genotypes (Abreu *et al.*, 2012). HPV types 16, 18, 31, 58, 52 and 52, 16, 18, 53, 66 are estimated to be the most common worldwide and in Eastern Africa, respectively (Sanjosé *et al.*, 2007). But some studies conducted in Ethiopia (Leyh-Bannurah SR, Prugger C, de Koning MN, Goette H, 2014; Derbie *et al.*, 2022a) showed that HPV genotypes 16 and 52 are predominant genotypes in Ethiopia. Since observational studies have demonstrated the distribution of HPV genotypes is heterogeneous among women from different populations (Castellsagué, 2008), the possession of population-based data is crucial to the development of new screening and management protocols for cervical neoplasms as well as to the assessment of the effect of future vaccination on HPV infections.

Currently, there are several evidences that suggest HPV testing is more effective and feasible method of cervical cancer screening (Ronco *et al.*, 2014a; Catarino *et al.*, 2015). Technology is advancing and highly sensitive tests have been developed and currently many countries are

introducing HPV testing for primary screening (Ronco *et al.*, 2014b). The detection of hr-HPV in the cervix has also been recommended by WHO in settings wherever possible (World Health Organization, 2013b). In the global market, there are more than 150 different HPV tests available for the detection hr-HPVs (Catarino *et al.*, 2015). Several studies conducted elsewhere support that HPV testing can be feasible in resource limited countries and appears to be the best strategy for CC in this context (Ogilvie *et al.*, 2005; Catarino *et al.*, 2015; Moses *et al.*, 2015). So far, the greatest challenges of HPV testing were the need for expensive laboratory infrastructure and the time to process the test (Catarino *et al.*, 2015). However, the innovation of rapid molecular methods for detecting hr-HPV DNA (*e.g.*, care HPV® - Qiagen, GeneXpert® - Cepheid) for screening is easing the challenges.

Therefore, the use of HPV testing at first triage would be the best option for developing countries (Haguenoer *et al.*, 2014). Studies suggested that only triaging women who tested positive at HPV test for VIA combined with the immediate treatment was cost saving and effective strategy (Goldie *et al.*, 2001; Haguenoer *et al.*, 2014). Moreover, HPV-based testing gives advantage of sample collection to be performed by the woman herself, not requiring trained personnel and infrastructure to perform a pelvic examination (Catarino *et al.*, 2015). This plays a key role in reducing the cultural barriers and burden on health system (Catarino *et al.*, 2015). Many studies also revealed that offering self-sampling for HPV testing (Self-HPV) can improve attendance to a CC screening program and it is well accepted among women (Dzuba *et al.*, 2002; Ogilvie *et al.*, 2005; Haguenoer *et al.*, 2014; Ronco *et al.*, 2014b; Giorgi Rossi *et al.*, 2015). This strategy assumed to be appealing and makes CC screening accessible to women in LMIC (Lazcano-Ponce *et al.*, 2011; Giorgi Rossi *et al.*, 2015).

As mentioned previously, approximately 80% of CIN1 lesions regress spontaneously (Follen and Richards-Kortum, 2000) and usually are managed conservatively. On the other hand, CIN2 and CIN3 have a considerable risk of progression toward invasive cancer and are therefore usually treated by conization or other less invasive procedures. As a result, the main challenge in CC screening and treatment is precisely determining which CIN1 cases will progress to CIN2 or CIN3 and it has been a focus of study. Investigators have attempted to identify biomarkers of risk that would enrich the CIN1 population for those in need of ablative therapy (Chen *et al.*, 2010).

In addition to the type of HPV, factors affecting the persistence of HPV and the cervical carcinogenesis include demographic, genetic, immunologic and environmental factors (Kim, Nam and Lee, 2011). Furthermore, studies have demonstrated that, cervicovaginal dysbiosis is also considered as an independent risk factor to the clearance and persistence of cervical HPV infection(Guo *et al.*, 2012; Usyk *et al.*, 2020). Some findings suggest that the presence and prevalence of specific vaginal microbiome community state types (CSTs) may be involved in the pathogenesis of CIN and cervical cancer. They have also identified specific bacterial species that could help to differentiate low- and high-grade disease (King *et al.*, 2011; Łaniewski *et al.*, 2018). The concept of manipulation of vaginal bacterial communities using pre- and probiotics is as an exciting prospect for the field of cervical pathology. Our research project determined the difference in the composition of microbiome HPV infection and different precancer lesion and cervical cancer.

The aim of this study was, therefore, to determine the circulating HPV genotypes at the population level, to investigate the persistence, clearance, and re-infection rates of genotype-based human papillomavirus infections and to associate them with their clinical outcomes. Furthermore, this study also characterized the vaginal microbiome structure and diversity in women with different CIN grades, cervical cancer, and normal women.

1.3. Significance of the study

The significance of this research project is to determine the prevalence and circulating HPV genotypes in the Ethiopian women since this study is a population-based study. This could enable public health policy makers to choose the right intervention strategy that suits our conditions. Moreover, based on the geographic distribution of these HPV genotypes, health related resources; diagnostic reagents, treatment options etc, will be allocated to the region in need efficiently. Consequently, the intervention strategies will minimize the morbidity and mortality rates of cervical cancer.

National cervical cancer screening only covers less than 1% of the female population in Ethiopia and 90% of women have never had a pelvic examination at all. Despite the presence of high unmet need of cervical cancer screening for eligible women in Ethiopia, still the coverage of screening service and the uptake of women for available service are very limited. In the current context of cervical cancer screening, the acceptability and safety of the screening method is crucial parameters to enhance the coverage and improve the uptake of women. Therefore, evidence from this study will help the society and policy makers by providing pragmatic evidence on the method of cervical cancer screening in Ethiopia while it is based in HPV DNA testing. In addition, this thesis project immediately benefited the women participants by giving cervical cancer screening and linking with appropriate treatment for those found to be positive with the screening test. Furthermore, since this study determined the unique features of the cervicovaginal microbiota in dysplasia and cancer in the Ethiopian context, these findings may lead to new strategies to identify patients with dysplasia at high risk who would benefit from intensified screening.

The other main significance of this research project was to build a capacity and technology transfer in HPV diagnostics in our country. As part of this thesis project, we established a PCR based HPV DNA testing laboratory in the Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University. This was done because of the strong collaborations we have made so far with different international institutions. Among these the collaboration we have with Martin Luther University Halle-Wittenberg Institute of Medical Immunology and Epidemiology, Halle and Charite University Medical Center, Berlin, from Germany and MD Anderson Cancer

Center, University of Texas, USA played a crucial role in the establishment of the lab. As a result, most of the experiments of this project like DNA extraction, amplification and genotyping of the cervical samples were performed in our country. The laboratory has also provided COVID-19 testing services using the already established infrastructure due to this thesis project.

Therefore, we strongly believed that the results and establishments made in this study will be used to improve the existing screening and diagnostic strategies for cervical cancer, and hence an effective cervical cancer prevention program in Ethiopia.

2. Literature Review

2.1. Global burden of cervical cancer

Cervical cancer is one of the most preventable cancers that occurs in the cells of the cervix. The cervix is the lower part or 'neck' of the uterus where it joins the inner end of the vagina (Pandey, 2017). All women are at risk for cervical cancer. It occurs most often in women over age 30. It is one of the major public health problems globally. In 2020, an estimated 604,237 women were diagnosed with cervical cancer globally, representing 6.5% of all female cancers (Sung *et al.*, 2021). If considerable interventions are not put in place, the annual number of new cases and death from cervical cancer has been projected to increase to 700, 000 and 400, 000, respectively in 2030 (World Health Organization, 2020) and this is representing an increment of 21% in the number of cases and 27% in the number of deaths.

Cervical cancer is the most common cancer among women in 36 low-and middle-income countries, mainly in sub-Saharan Africa (Sung *et al.*, 2021) and killed an estimated 341,843, women in 2020, 90% of whom were in less-developed regions of the world (Ferlay *et al.*, 2010; Sankaranarayanan *et al.*, 2012; Torre, Bray, Siegel, Ferlay, *et al.*, 2015; International Agency for Research on Cancer (IARC), 2020). All but one of the top 20 countries worldwide with the highest burden of cervical cancer in 2018 were in Africa (World Health Organization, 2019). A woman diagnosed with cervical cancer is almost twice as likely to die than a woman diagnosed with breast cancer and a woman living with HIV is six times more likely to develop cervical cancer than her HIV-negative peers. It is, therefore, a matter of public health, as it affects women within the reproductive age groups (Sung *et al.*, 2021).

In most of the developing countries, cervical cancer is the leading cause of cancer death among women (Bleotu and Anton, 2010). Incidence rates are highest in sub-Saharan Africa, Latin America and the Caribbean, and Melanesia and lowest in Western Asia, Australia/New Zealand, and Northern America (Sankaranarayanan *et al.*, 2012; Torre *et al.*, 2015). Cervical cancer is one of the diseases that clearly show the country's economic status because there is high disparity between higher and lower income regions. It was indicated that a 0.2 unit increase in human development index (HDI) was associated with a 20% decrease in cervical cancer risk and a 33%

decrease in cervical cancer mortality risk. Furthermore, a 0.2 unit increase in gender inequality index (GII) increased the risk of a cervical cancer diagnosis by 24% and of cervical cancer death by 42% (Singh, 2012). This is primarily due to the difficulty in implementing effective screening programs like the cytology-based screening.

2.1.1. Cervical cancer in Ethiopia

With the population of 33.7 million women ages 15 years and older who are at risk of developing cervical cancer, the current Ethiopian cervical cancer estimates indicate that every year 7445 women are diagnosed with the disease and 5338 die from the disease (ICO/IARC Information Centre on HPV in ETHIOPIA, 2021). In Ethiopia, cervical cancer ranks as the 2nd most frequent cancer among women and the 2nd most frequent cancer among women between 15 and 44 years of age. The prevalence of cervical cancer in Ethiopia was 18.2 % with the incidence and death rates of 17.3% and 16.5% respectively (ICO/IARC Information Centre on HPV in ETHIOPIA, 2021; Sung *et al.*, 2021).

Despite this burden, however, the national cervical cancer screening only covers less than 1% of the female population in Ethiopia, where invasive cervical cancer (ICC) is associated with the highest cancer mortality among women. According to 2012 estimate, the estimated age standardized incidence and mortality rates of Ethiopian women were 4 and 9 times higher incidence and mortality rate than in western Europe (Leyh-Bannurah *et al*, 2014). According to the Ethiopian ministry of health, about 21 million women were at risk of developing cervical cancer in 2010 (FMoH, 2015). In developing countries, including Ethiopia, almost all women with cancers present to health care facilities at late stages with advanced disease and low probability of recovery (FMoH, 2015). Moreover, nearly 84% women in Ethiopia are residing in rural area where access to screening and treatment facilities is limited or lacking, this makes the prevention and control efforts cumbersome.

Data on Ethiopian cervical cancer and HPV genotype prevalence and distribution are none or rare. However, data on the genotype of HPV circulating in the different part of the country is essential as pre-vaccine baseline data to monitor changes after initiating HPV vaccination is crucial.

2.2. HPV and cervical cancer

In 1842, an Italian physician postulated for the first time the association between a sexually transmitted agent and ICC. He noted a high frequency of cervical cancer among married women, widows, and prostitutes, but was a rare occurrence among virgins and nuns (Kelly, 2017). Later on, different studies indicated the sexual behaviour of a woman and that of her male partner was associated with the risk of ICC (Brinton, 1992).

Later in 1990s and 2000s, prospective studies established the temporal association between exposure to hr-HPV and the subsequent development of cervical intraepithelial neoplasia and cervical cancer. These data, combined with strong biological plausibility derived from the basic sciences, led to acceptance of hr-HPV as a necessary, but insufficient cause of nearly 100% of cervical cancers (Gravitt and Winer, 2017). The finding was universally consistent, and to date there are no documented alternative hypotheses for the aetiology of cervical cancer (Bosch F. *et al.*, 2002). In addition, these data consistently fulfil the Bradford–Hill criteria for causality, the well-accepted temporal pathway from HPV infection to invasive cervical cancer (ICC) (Gravitt and Winer, 2017). In the meantime, the HPV was reported to be localized in cervical tumour tissue using a nucleic acid hybridization technique in the 1970s and later retrospective studies demonstrated the presence of persistent HPV DNA precedes cervical lesion development (Wallin *et al.,* 1999).

It is now reported that CC is by far the most common HPV-related disease and nearly all cases of CC can be attributed to hr-HPV infection (World Health Organization, 2020). HPV infection is a risk for women that may become chronic and pre-cancerous lesions progress to invasive cervical cancer. From a worldwide meta-analysis of hr- HPV genotype distribution study, (Guan *et al.*, 2012) the HPV prevalence among women with atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) was 12.4%, 52.5%, 75.7% and 85.4%, respectively. From histological examinations, women with cervical intraepithelial neoplasia grade 1 (CIN1), CIN2, CIN3 and ICC was 73.4%, 85.6%, 92.6% and 89.4%, respectively **(Figure 2.1)**. Despite the fact that HPV is a necessary cause of ICC (Walboomers *et al.*, 1999; Bosch F. *et al.*, 2002), reports have also

indicated patients with ICC who are HPV negative (de Sanjose *et al.*, 2010) reported from biopsies of invasive cervical cancer around the world that only 85% were positive for any HPV. Studies estimated that approximately 5.5–11% of cervical cancers worldwide are HPV-negative. These were attributed to either truly negative or false-negative results (Xing *et al.*, 2021).

Among the HPV types that infect humans, around 30-40 infect the anogenital tract (Stanley, Pett and Coleman, 2007; Doorbar *et al.*, 2012). Of these, 13 types are recognized as oncogenic to humans: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Twelve of these HR-HPV types have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic to the cervix and type 68 is classed as probably carcinogenic (IARC, 2012). From a meta-analysis in 30,000 ICC cases (Li *et al.*, 2011), HPV 16 was the most frequent HPV type in ICC in all regions of the world (57%, 95% CI, 54.3-58.9), followed by HPV 18 (16%, 95% CI, 14.6-17.4). Together they accounted for about 73% of all ICC. The next most common HPV types were HPV 58, 33, 45, 31, 52, 35, 59, 39, 51 and 56 in descending order. However, the relative order of the HPV genotypes in ICC varied in different regions of the world (Jalil and Karevskiy, 2020). The distribution of HPV also differed between adenocarcinoma (ADC) and squamous cell carcinoma (SCC). HPV 18 was most frequent in ADC (36.8%, 96% CI 34.9-39.7), while HPV 16 was most common in SCC (59.3, 95% CI 56.8-61.7) (Alder, 2018).

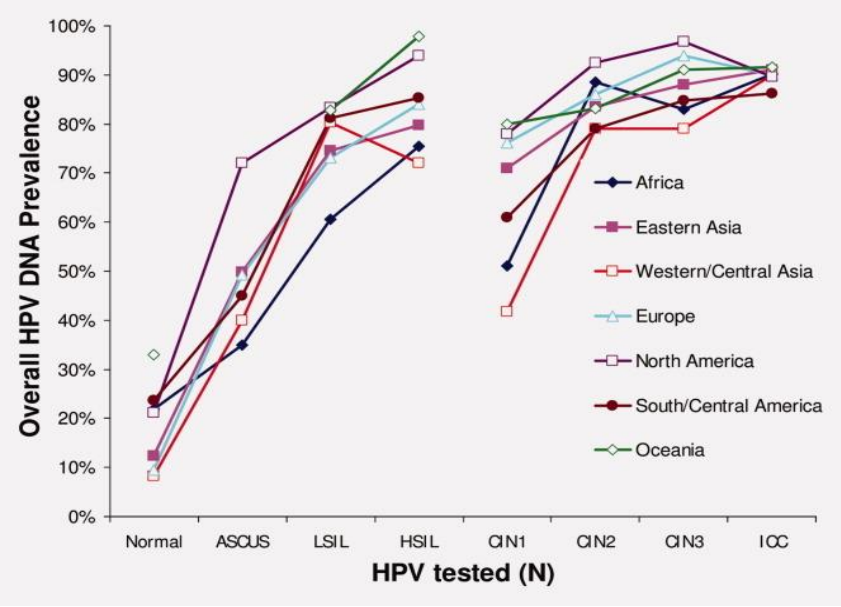


Figure 2.1: Prevalence of HPV DNA by cervical disease grade and region (Guan *et al.*, 2012).

2.3. Virology of Human Papillomavirus

Human Papillomaviruses are small, double-stranded DNA viruses. They are members of the *Papillomaviridae* family, a large family with a tropism for squamous epithelium (Ferlay *et al.*, 2010). HPVs are relatively stable and resistant to desiccation, organic solvents, and heat treatment to 56°C. Since humans are the only known host for HPV, they are difficult to culture in vitro and hence often detected by molecular methods (Harden and Munger, 2017).

The HPV virion is 50 to 60nm naked icosahedral DNA tumour virus (Doorbar, 2006). They have icosahedral capsids composed of 72 capsomeres, surrounding a circular DNA genome of ~7900 base pairs (Moody and Laimins, 2010). The capsid is composed of mainly one type of protein, called major protein, L1, and a minor protein, L2, which is found in small amount (Doorbar *et al.*, 2015). The L1 protein polypeptide has unique self-assembly property (Stanley, Lowy and Frazer, 2006) where 5 L1 peptides interact and stabilize via hydrogen bond to form individual pentameric capsomers (Doorbar *et al.*, 2015). When the 5 L1 peptides assembled into a capsomer, a groove is created at the centre, a site where the L2 protein situates itself from inside. Moreover, the L1 protein at its C-terminal loops out and forms two contacts with adjacent capsomers at their bases via disulfide bonds (Wolf *et al.*, 2010). These contacts help firm assembly of the pentamers in the icosahedral capsid symmetry (De Villiers *et al.*, 2004). Such 72 capsomers, 360 L1 molecules, form the viral icosahedral shell encapsidating a double stranded ~8kbp DNA (dsDNA) genome forming an intact HPV particle (Bienkowska-Haba, 2010).

As of 2016, more than 200 different HPV types have been identified (http://www.hpvcenter.se/), (Mühr, Eklund and Dillner, 2018). The HPV types are classified as cutaneous and mucosal HPVs depending on their tissue tropism. The different HPV types have also been categorized into five genera which include: α-papillomavirus, β-papillomavirus, γ-papillomavirus, mu-papillomavirus and nu-papillomavirus (De Villiers *et al.*, 2004; Bernard *et al.*, 2010). There are 65 *Alpha papillomaviruses*, 51 *Beta papillomaviruses*, 84 *Gamma papillomaviruses*, 4 *Mu papillomaviruses* and a single *Nu papillomavirus* (Doorbar, 2013).

HPVs are classified based on the nucleotide sequence of the ORF coding for the capsid protein L1(Bzhalava *et al*., 2015). HPV types belonging to different genera have less than 60% similarity within the L1 part of the genome. Different viral species within a genus share between 60 and 70% similarity. A novel HPV type has less than 90% similarity to any other HPV type (De Villiers *et al.*, 2004; Bernard *et al.*, 2010). This nomenclature at the species level and above is determined by the papillomavirus study group of International Committee on Taxonomy of Viruses (ICTV) (Chen, de Freitas and Burk, 2015). Thus, de Villiers *et al* proposed a classifications system to represent Papillomaviridae Family, Genus, Species, Type, Subtype, and Variants (De Villiers *et al.*, 2004) (**Figure 2.2**).

The numbering system that we are using nowadays in HPV typing which is depending on the L1 sequence was based on the agreement made in the 1995 Papillomavirus Workshop held in Quebec (Harari, Chen and Burk, 2014). However, many new HPVs are being discovered that create instability to the classification system (de Villiers, 2013). For instance, after 2004, 13 new distinct genera were discovered from different hosts (totally 29 genera) exceeding the 24 Greek letters; that demanded amendment of classification and nomenclature system (Bernard *et al.*, 2010).

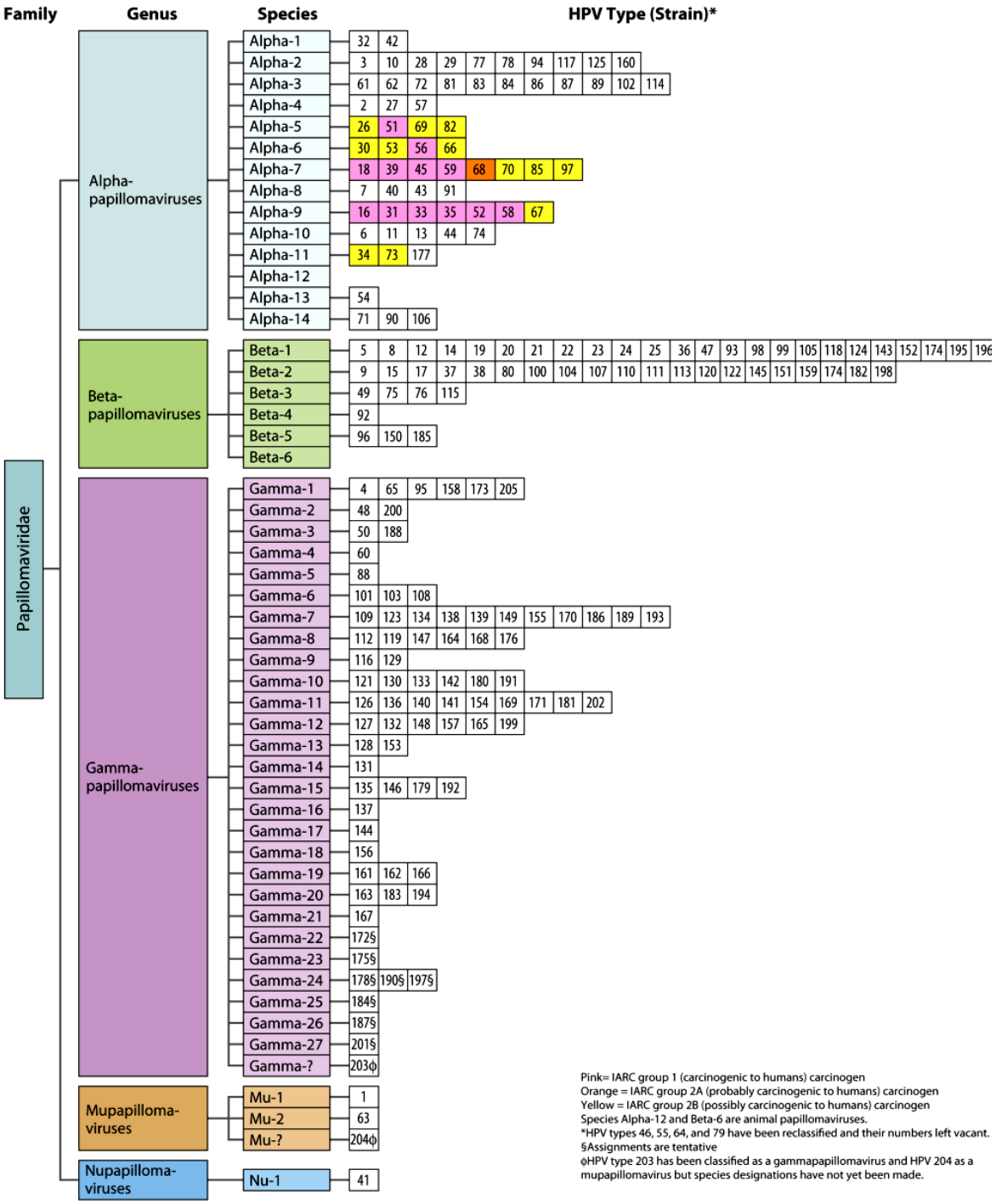


Figure 2.2: HPV classification based on the nucleotide sequence of the capsid protein L1 gene adapted from (Burd, 2016)

2.3.1. Human Papillomavirus genome organization

All HPVs have a common gene organization; a single circular double stranded (ds) DNA about 8k base pair (bp) (Motoyama *et al.*, 2004) long with minor differences (Burk, Chen and Van Doorslaer, 2009). The HPV genome can be functionally divided into two regions: Upstream Regulatory Region (URR) or Long Control Region (LCR) and Open Reading Frames (ORFs). URR does not code for proteins but contains cis-elements required for the regulation of the gene expression, replication of the genome, and its packaging into virus particles. ORFs can be divided into the Early Region (E), necessary for the replication, cellular transformation and the control of viral transcription, and Late Region (L) that codes for the capsid proteins that comprises the outer coat of the virus (Zekan, Sirotkovic-Skerlev and Skerlev, 2011). Each ORF in the early region is designated "E" followed by a numeral, indicative of the length of the ORF (Harden and Munger, 2017).

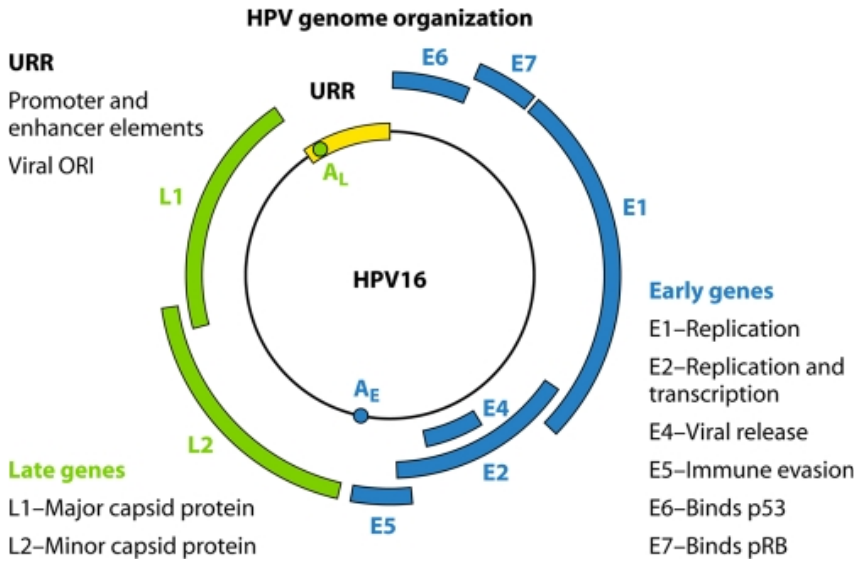


Figure 2.3: Schematic presentation of the HPV 16 genome. *The non-coding region is the URR. The ORFs encode the early (E), and late (L) viral proteins. The early region of the HPV genomes covers more than 50% of the virus genome and contains the E1, E2, E4, E5, E6, and E7 genes that are carefully expressed at the early and late stages of infection. The early genes are expressed in the infected basal cells while the late proteins are synthesized only in well differentiated cells. Adapted from* (Stanley, 2010)

The early proteins E1 and E2 are involved in viral DNA replication and viral RNA transcription, E4 is involved in cytoskeleton reorganization and E5, E6, and E7 are responsible for cellular transformation, and immortalization (Zheng and Baker, 2006). The two late genes *L1* and *L2* encode structural proteins that form the viral icosahedral capsid consisting of 72 capsomers (Ganguly and Parihar, 2009). E5 decreases intercellular communication and isolates the transformed cells and interacts with the growth factor's receptors and encourages cellular proliferation. It also stimulates the expression of E6 and E7. E6 is oncogenic, stimulating the growth and transformation of the host cell by the inhibition of protein p53's normal tumour-suppressor function. E7 also acts as an oncogene, inducing cellular proliferation by inhibition of protein pRb (Zekan, Sirotkovic-Skerlev and Skerlev, 2011).

Despite the above organization is shared among all alpha HPVs, only four ORFs (those of E1, E2, L1, and L2) are necessary to fulfil the requirements to ensure the viral replication and shedding of the virus, and are present in all known PVs (Van Doorslaer and McBride, 2016). Certain HPV genera and types lack an ORF. For example, the E5 ORF is lacking from HPV types that belong to the genera beta, gamma, and mu, and both the E5 and E6 ORFs are lacking from three gamma HPV types (HPV101, 103, and 108) (Chen *et al.*, 2007; Nobre *et al.*, 2009). Beta and gamma HPV types have the particularity of harbouring a shorter LCR compared with members of other genera (Gheit, 2019).

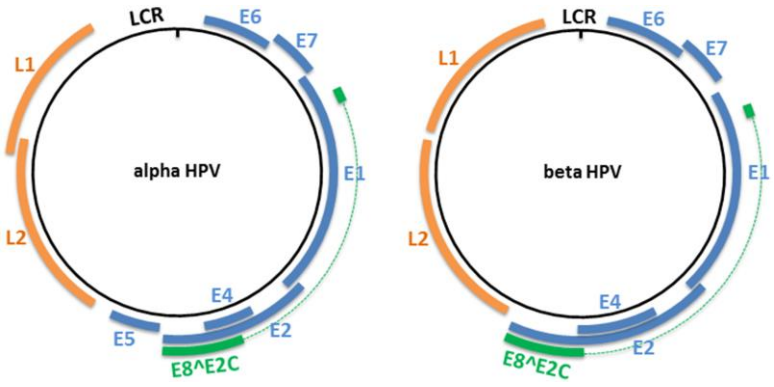


Figure 2.4: Genome organization of alpha and beta HPV types (Gheit, 2019)

Table 2.1: HPV gene functions, adapted from (Kajitani *et al.*, 2012)

Function in viral lifecycle	Activities	Target factor
E1		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
E2		
Transcription of viral genes Replication of viral genome Maintenance of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChIR1
E6		
Reactivation of cellular replication mechanisms Proliferation, immortalization, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	p53, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
E7		
Reactivation of cellular replication mechanisms Proliferation, genomic instability, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	RB, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
E4		
Unknown	Destruction of keratin network, induction of G_2M arrest of cell cycle	Cytokeratin 8/18
E5		
Possibly involved in proliferation and/or inhibition of apoptosis	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
L1		
Major capsid protein		
L2		
Minor capsid protein		

2.3.2. Human Papillomavirus Life cycle

The target of HPV infection is the stratified epithelium and its cycle is linked to the differentiation status of the host cell keratinocyte (Egawa *et al.*, 2015) and is characterized by distinct phases of replication (Longworth and Laimins, 2004; McBride, 2013). So, the hallmark of the HPV lifecycle is its close association with the differentiation program of the infected host squamous epithelium. HPVs firstly infect undifferentiated basal epithelial cells and then viral progeny are produced in differentiated daughter cells in the uppermost epithelial layers (Hong and Laimins, 2013). They initiate infection by gaining access to the proliferating basal cells of the stratified epithelium through a micro abrasion (Pyeon *et al.*, 2009) **(Figure 2.5).**

Viral cell entry

Given that basal epithelial cells are shielded by several layers of differentiated cells, they are not easily accessible, and the virus must infect these protected cells through micro wounds or abrasions that expose lower epithelial layers (Kajitani *et al.*, 2012). These abrasions are created due to many activities including sexual intercourse for hr-HPV infections. Thus, the virus, deposited on the epithelium from infected partner, may fall into the micro-trauma and gets access to the basal cells (Graham, 2010). Furthermore, cells located in the squamous columnar transformation zone in the cervix and anus has been shown to be particularly accessible and vulnerable to HPV infection (Herfs *et al.*, 2012).

The two HPV capsid proteins (L1 and L2) play a major role in the attachment and entry of the virus to the host cell. During HPV entry, L1 capsid protein bind to the cellular receptors on the basal membrane (Brianti, De Flammineis and Mercuri, 2017). The primary cellular receptor is Heparin sulphate proteoglycans (HSPGs), a ubiquitous polysaccharide and laminin on the surface of basal cellular layers (Shafti-Keramat *et al.*, 2003). The HSPGs attach through their glycosaminoglycan (GAG) chains on basal cells or exposed basement membrane resulting from epithelial trauma or permeabilization (Schäfer, Blumenthal and Katz, 2015). Initial L1 attachment to HSPGs induces conformational changes in the virus capsid ultimately resulting in loss of affinity for the primary receptor. Ultimately, the L2 amino terminus is exposed, making it susceptible to cleavage by furin-related proteases, which is necessary for infection by some HPVs (Cruz *et al.*, 2015). The receptor strategy used may be dependent upon the HPV genotype, the cell type to be infected, or several different receptor strategies may be applicable in a single infection (Graham, 2017).

The structural conformation process achieves the transfer of viral capsid to the secondary receptor (alpha 6 integrin) and for the necessary transfer of the viral genome to the nucleus and internalization of the virus (Horvath *et al.*, 2010). In addition to alpha 6 integrin, epidermal growth factor, tetraspanin enriched membrane microdomains, syndecan 1, annexin A2 heterotetramer, and vimentin serves as the entry receptors for HPVs (Abban and Meneses, 2010).

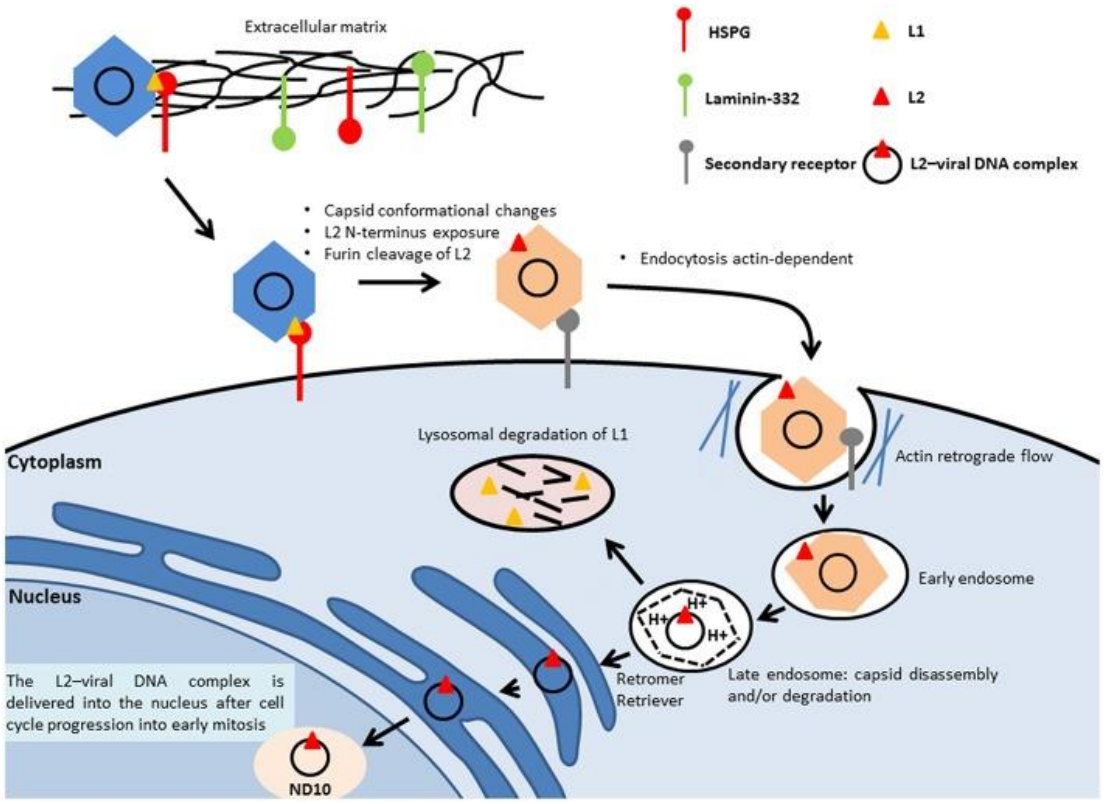


Figure 2.5: HPV binding and internalization. *The virus binds to heparan sulphate chains of proteoglycans (HSPGs) though the major capsid L1 protein and internalization through conformational change of the minor L2 capsid protein. Adapted from* (Gheit, 2019).

Uncoating

Once in the cytoplasm after endocytosis, the L1 major coat protein starts to dissociate from its partner L2, the minor capsid protein. Low pH and cellular proteins, cyclophilins, mediate the dissociation process (Bienkowska-haba *et al.*, 2012; Sapp and Weiller, 2013). Then, the L2-DNA complex is sorted out from L1 and escapes from the endocytic vesicle (**Figure 2.5**) (Ka *et al.*, 2006). Then, it was found that the L2-DNA composite trafficking to the trans Golgi network (TGN) employs retromer subunits (Lipovsky *et al.*, 2013; Sapp and Weiller, 2013). It was studied that HPV reaches the nucleus approximately 24 h following cellular attachment where the capsid disassembles and incoming L2 and the viral genome associated with PML (promyelocytic leukaemia) nuclear bodies. This nuclear location is commonly used by DNA viruses to initiate

viral transcription but while many viruses disrupt PMLs upon nuclear infection, HPV seems to require PML integrity to establish nuclear infection (Day *et al.*, 2004)

Early phase of the viral replication cycle

Immediately after nuclear entry into the dividing cells of the basal layer or ectocervix viral early transcription is initiated. Immediate transcription leads to the detection of early transcripts E1 and E2 proteins as early as 4hrs post nuclear entry while early replication is detectable after 8hrs (Ozbun, 2002). The E1 and E2 proteins cooperatively bind to specific binding sites in the replication origin to initiate replication (Ustav and Stenlund, 1991). E1 is the only HPV encoded protein that has enzymatic activity; helicase and ATPase (Bergvall, Melendy and Archambault, 2013) while E2 does not have enzymatic activity. But it has multitude of functions in the HPV life cycle. E1 an ATP dependent helicase binds and unwinds DNA to allow access of the cellular replicative machinery while E2 functions as a helicase loader to increase specificity, and facilitate binding of E1 to the replication origin (Stenlund, 2003).

The E1:E2 dimer complex interacts with high affinity and specificity at the HPV origin (Ori) of replication (Bergvall, Melendy and Archambault, 2013). Furthermore, it recruits cellular replication mediators (polymerases) to begin DNA amplification and maintenance (Longworth and Laimins, 2004; Ammermann *et al.*, 2008). Thus, early amplified HPV genome copy number per infected basal cell reached between 50 and 100 copies (Doorbar *et al.*, 2015). Then, HPV DNA may replicate in an ordered manner once per S-phase (Hoffmann *et al.*, 2006) with the basal cell. That is, when the basal cell divides into two, similarly the HPV DNA doubles, segregate and shared among the daughter cells (Jr *et al.*, 2015). The papillomavirus replication origin encompasses an E1 binding site, at least one E2 binding sites and an A/T rich region (Ustav and Stenlund, 1991). Although one E2 binding site is often sufficient for initiation of replication, additional E2 sites will enhance this process.

E2 has four binding sites; two at proximal to the early promoter, one at the Ori and one at the enhancer regions of the LCR (Graham, 2016) and therefore, it serves as replication factor that recruits E1 to the Ori of replication and controls the expression level of E6 and E7 oncogenic proteins. At high concentration, E2 binds to a specific palindrome (5'-ACCG (N4) CGGT-3')

sequence in the early promoter P97 that blocks E6 and E7 expression. Hence, it acts as transcription repressor. E2 also distributes viral genome among daughter cells during mitosis (McBride, 2013; Graham, 2016).

As the infected cells undergo differentiation, late gene expression and viral genome replication are induced. E4 and E5 are both required for viral amplification. HPVs do not encode any other replication enzymes and must hijack the host DNA synthesis machinery to accomplish replication of the viral genome. E1 and E2 recruit cellular DNA polymerases and other essential accessory enzymes to enable viral genome replication. Normally, differentiating cells would not be capable of supporting DNA synthesis given they have withdrawn from the cell cycle upon exiting the basal layer of the epithelium. However, HPVs can activate cellular DNA replication machinery to allow vegetative viral DNA synthesis through the actions of E6 and E7 (Maglennon, McIntosh and Doorbar, 2011).

To maintain the cellular replication machinery active, the viral proteins E6 and E7 are expressed and uncouple cell growth arrest and differentiation primarily through the inactivation of p53 and pRb, respectively. Many papillomavirus E7 proteins target the retinoblastoma tumor suppressor pRB and the related "pocket proteins" p107 and p130. The inactivation of pRb by E7 forces infected cells to remain in a proliferative state and escape cell cycle exit, while abrogation of p53 by E6 ensures cell survival by preventing apoptosis triggered by this aberrant growth signal (Barrow-Laing, Chen and Roman, 2010).

Late phase of the viral replication cycle

The late phase of the viral life cycle involves vegetative viral DNA replication and subsequently, virion formation. Increased expression of the viral E1 and E2 proteins is required to accomplish this phase. This late stage is marked by activation (due to changes in cell signaling) of the viral major late promoter (HPV16: P670; HPV18: P811) that is situated in the E7 gene region (Spink and Laimins, 2005). The productive phase of the viral life cycle is activated further upon epithelial differentiation, resulting in the amplification of viral genomes to thousands of viral copies per cell in the suprabasal layers, as well as activation of late gene expression. The amplified genomes are then packaged into infectious virions by the L1 and L2 proteins, which form the subunits of the

icosahedral capsid. Finally, viral escape probably occurs by natural tissue desquamation and may be facilitated by the keratin network disrupting ability of E4 (Maglennon, McIntosh and Doorbar, 2011). Completion of the viral lifecycle involves cell cycle exit and expression of L1 and L2 to allow genome packaging. Virion assembly occurs in the nuclei of terminally differentiated keratinocytes, in which viral genome replication and expression of viral RNA has occurred. Nuclear entry of L1 and L2 is mediated by cellular karyopherins, which transport molecules between the nucleus and the cytoplasm. L1 can assemble into VLPs and L2 may increase the efficiency of this reaction (Darshan *et al.*, 2004).

The regulation of viral life cycle in this manner allows HPV to avoid detection by the immune response as high levels of viral gene expression as well as virion production are restricted to the uppermost layers of the epithelium, which are not under immune surveillance. Due to small coding capacity of the viral genome, HPV depends on the host DNA replication machinery to synthesize its DNA. To support productive replication, HPV employs numerous mechanisms to subvert key regulatory pathways that regulate host cell replication, maintaining the differentiating cells active in the cell cycle. As such, HPV can reactivate cellular genes and signaling pathways necessary to support late gene expression and amplification of viral DNA (Stanley, 2010).

2.4. Pathogenesis of cervical cancer

HPV infection is one of the most prevalent sexually transmitted infectious agents. It establishes infection in the basal cells of the cervix. Then, after entry to the host cell's nucleus, it starts replicating in line with the natural biology of the epithelial cells with minor damage (Horvath *et al.,* 2010). However, deviation from the normal productive infection, HPV infection is usually associated with several disease conditions. Of all, initiation of cervical cancer is the major one (Jo and Kim, 2005). There are also other conditions due to HPV infection including cancers of the vagina, vulva, penile, anal, a fraction of head and neck cancers, genital warts etc (Mclaughlin-Drubin and Münger, 2010).

To better understand how HPV associated diseases develop, understanding of the biological niche of hr-HPVs is required. The cervix is approximately 4 cm in length and 3 cm in diameter that connects the vagina and the uterus and is divided in the ectocervix, covering the surface of the vagina, and the endocervix, bordering the endocervical canal of the uterus (Prendiville and Sankaranarayanan, 2017). The ectocervix is lined with squamous epithelial tissue, while the endocervix is lined with columnar epithelial. Ecto- and endocervix meet at an area called the squamocolumnar junction (SCJ) (Pierre, Raluca and Rosa, 2016). The location of the SCJ on the cervix varies over a woman's lifetime and is dependent on age, hormonal status, oral contraceptive use, birth trauma and pregnancy. Among the three distinct epithelial types, the cervical Transformation Zone (TZ) is the most important regarding cancer risk. This is an area of high cellular turnover, as columnar cells change into squamous cells. The location of the transformation zone varies among women. In teenage girls, the transformation zone is on the immature cervix's outer surface and is more susceptible to infection than in adult women. In older women, the transformation zone may be higher in the cervical canal with decreasing levels of estrogen (Doorbar and Griffin, 2019).

Sexual contact with an infected partner is necessary for transmission of HPV and it is thought that this occurs through microscopic abrasions in the mucosa (Zhang *et al.*, 2015). In addition to peno–vaginal intercourse, HPV infections can also be transmitted by other sexual practices such as oral sex, peno–anal intercourse, digital–vaginal sex, and use of insertive sex toys (Edwards and Carne, 1998; Sonnex, Strauss and Gray, 1999). Free HPV virions (possibly

deposited as a consequence of intercourse) can then reach and enter the basal cells, initiating a HPV infection. Infections can be either transient, meaning clearance will occur within 12–18 months depending on the HPV type, or persistent (Rositch *et al.*, 2013). It is estimated that around 80% of all HPV infections are transient, while the remaining 20% persist within the host, of which a small subset (1–3.5%) can eventually cause lesions and possibly cervical cancer (Schiffman *et al.*, 2007).

Histologically, there are two types of cervical cancers and cervical pre-cancers, squamous cell carcinoma (SCC) and adenocarcinoma. SCC develops from the transformation zone and adenocarcinoma develops from the mucus-producing cells of the endocervix. More than 70% of cervical cancer are SCC (Vizcaino *et al.,* 2000) while adenocarcinoma and adeno-squamous cell carcinoma represent 10–15%, and other or unspecified histology represent the remaining 10–15% (Vizcaino *et al.*, 1998).

Prior to the development of cervical cancer, pre-malignant pre-invasive lesions known as cervical intraepithelial neoplasia (CIN) are formed (**Figure 2.6**). These pre-cancer lesions could develop in three grades: CIN1 corresponds to mild dysplasia that consists of abnormal cells involving the lower third of the epithelium covered with differentiated epithelia. CIN2 corresponds to moderate dysplasia at which the abnormal cells involve more than one third and CIN3 includes severe dysplasia that involve the full thickness of the epithelium (Anderson *et al.*, 1991; Woodman, Collins and Young, 2007). However, when the abnormal cells invade the dermis by breaking through the basal lamina of the epithelium, this is called invasive cervical cancer (**Figure 2.6)**. Accordingly, lesions with CIN1 are classified as low-grade squamous intraepithelial lesions (LSIL) and lesions with CIN2 or CIN3 are combined as high-grade squamous intraepithelial lesions (HSIL) (Alrajjal *et al.*, 2021).

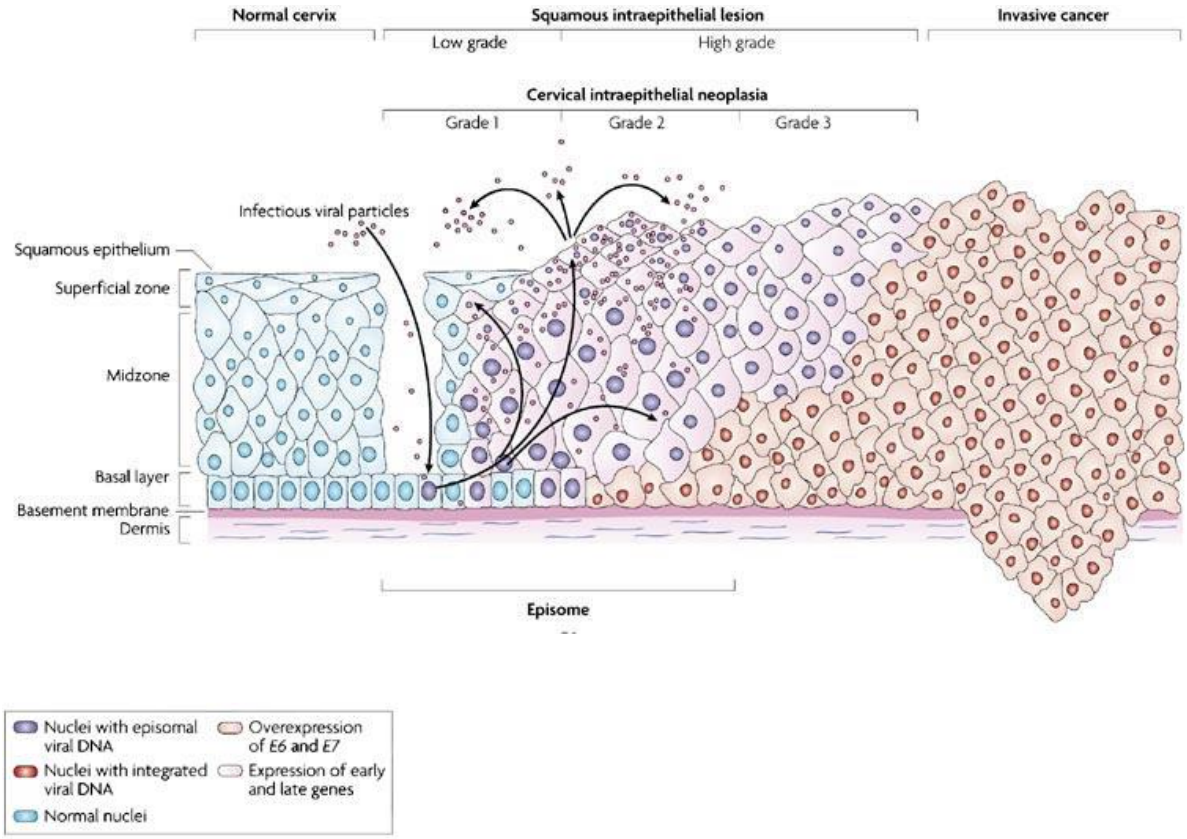


Figure 2.6: Schematic representation of HPV infection, progression, and carcinogenesis

The two main proteins of hr-HPVs which have an oncogenic capacity and play a major role in the carcinogenesis process are E6 and E7. These proteins act by modifying the control of the cell cycle and by regulating apoptosis (Sano and Oridate, 2016). The incorporation of viral DNA disrupts the activity of the E2 protein. The E2 protein is recognized as having the ability to repress the transcription of E6 and E7, and thus its interruption causes dysregulated expression of these oncoprotein. these proteins are able to immortalize cells, so that cells retain their mitotic ability to generate clones that also have the immortalized phenotype and do not experience terminal differentiation (Howley, 2006).

E6 and E7 proteins of the high-risk human papillomaviruses can inactivate tumour suppressors p53 and retinoblastoma protein (pRb), respectively. Although E6 protein itself cannot bind to p53, it can bind to a cellular ubiquitin ligase named E6AP and make ternary complexes with p53 so that it becomes ubiquitinated. E6 protein also has functions independent of p53

inactivation. It is likely that ubiquitin ligase E6AP is a key player not only in the degradation of p53 but also in the activation of telomerase and cell transformation by E6. (Yugawa and Kiyono, 2009).

Most biological functions of E7 are achieved by inactivating pRb family proteins. E7 is known to bind to the retinoblastoma tumour suppressor gene product, pRb, and its family members, p107 and p130. In the hypophosphorylated state, pRb family proteins can bind to transcription factors such as E2F family members and repress the transcription of particular genes involved in DNA synthesis and cell-cycle progression (Dyson, 1998). Phosphorylation of pRb by G1 cyclin-dependent kinases releases E2F leading to cell cycle progression into the S phase. Because E7 is able to bind to unphosphorylated pRb, it may prematurely induce cells to enter the S phase by disrupting pRb–E2F complexes (Narisawa-Saito and Kiyono, 2007) (**Figure 2.7)**.

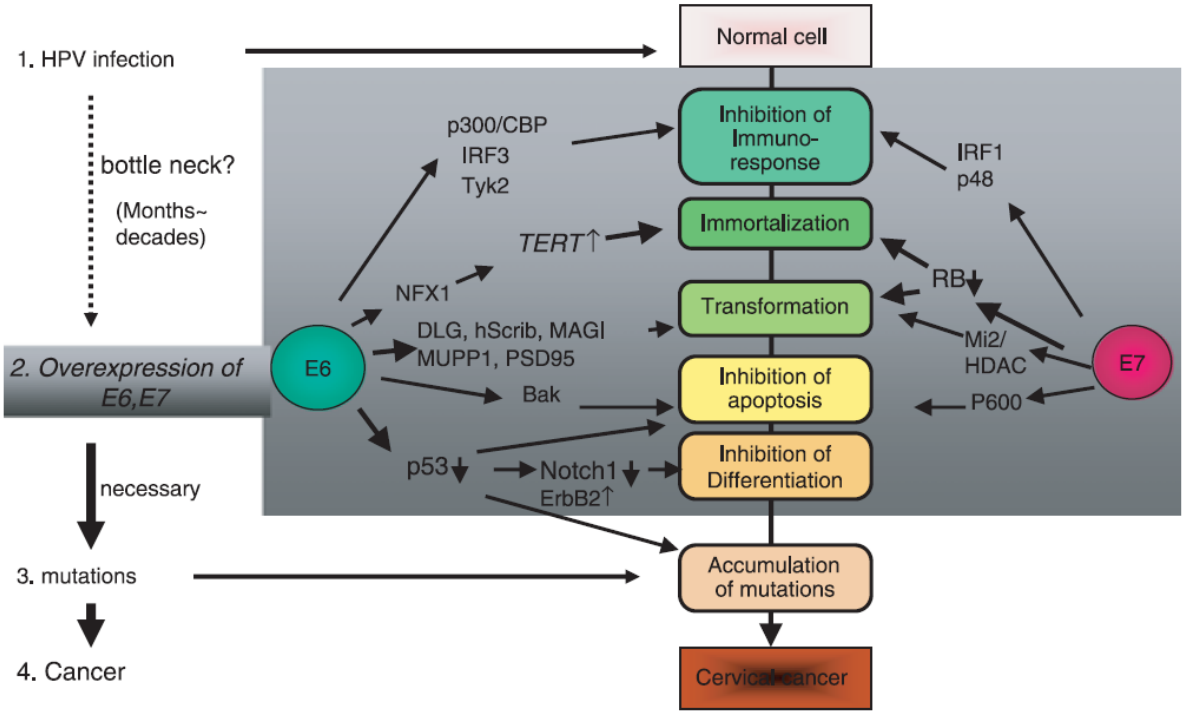


Figure 2.7: Multi-step carcinogenesis for HPV-induced cervical cancer. E6 and E7 cooperatively function in the development of cervical cancer adapted from (Narisawa-Saito and Kiyono, 2007)

HPV DNA integration into the host genome is also one of the important risk factors for cervical carcinogenesis (Stanley, Pett and Coleman, 2007). In the initial infection, HPV genome is found in the nucleus as an episome. But, during progression to high grade dysplasia, integration of the genome into the host genome occurs. HPV viral integration into host genome DNA is now thought to be associated with the progression from low- to high-grade CIN (Arias-Pulido *et al.*, 2006). Furthermore, the DNA double-strand breaks resulted from high-risk E6 and E7 expression are important requirements for the initiation of virus integration into the host genome (Arias-Pulido *et al.*, 2006).

Therefore, most CIN1 lesions mainly contain episomal HPV while both episomal and integrated HPVs are observed in CIN2/3 lesions. Due to this integration phenomenon, the pure episomal form is rarely observed in the invasive stages **(Figure 2.8)**. HPV integration frequently disrupts E2 expression, leading to the elevated expression of E6 and E7 resulting from the subsequent inability to suppress the expression of viral oncogenes (Sano and Oridate, 2016).

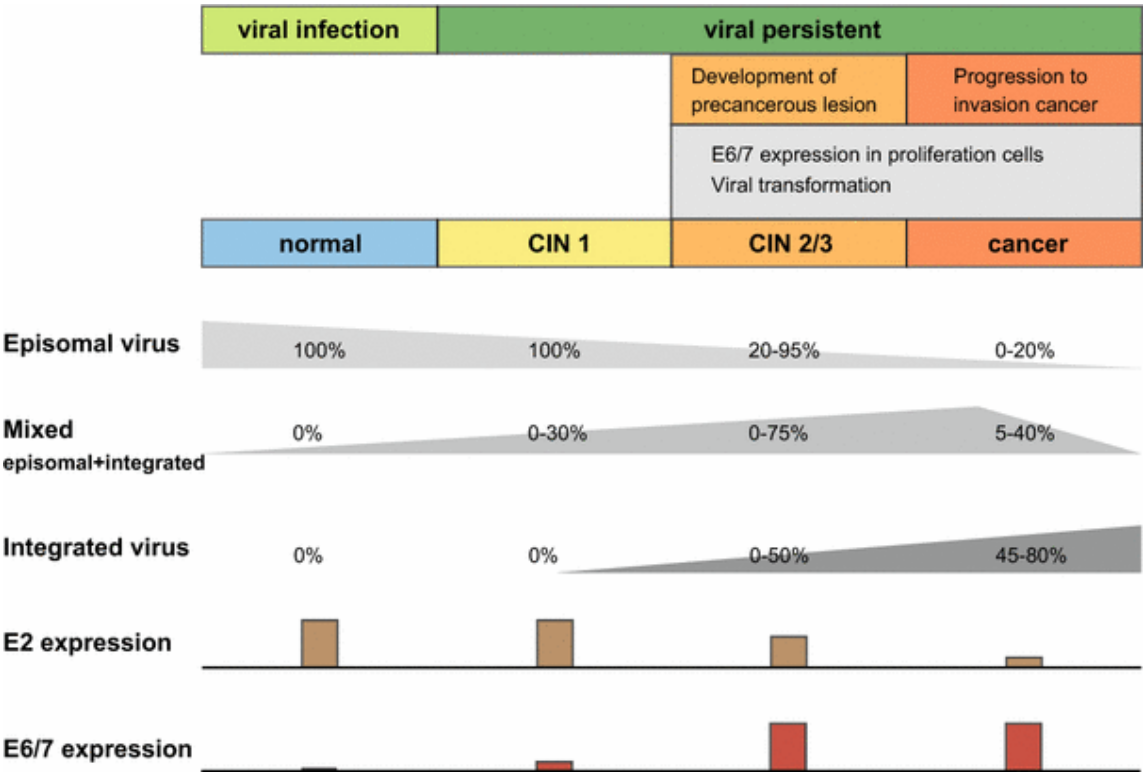


Figure 2.8: Changes in the physical state of human papillomavirus on progression of transforming cervical intraepithelial neoplasia (CIN) to cervical cancer, adapted from (Sano and Oridate, 2016)

2.4.1. HPV persistence and clearance

Nearly all precancerous cervical lesions and cervical cancers are caused by persistent hr-HPV infections (J. M. Walboomers *et al.*, 1999). Thus, persistent hr-HPV infection is an important intermediate phenotype to cervical cancer (Sudenga and Shrestha, 2013). However, most HPV infections are transient after detection of the first infection. More than half of the HPV infections clear within 6 months and up to 90% are spontaneously cleared in 24 months (Plummer *et al.*, 2007; Rodriguez *et al.*, 2007; Oh *et al.*, 2008; Philip E. Castle *et al.*, 2009). However, the same woman may be re-infected with the same HPV type, although most of the type-specific HPV infections are cleared. Re-infection of new or the same HPV type may occur from a new partner, re-infection of the same partner, or reactivation of the initial infection (Richardson *et al.*, 2003).From different studies, only a small fraction of the cervical intraepithelial lesion (CIN) grade II lesions progressing to invasion or about 22% to carcinoma in situ. In addition, it was indicated that probably less than 50% of women with CIN3 develop invasive cervical cancer within 30 years (McCredie *et al.*, 2008).

In the development of cervical cancer, infection of the cervical epithelium with carcinogenic HPV genotypes is the first step. However, high risk HPV infection by itself is not enough for the cancer to arise. The most important step of HPV infection is, therefore, the persistence of HPV infection, progression to precancerous lesions, and invasion (Gravitt, 2011; Schiffman *et al.*, 2011). As a matter of this, cervical cancer is a relatively rare and late consequence of persistent infection with oncogenic HPV types. Therefore, persistent HPV infection could also be a useful screening marker for cervical cancer and pre-cancer. For example (Iacobone *et al.*, 2021) in their follow up study confirmed that HPV same genotype persistence has 30-fold increased odds of developing CIN2+ recurrence after treatment.

Despite lack of general consensus, persistent infection is defined as “finding the same genotype positive in two consecutive HPV tests”; however, the screening interval of type-specific infection may widely vary among different reports (Mũoz *et al.*, 2009; Rositch *et al.*, 2013). The time interval between two measures affects the persistence estimates as many infections will be cleared by year 2 (Marks *et al.*, 2012).

2.5. Risk factors associated with the natural history of HPV

Risk factors that influence the pathway from HPV acquisition to persistence and the development of CIN and ICC includes two groups of factors, corresponding to the key stages in HPV natural history.

2.5.1. Socio-demographic and behavioural factors

These factors determine the acquisition of hr-HPV infection.

Age: The relationship between age and HPV prevalence is similar worldwide with the highest prevalence among women <25 years and a decline at older ages (Smith *et al.*, 2008; Forman *et al.*, 2012). Cervical ectopy, which is more likely among young women has been shown to increase the risk of acquisition of other STIs (Kleppa *et al.*, 2015), including HPV (Monroy *et al.*, 2010). The single layer columnar epithelium, indicative of ectopy, is a weak physical barrier and may facilitate entry of HPV and other STIs. Furthermore, oral contraceptive use - another risk factor for HPV infection, which is frequently used among younger women- can increase the likelihood of ectopy (Bright *et al.*, 2011).

Smoking: Studies showed that cigarette smoking is associated with HPV prevalence, incidence and persistence (Vaccarella *et al.*, 2008). Compared to HPV-positive women who never smoked, HPV-positive women who have ever smoked tobacco have increased odds of CIN2/3 (Louie *et al.*, 2011; Roura *et al.*, 2014). The biological plausibility for this association is that tobacco smoke contains known carcinogens such as polycyclic aromatic hydrocarbons that could have a direct transformation effect on the cervix (Louie *et al.*, 2011). It is also known to suppress the immune response by reducing the number of Langerhans cells and other immune markers, thereby allowing HPV to persist and cervical lesion to develop (Koshiol *et al.*, 2006). Furthermore, since the cervical mucus of smokers contains measurable amounts of cigarette constituents and their metabolites, these substances damage the DNA of cervix cells, increase cell proliferation and may contribute to the development of cervical cancer (Xi *et al.*, 2009).

Age at first pregnancy and parity: The increase in ICC risk among women with younger age at first pregnancy may be attributed to cervical trauma during delivery, which can result in cervical ectopy, thereby increasing exposure of the SCJ to HPV infection. High concentrations

of oestrogen and progesterone during pregnancy could facilitate cervical carcinogenesis, a mechanism similar to that reported for hormonal contraceptive users. Immunosuppression during pregnancy can also facilitate acquisition and persistence of HPV (ICESCC, 2006).

Age at first sexual intercourse and number of lifetime sex partners: Early AFSI may be a surrogate marker for early HPV infection which may have persisted. Early AFSI has shown to be associated with riskier sexual behaviour, such as unprotected sex, a higher number of lifetime sex partners, or concurrent sex partners and a woman's partner having multiple sex partners (Louie *et al.*, 2009). The peak transmission of HPV infections is typically among young women following sexual debut and it has been suggested that the immature cervix during adolescence may be more susceptible to HPV acquisition and persistence (Watson-Jones *et al.*, 2013).

2.5.2. Biological factors

These are risk factors that determine the persistence of infection and cervical precursor lesion development/carcinogenesis.

Male circumcision: The reduction in HPV acquisition and ICC among women is linked to the reduction in transmission of HPV from male partners, which result from: a.) an overall reduction in HPV prevalence among men, and b.) reduction in likelihood of transmission from infected males to their female partners. The foreskin is susceptible to tearing during the intercourse, which would give viruses an easy pathway into your body. It could also be that the folds in foreskin provide an environment for viruses and bacteria to thrive (Zitkute and Bumbuliene, 2016). The glans of the circumcised penis has a thicker epithelium making it more resistant to abrasions and therefore less susceptible to HPV viral entry. In these men, the distal urethra is the only mucosal epithelium, which is known to contain relatively fewer HPV-related lesions (Aynaud *et al.*, 1999).

Hormonal contraception: long-term use of both oral and injectable contraceptives have been considered as important risk factors for cervical cancer and pre-cancer but not independently rather as cofactors that interact with HPV to induce cervical carcinogenesis (Roura *et al.*, 2016). The cervix is an estrogen-responsive tissue, highly sensitive to steroid hormone contraceptive usage. Estrogen has been suggested as a synergistic factor that is

required to induce HPV-dependent cervical carcinogenesis (Ramachandran, 2017). Therefore, hormonal contraception influences the differentiation and maturation of the cervical epithelium which can lead to thinning of the mucosal epithelium and micro tearing, acting as portal of entry for HPV (Lavreys *et al.*, 2004). Furthermore, hormonal contraceptives can regulate cytokine and immunoglobulin expression which may facilitate HPV persistence. Both oestrogen and progesterone increase the expression of the HPV oncoproteins E6 and E7 linked to ICC development (De Villiers, 2003).

Other Sexually Transmitted Infections (STIs):

Sexually transmitted infections (STIs) are the most common infectious diseases worldwide and include bacterial, viral, or protozoan infectious agents that are transmitted via sexual activity. The common agents include *Chlamydia trachomatis, Neisseria gonorrhea, Trepanoma pallidum*, *Trichomonas vaginalis*, Herpes Simplex Virus (HSV-2), HIV etc (Mu, 2014). STIs are associated with serious reproductive tract health complications, such as ectopic pregnancy, pelvic inflammatory diseases, and infertility. They are also linked to several types of cancers, including cervical, anal, and oropharyngeal cancers (Caini *et al.*, 2014).

The presence of other STIs enhances the risk of acquiring HPV infection and its consequences directly or indirectly, possibly due to disruption of the mucosal epithelial barrier caused by inflammation and ulceration, thereby facilitating entry of the HPV virus (Horvath *et al.*, 2010). An STI may facilitate the entry of multiple hr-HPVs as well as decrease the host's ability to resolve the HPV infection. However, the link with ICC is less clear, as STI positivity is a marker of past sexual behaviour and may act as a confounder for hr-HPV infection (Paba *et al.*, 2008b). Several STIs induce inflammatory responses which are linked to hr-HPV persistence and carcinogenesis, like Herpes simplex type 2 virus (HSV-2), Chlamydia trachomatis, Trichomonas vaginalis, Bacterial vaginosis (BV), Human immunodeficiency virus (HIV) (Yin *et al.*, 2013).

2.6. Control of HPV related cervical disease

Cervical cancer is by far the most common HPV-related disease, more than 95% is due to HPV. Most HPV infections clear spontaneously and most of the pre-cancerous lesions can also resolve spontaneously in immunocompetent women. Despite this fact, there is a risk for all women that HPV infection may become chronic and pre-cancerous lesions progress to invasive cervical cancer (World Health Organization, 2020). For cervical cancer to develop, it takes 15 to 20 years women with normal immunity and 5 to 10 years in women suppressed immune systems (World Health Organization, 2020). Therefore, it can often be prevented by having regular screenings to find any precancers and treat them and by receiving the HPV vaccine.

Comprehensive cervical cancer prevention and control programme should be applied to decrease the burden of cervical cancer. This comprehensive approach is to reduce HPV infections, to detect and treat cervical, pre-cancer lesions, and to provide timely treatment and palliative care for invasive cancer (World Health Organization, 2013a). The components of cervical cancer prevention and control comprises primary prevention, secondary prevention and tertiary prevention(World Health Organization, 2013b).

2.6.1. Cervical cancer screening strategies

Cervical cancer screening is used to detect precancerous changes before signs or symptoms of cancer occur. It has dramatically reduced the incidence of cervical cancer in those countries that have implemented screening programs; however, most cervical cancers continue to occur in women who do not attend regular screening (under screened) or who have never been screened (Racey, Withrow and Gesink, 2013).

The World Health Organization (WHO) proposed a global strategy to eliminate cervical cancer through the 90-70-90 targets for 2030 (i.e., vaccinating 90% of girls by age 15, screening 70% of women with high performance tests by 35 and 45 years of age, and treating and/or manage 90% of women who tested positive for pre-cancer and invasive cancer). Thus, population-based screening remains important for both the vaccinated and more so the non-vaccinated population (Roden, Stern and Sciences, 2019).

2.6.1.1. Cytology

The Papanicolaou (Pap) test is one of the standard screening tests used for the detection of cervical cancer. The Pap test, which is administered during a pelvic examination, involves the examination of cervical cells for abnormal histological changes consistent with cervical cell dysplasia and cervical cancer (Racey, Withrow and Gesink, 2013). In developed countries, regular screening with a Pap smear has been shown to effectively lower the risk for developing invasive cervical cancer, by detecting precancerous changes (Forman *et al.*, 2012). However, it is not perfect. Even in high-income countries, more than half of the women who are diagnosed with cervical cancer had never had a Pap test or were infrequently screened.

Although a single Pap smear has relatively low sensitivity, frequent testing over the course of a woman's lifetime assures the identification of the precursor lesions. The requirement for repeat testing and multiple follow-up visits makes cytology screening strategies virtually impossible to implement in developing nations, which bear the major burden of cervical cancer (Sowjanya *et al.*, 2009). Evidence showed that only 5% of women have undergone cytological screening in 5 years period in developing countries. This is because the method requires highly educated professionals and resources (Blumenthal *et al.*, 2004). Moreover, delays in reporting cytology results and access of service point have been big challenges in developing countries to sustain the service (Goldie *et al.*, 2001).

In low resource settings as women have limited access to routine primary healthcare and even implementation of Pap testing in higher-level facilities has not been effective at reducing cervical cancer incidence and mortality (Denny, Quinn and Sankaranarayanan, 2006). Pap testing has not been a popular choice for screening in Africa, likely because of the many resources required for its successful use (e.g., pathologist, laboratory equipment, and delay in follow-up /treatment).Many industrialized countries have revised their guidelines and are implementing HPV based screening as primary screening test of cervical cancer (Arbyn *et al.*, 2012).

2.6.1.2. 'See and Treat' Method

Two visual methods are available: Visual inspection with acetic acid (VIA); Visual inspection with Lugol's iodine (VILI) (WHO, 2011). It has been demonstrated that VIA is an alternative sensitive cervical cancer screening method (Gaffikin *et al.*, 2007; Sankaranarayanan *et al.*, 2007). It is relatively cheaper and can be done at lower health facility as the method does not require highly trained professionals (Gaffikin *et al.*, 2003). More importantly, the result can be available at the same time and those who found positive can be treated at the same time and places (Forman *et al.*, 2012). WHO Guidelines recommend visual inspections with acetic acid (VIA) for population-based screening or HPV testing if it can be afforded (World Health Organization, 2013b).

Cryotherapy is a first step modality to treat the precancerous lesion, which is effective and easier to implement than other treatment modalities such as loop electrosurgical excision procedure (LEEP), loop excision of the transformation zone (LETZ) and cone biopsy (Chirenje *et al.*, 2001) Furthermore, the modality does not require complicated equipments and can be done by lower trained health professionals. However, maintaining quality assurance, the invasiveness of a pelvic examination and user variability of the test remain barriers (Moses *et al.*, 2015).

Moreover, the short comings of this method include having of low positive predictive value and lack of evidence on periodic screening performance (WHO, 2002; Moses *et al.*, 2015). In additions, the low uptake of the method is become a big challenge and required innovative approach to assure the high coverage of cervical screening among the eligible women.

2.6.1.3. High risk HPV DNA-based screening methods

Infection of the genital tract with high-risk HPV is the primary cause of cervical cancer. This has led to the investigation of using HPV assays as an alternative to cytology in screening for cervical cancer either by way of co-testing with the Pap test or as a primary self-sample HPV screening test used to triage women for subsequent Pap testing (Racey *et al.*, 2013). It is found that the presence of hr-HPV in cervical swabs has a higher sensitivity, but lower specificity, for the detection of cervical cancer precursors when compared with cervical cytology (Cuzick *et al.*, 2008). But

cytology still requires a clinic visit by the woman, and a speculum-assisted pelvic exam by a health care provider. These requirements limit broad access to and acceptability of the method in many regions of the developing world (Sowjanya *et al.*, 2009).

An alternative strategy is screening for HPV from a vaginal swab taken by the women herself, so that the first stage of screening can occur outside of a clinical setting (Virtanen *et al.*, 2011). Studies evaluating the comparability of self-collected vaginal versus clinician collected cervical swabs have generally shown good agreement for their ability to detect HPV (Racey *et al*., 2013), but the two methods have not been adequately compared for their performance in detecting cervical neoplasia in a screening setting (Hellsten *et al.*, 2021).

Beyond the efficiency and effectiveness of HPV testing as a diagnostic test, studies have also considered women's attitudes toward self-collection and found that women have a high acceptance of and positive attitudes toward the use of self-collected HPV testing (Igidbashian *et al.*, 2011; Sossauer *et al.*, 2014). In the Ethiopian context, in a cluster randomized clinical trial we have conducted among women living in Butajira, we have proved that HPV based screening with self-collected sample had a superior acceptability and adherence to procedures compared to VIA (Muluken Gizaw *et al.*, 2019).

HPV testing is becoming a reality in several countries either adopting this modality at a program level or introducing it through execution of regional pilot studies (Wentzensen *et al.*, 2017). This screening method has higher sensitivity for cervical precancers and allows extending screening intervals. Recognizing HPV as necessary cause for most cervical cancers has led to development of new HPV assays as well as other biomarkers for screening and management *(*Sahasrabuddhe *et al.*, 2011; Schiffman *et al.*, 2011; Wentzensen *et al.*, 2016).

The abundance of available assays (>200) has led to considerable confusion about the best approaches to screening among providers. In addition, many developed countries validated the assays available and suitable for their programs and developed their screening guidelines which is critical in the prevention of cervical cancer. Besides, as primary HPV based screening becoming the standard of care globally, additional focus on evidence of clinical performance of HPV assays

for use in cervical cancer screening programs is required (de Sanjose and Holme, 2019).

The current WHO cervical cancer screening test recommendation is to use HPV DNA or HPV mRNA as primary screening test either with or without triage to colposcopy or treatment to prevent cervical cancer among the general population of women (World Health Organisation, 2021). In addition, genotyping HPV tests are widely used in epidemiological studies, HPV surveillance, and vaccination impact monitoring (Poljak *et al.*, 2020). Furthermore, HPV genotyping is becoming crucial in risk stratification due to more or less carcinogenic types and type-specific persistence (Elfgren *et al.*, 2017) as risk for dysplasia development. Consequently, several commercial HPV genotyping assays are being successfully introduced for population-based HPV screening as well as for research purposes.

2.6.2. Cervical Cancer screening experience in Ethiopia

There is very low (2.9%) cervical cancer screening rate in Ethiopia (Gelibo *et al.*, 2017). Routine access to cervical cancer screening and treatment for precancerous lesion was not available until implementation of the Addis Tesfa project in 2009 for HIV infected patients by Pathfinder (Pathfinder, 2010; FMoH, 2015). VIA screening combined with access to cryotherapy was piloted in Ethiopia by the FMoH in collaboration with Pathfinder in 2009 only (FMoH, 2015). The service was introduced as a single-visit approach to cervical cancer prevention integrated into a comprehensive care package for people living with HIV at 14 Hospitals. The service was subsequently initiated in eleven additional health facilities (clinics of the Family Guidance Association of Ethiopia (FGAE), military hospitals, and some other facilities) making the service available to a total of 25 health institutions. Moreover, the estimated coverage of cytology-based cervical cancer screening in Ethiopia is 1.6% in urban settings and 0.4% in rural areas (FMoH, 2015).

2.6.3. Challenges of cervical cancer screening in low-income countries

The success of cervical cancer control programs in high income countries were due to the placement of extensive high-quality screening for decades (Geldsetzer and Lemp, 2021). However, the disease is still a leading cause of cancer death among women in low and middle-income

countries (LMIC). Approximately, 90% of deaths from CC occur in low- and middle-income countries, especially in areas of high HIV prevalence, and largely due to limited prevention and screening opportunities and scarce treatment options. Screening methods such as cytology – although widely used in high-income countries – have limited relevance in many low-resource settings. The World Health Organization recommends screening using HPV testing wherever possible; however, although HPV primary testing is more sensitive and detects precancers earlier than cytology, there are currently costs, infrastructure considerations and specificity issues that limit its use in low- and middle-income countries (Cubie and Campbell, 2020).

The other screening method recommended for LMIC is VIA, identification of abnormalities by inspection of the cervix without the benefit of magnification. The main advantages of VIA are its low use of resources – it can be undertaken by trained nurses and midwives; it does not utilize laboratories and it gives an immediate result allowing screening and treatment to be completed in a single visit. However, VIA is subjective, with often considerable variability between providers even in settings where quality measures have been introduced. Loss of skill set and misinterpretation of temporarily acetowhite lesions lead to false positives and overtreatment (Sankaranarayanan *et al.*, 2012; Maseko, Chirwa and Muula, 2015).

The other challenge of the recommendations for LMIC i.e support HPV testing alone (followed by treatment with cryotherapy) or in conjunction with visual inspection with acetic acid (VIA) triage testing is the cost-effectiveness of visual triage of HPV positive women. The cost-effectiveness study from three LMIC indicated that VIA triage of HPV-positive women is not likely to be cost-effective in settings with high cervical cancer burden. HPV testing alone followed by treatment may achieve greater health benefits and value for public health dollars (Campos *et al.*, 2017). Most of the successful screening programs so far have been using repeated screening of a large fraction of women with cervical cytology and referral to colposcopy and treatment of cervical cancer precursors. However, it has proven extremely difficult to successfully implement such programs in developing countries (Geldsetzer and Lemp, 2021).

2.7. HPV infection and the cervicovaginal Microbiome

The female genital tract is an important bacterial habitat of the human body. The microbiome in the vagina is a dynamic microecosystem that fluctuates constantly during the women's entire life (Chen *et al.*, 2021). The cervicovaginal microbiota plays a crucial role in vaginal health and its alteration affects millions of women annually, and is associated with numerous adverse health outcomes, including HPV infection (Gao *et al.*, 2013). In the vaginal tract, more than 50 microbial species have been described. But the Lactobacillus species are the pretty dominant species (up to 70% of the total) including *L. crispatus, L. gasseri, L. inners, and L. jensenii* (Miller *et al.*, 2016).

Lactobacilli protect the host against genital infections by producing lactic acid (which lowers vaginal pH below 4.5), secretion of antimicrobial compounds and competitive exclusion (Ravel *et al*, 2011) and therefore this Lactobacilli dominance has been associated with vaginal health (Hickey *et al.*, 2012; Martin and Marrazzo, 2016; Nunn and Forney, 2016). Furthermore, there are also other key microbial species found in the vagina including anaerobes (Gardnerella, Atopobium, Mobiluncus, Prevotella, Streptococcus, Staphylococcus, Ureaplasma, and Megasphaera) and commensal microorganisms, such as the opportunistic fungus, Candida albicans (Li *et al.*, 2012).

There are different factors that are known to affect the overall structure or function of vaginal microbiome and as well as delicate state of equilibrium including genetic disposition, ethnicity, diet and lifestyle, hygiene status, infections, human behaviors such as antibiotics use and sexual activity, physiological status of the vagina, and particularly estrogen (Ma, Forney and Ravel, 2012). It has also been observed that the vaginal microbiota was having distinct microbial community structure and composition at different stages of women's reproductive lifecycle and menopause (Kaur *et al.*, 2020).

Although several studies focused on the association between bacterial vaginosis and HPV infection, little is known about the composition of vaginal microbial communities involved in HPV acquisition, persistence, and the development of cervical cancer (Kyrgiou *et al*., 2017). It was expected that the vaginal microbiota composition may participate in human host susceptibility to

HPV infection, persistence, subsequent development of dysplastic and neoplastic lesions ultimately (Arokiyaraj *et al.*, 2018a) .

From several studies so far, the typical pattern of HPV-mediated cervical cancer progression involves, HPV acquisition, persistence, progression to pre-cancer (CIN 1, 2 and 3), and invasive cancer (Arokiyaraj *et al.*, 2018b). There are also evidences that most cases of HPV infection are transient, likely to regress naturally (Oh *et al.*, 2008). Mechanisms associated with clearance or persistence of HPV infection is not well understood. Currently, different factors have been found to be associated with the development of CC and persistence of HPV infection (Veldhuijzen *et al.*, 2010). Recently, there are emerging data that support the concept that the complex interactions of the host with the vaginal microbiome may be involved in the natural history of cervical cancer. As a result, along with higher rates of HPV infection, Bacterial Vaginosis (BV) has been associated with delayed clearance of the virus and with cervical intraepithelial neoplasia, suggesting that a diverse, *Lactobacillus*-depleted microbiome may play a mechanistic role (King *et al.*, 2011; Guo *et al.*, 2012).

As the microbiome influences acquisition and pathogenicity of viral infections in the gut, the same may occur in the genital tract. As a result, BV, a condition characterized by a decrease in *Lactobacillus* and a consequent increase in anaerobic bacteria (e.g., Gardnerella, Prevotella, and Clostridiales) (Fredricks, Fiedler and Marrazzo, 2005), is associated with increased risk of acquisition, reactivation or delayed clearance of HPV infection (King *et al.*, 2011). It has been shown that reduction of *Lactobacillus species*, combined with increased diversity of cervicovaginal microbiota, are risk factors for HPV acquisition, persistence, development of cervical intraepithelial neoplasia and cervical cancer (A Mitra *et al.*, 2015).

Several cross-sectional studies evidenced that there is an association between BV and HPV infection and persistence (Arokiyaraj *et al.*, 2018b). Women infected with HPV without cervical disease have been shown to exhibit more diverse vaginal microbiome relative to HPV-negative women. Furthermore, it was indicated that the presence of BV was associated with higher rates of HPV infection suggesting a diverse, Lactobacillus-depleted microbiome may contribute to HPV persistence (Gillet *et al.*, 2011). Thus, Gao *et al* found that there was significantly greater diversity

in the microbiota of HPV-positive women and that *L. gasseri* and *Gardnerella vaginalis* are found in significantly higher frequencies in HPV-positive women respectively (Gao *et al.*, 2013).

Studies also showed that an elevated vaginal pH, which is determined by the microbiota composition and HPV infection, is associated with decreased abundance of *Lactobacilli*, and it in turn is associated with a 30% greater risk of infection with LSIL and with multiple HPV types (Clarke *et al.*, 2012).

According to Ravel and his colleagues, the vaginal microbial profile is classified into a total of five 'community state types' (CSTs) (Ravel *et al.*, 2011). The CSTs I, II, III and V are characterized by dominance of *Lactobacillus crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* respectively. In addition, these community state types are likely to have low species diversity and evenness. On the other hand, CST IV is typically devoid of *Lactobacillus* species and instead enriched with strict anaerobic species including *Gardnerella*, *Megasphera*, *Sneathia* and *Prevotella* (Mitra *et al.*, 2016). However, the structure of the vaginal microbiota is dynamic so that there are transitions between CSTs with the most common transition observed from CST III to CST IV (Romero *et al.*, 2014) which suggests that *L. iners* may be less able to inhibit colonization of strict anaerobes and pathobionts compared to other *Lactobacillus* species.

Longitudinal studies have shown that CST I and a high relative abundance of *L. crispatus* may be protective against HPV acquisition. Transition states such as CST III and a BV-like state, CST IV-B, likely lead to pro-inflammatory states which cause tissue damage and promote E6/E7 expression, genomic instability and viral integration which ultimately promotes development of HSIL. Increasing lack of *L. crispatus* has also been associated with increasing SIL severity, and various other species have been associated with both presence of HPV infection and SIL disease states **(Figure 2.10)** (Kyrgiou, Mitra and Moscicki, 2017).

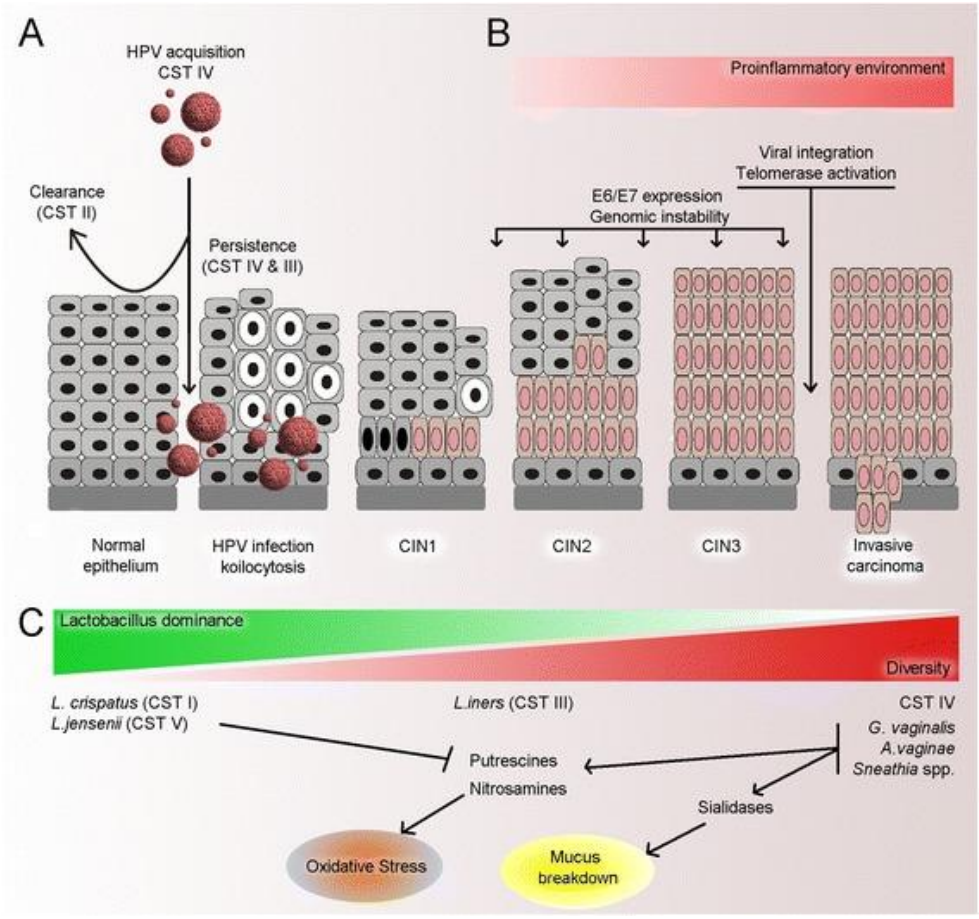


Figure 2.10: Potential mechanisms associating the VMB with cervical disease. **a** VMB structure appears to be associated with acquisition and persistence of HPV infection, and CST II is associated with most rapid clearance of an acute HPV infection. **b** Dysbiosis can result in a proinflammatory environment, which can facilitate several of the necessary steps in viral transformation including E6 and E7 expression, genomic instability, viral integration, and telomerase activation, which are necessary for carcinogenesis. **c** Higher diversity with lower *Lactobacillus* spp. content has been associated with increasing severity of CIN. Species associated with high diversity VMBs may produce sialidases which cause mucus breakdown, predisposing the cervical epithelium to tissue damage, as well as producing biological amines which are responsible for oxidative stress, a key mechanism in carcinogenesis. Certain species *Lactobacillus* spp. have been shown to mop up these amines, and therefore their presence may reduce the risk of oxidative damage. *L. iners* does not appear to share many of the protective mechanisms of other *Lactobacillus* species and therefore appears intermediate in its ability to prevent cervical disease, adapted from (Mitra *et al.*, 2016)

2.7.1. Microbiome in cervical cancers

Culture-based studies have shown that cervical cancers are frequently infected with enteric pathogens, such as *E. coli, Enterococcus and Enterobacter* (Mubangizi, Namusoke and Mutyaba, 2014). The composition of the vaginal microbiome has been shown to correlate with changes in HPV status, suggesting that the microbiome may play a role in HPV persistence (Chase *et al.*, 2015). However, there are scarce published studies reporting on the microbiome of cervical cancers as compared to dysplasia or correlation of microbiome with cervical cancer response to radiation therapy.

Since only a small proportion of women infected with hr-HPVs develop CIN 2+, the precursor of invasive cervical cancer, it is important to identify the factors that may alter the natural history of hr-HPV infection. As mentioned in the above sections, there are likely other factors within the local microenvironment that contribute to cervical carcinogenesis (Łaniewski *et al.*, 2018). An abnormal vaginal microbiota has been associated with the acquisition of HPV infection and both, the vaginal microbial communities and the cytokine profile may play a role in promoting cervical dysplasia (Gao *et al.*, 2013). Therefore, additional factors act in conjunction with HPV to influence the risk of cervical cancer development.

Studies have demonstrated that microbiota influence cancer susceptibility and progression by modulating inflammation, inducing oxidative stress, and promoting genomic instability of the host cells (Mangerich *et al.*, 2012). However, few studies have been carried out on the cervical microbiome as a modifier of the HPV natural history, particularly with respect to the development of cervical lesions and cervical neoplasm (Smith *et al.*, 2012). Nowadays many gaps remain in the knowledge regarding the association between vaginal and cervical microbiomes and cervical cancer development (Chase *et al.*, 2015).

As described by Łaniewski and his colleagues in 2018, increasing severity of cervical neoplasm is associated with decreasing relative abundance of *Lactobacillus* species and increasing abundance of a variety of microaerophiles and anaerobes. Rates of *Lactobacillus*-dominant (LD) VMB (defined as ≥80% relative abundance of *Lactobacillus species*) were significantly decreased, whereas dysbiotic non-*Lactobacillus*-dominant (NLD) VMB was increased in low-grade cervical

dysplasia (LGD) and invasive cervical carcinoma when compared to controls, both HPV positive and HPV negative (Łaniewski *et al.*, 2018). Therefore, Lactobacillus species is highly abundant in the cervix of women without alterations in Papanicolaou; with *Lactobacillus crispatus* and *Lactobacillus iners* as the most abundant species in HPV-negative and HPV-positive women without lesions, respectively (Ravel *et al.*, 2011; Chase *et al.*, 2015).

A growing body of evidence has shown that SIL disease progression is associated with increased vaginal microbiome diversity (A Mitra *et al.*, 2015). The diversity and composition of the cervix microbiota have been found extremely different in each stage of the natural history of cervical cancer. Thus, microbiota diversity is higher in cervical cancer cases than in the non-cervical lesions (Audirac-Chalifour *et al.*, 2016; Chen *et al.*, 2020; Sims *et al.*, 2020; Wu *et al.*, 2021). In one study that identified the differential taxa, it was found that *Sneathia species* were significantly enriched, whereas *Lactobacillus species* were underrepresented in invasive cervical cancer, as well as, in the precancerous groups (Love, Huber and Anders, 2014).

In other similar studies, other BV-associated bacteria, *Atopobium* and *Parvimonas* were significantly enriched in both precancerous groups (the low grade and high grade), whereas other BV-associated bacteria (e.g., *Gardnerella*, *Prevotella*, *Megasphaera*, and *Shuttleworthia*) were enriched only in the high-grade lesion and *L. iners* are enriched in HPV+, low grade and high-grade groups. Therefore, it is indicated that BV-associated bacteria might play a role in cervical carcinogenesis (Łaniewski *et al.*, 2018).

Research questions

In this PhD research project, the following research questions were addressed:

1. Is the acceptance and quality of samples collected with self-sampling in rural Ethiopia the same with other countries?
2. Are the HPV genotypes circulating at a community level in women from Butajira the same with other community level genotypes?
3. Is the community level age specific prevalence of hr-HPV in Butajira the same with elsewhere?
4. Is the persistence and clearance of hr-HPV infection influenced by the specific HPV genotypes?
5. Is the composition and diversity of cervical microbiome varying across different groups (cervical cancer patients, women with cervical dysplasia and normal cervix)?

3. Objectives

3.1. General Objective

To determine the molecular epidemiology of circulating HPV genotypes, their persistence, clearance, and re-infection rates at population level in South Central Ethiopia. Furthermore, we characterized the cervicovaginal microbiota in women with premalignant dysplasia or invasive cervical cancer compared with that of healthy women from Butajira and Addis Ababa.

3.2. Specific Objectives

1. To determine the population level HPV prevalence in Butajira, South Central Ethiopia
2. To determine the circulating HPV genotypes at population level in Butajira, South Central Ethiopia.
3. To determine the persistence, clearance, and re-infection rates of hr-HPV infections after 6 and 24 months of baseline screening
4. To determine the prevalence of sexually transmitted infections from hr-HPV positive women
5. To determine the extent of abnormal Pap cytology from hr-HPV infections in Butajira
6. To characterize the cervicovaginal microbiota in women with premalignant dysplasia, invasive cervical cancer compared with that of healthy women.
7. To evaluate the performance of three HPV genotyping assays as a primary screening method of cervical cancer in Ethiopian context

4. Materials and Methods

This PhD study was a combination of two interrelated studies and involved cross-sectional and prospective cohort study designs. The study was conducted from October 2017 to February 2020 and involved two cohorts:

4.1. HPV genotype distribution, persistence, clearance, and re-infection rates: A community- based follow up study.

4.1.1. Population

For this cohort, women aged 30- 49 was recruited from Butajira, south-central Ethiopia in the Gurage Zone of the Southern Nations, Nationalities and Peoples' Region of Ethiopia. The target population for this study is around 16.2% of the population. The study is conduct in the Health and Demographic Surveillance Site (HDSS) of Addis Ababa University.

4.1.2. Study setting

This study was conducted in the Health and Demographic Surveillance Site (HDSS) of Addis Ababa University, which is in Butajira district. Conducting this kind of study in HDSS gives the possibility to easily identify the required population group as it has updated defined population with basic demographic backgrounds. It provides also additional advantage for population-based follow up, thus, to estimate the proportion of women reached with the program.

Butajira is situated in the south-central Ethiopia in Gurage Zone and is located 135 km from the capital Addis Ababa. The district has an estimated population of 175,682. The HDSS covers a sample within the district, following ten kebeles/villages initially sampled from the entire district using a probability proportional to size technique. Currently, the HDSS covers about 75,000 individuals. Nine of the ten kebeles are rural while one is an urban kebele located in Butajira town. In the HDSS each household is identified by unique number within its village, and everyone within their household.

4.1.3. Sample size calculation

Since this PhD study was part of a two arms (VIA and HPV+VIA) cluster-randomized trial, the total number of ten kebele's were randomly classified into Health posts will be used as a cluster for randomization. A gain from 60% adherence to the standard procedure Arm A to 70% with Arm B is considered clinically relevant and realistic. Sample size has been calculated based on the two-sided Chi-Square-test (α=0.05) with a Bonferroni-Holm-correction to compare two independent proportions with equal sample size per group. To detect a difference proportion of 10% between procedure 2 and control group with a power of 80% we estimate a necessary sample size of 84 women per cluster (together 756 women in nine clusters). To allow a correction for the design effect in the planned cluster-randomized trial we inflated the number of patients using an intra-cluster correlation coefficient of 0.01 as recommended for subunit analysis in general practice process outcomes as adherence. 420 women will be recruited in each nine health posts and one health center and a total of 3780 will be included. A total of 20 clusters will be developed using the week of data collection in the HDSS. Sample size calculations were carried out based on the formula by n Query Advisor 4.0

4.1.4. Inclusion criteria

Women eligible for inclusion in this study were those who reside permanently in the selected study area and those who were willing to participate in the study by providing oral informed consent.

4.1.5. Exclusion Criteria

Pregnant women and women at menstruation during the sample collection period were excluded from sample collection and invited for another sampling time. In addition, women who had history of hysterectomy, chemotherapy and radiotherapy, women who had cognitive or physical impairment that prevent them to give informed consent/participation were also excluded.

4.1.6. Strategy

There was an HPV screening cluster-randomized trial which is registered in clinical trial.gov (NCT03281135) with two arms (VIA and HPV arm). In this trial, the total number of ten kebele's in the Butajira HDSS were randomly classified in to two arms (VIA and HPV+VIA). Health posts were used as a cluster for randomization. Community sensitization and awareness creation was performed at health facilities and in communities at social, business, or religious gatherings, about cervical cancer and its prevention for eligible women in both arms.

This specific PhD study was focused on the one arm (HPV+VIA). Women were instructed to undergo HPV self-sampling at nearby health posts using Evalyn Brush®. Self-collected brushes were sent to the Molecular biology laboratory in the Department of Microbiology, Immunology and parasitology, College of Health Sciences, Addis Ababa University, Addis Ababa, where the laboratory service is available for the HPV DNA testing. Only women with samples positive for hr-HPV were contacted and informed to go to the Hospital offering VIA. In Butajira hospital offering VIA, samples (Thip prep, Evalyn Brush self-sample, Pap smear) were taken for further characterization of the hr-HPV positive women. An aliquot of the samples was sent to collaborator's HPV laboratory, Charité, Berlin, Germany for HPV genotyping, HPV assays performance evaluation and quality control.

The women found positive for hr-HPV were invited for VIA and then received Cryotherapy at Butajira Hospital if they were found positive. But before treatment, Self-collected Evalyn Brushes, Thin prep and cervical swab using PapCone® (Otto Bock, Germany) was taken for HPV genotyping, STI testing and Pap smear examination. To evaluate the persistence, clearance and reinfection rates, all hr-HPV positives women were invited after 24-months and asked for a self-sampled brush for HPV-DNA testing.

4.1.7. Sample collection device and training of health workers

Community sensitization and awareness creation was performed at health facilities and in communities at social gatherings, about cervical cancer, and its prevention for eligible women in the selected districts. Community health workers, in our case Health Extension Workers (HEWs)

and Health Development Armies (HDAs) were pre-trained about cervical cancer and cervical cancer prevention and the sample collection. Cervicovaginal samples were taken by self-collection using the Evalyn Brush® device (Rovers Medical Devices, Oss, The Netherlands). Women were assisted by trained HEWs. Training consisted of local language translated video and a step-by-step demonstration of the self-collection device.

Evalyn Brush® is a user-friendly and easy to use cervicovaginal self-sampling device. It has a standard depth of insertion and number of rotations after insertion. The depth of insertion is controlled by the wings and needs to be rotated five times, and at each rotation, there is an audible click indicating the number of rotations (**Figure 4.1**).

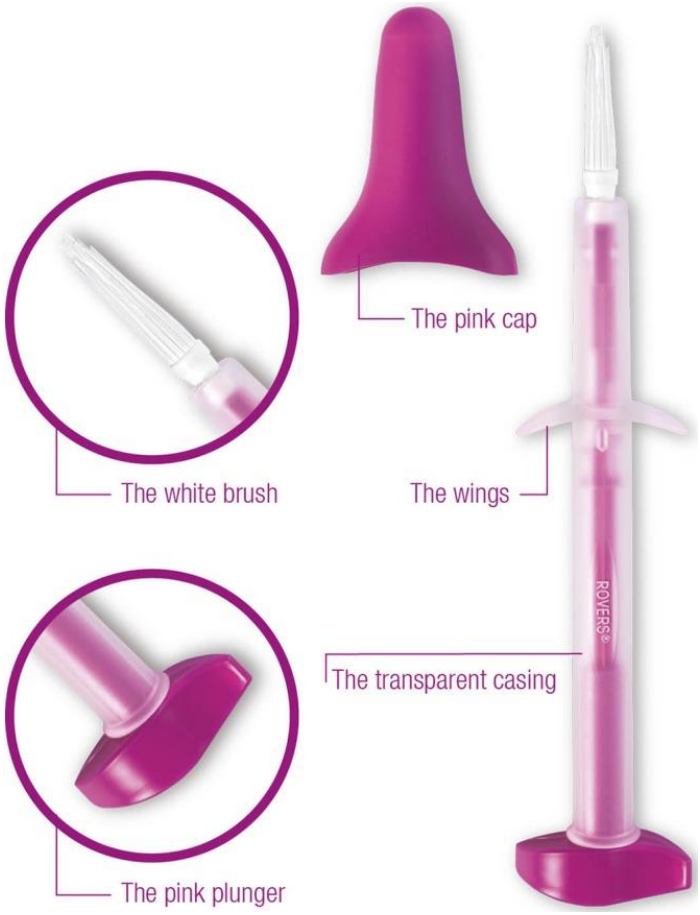


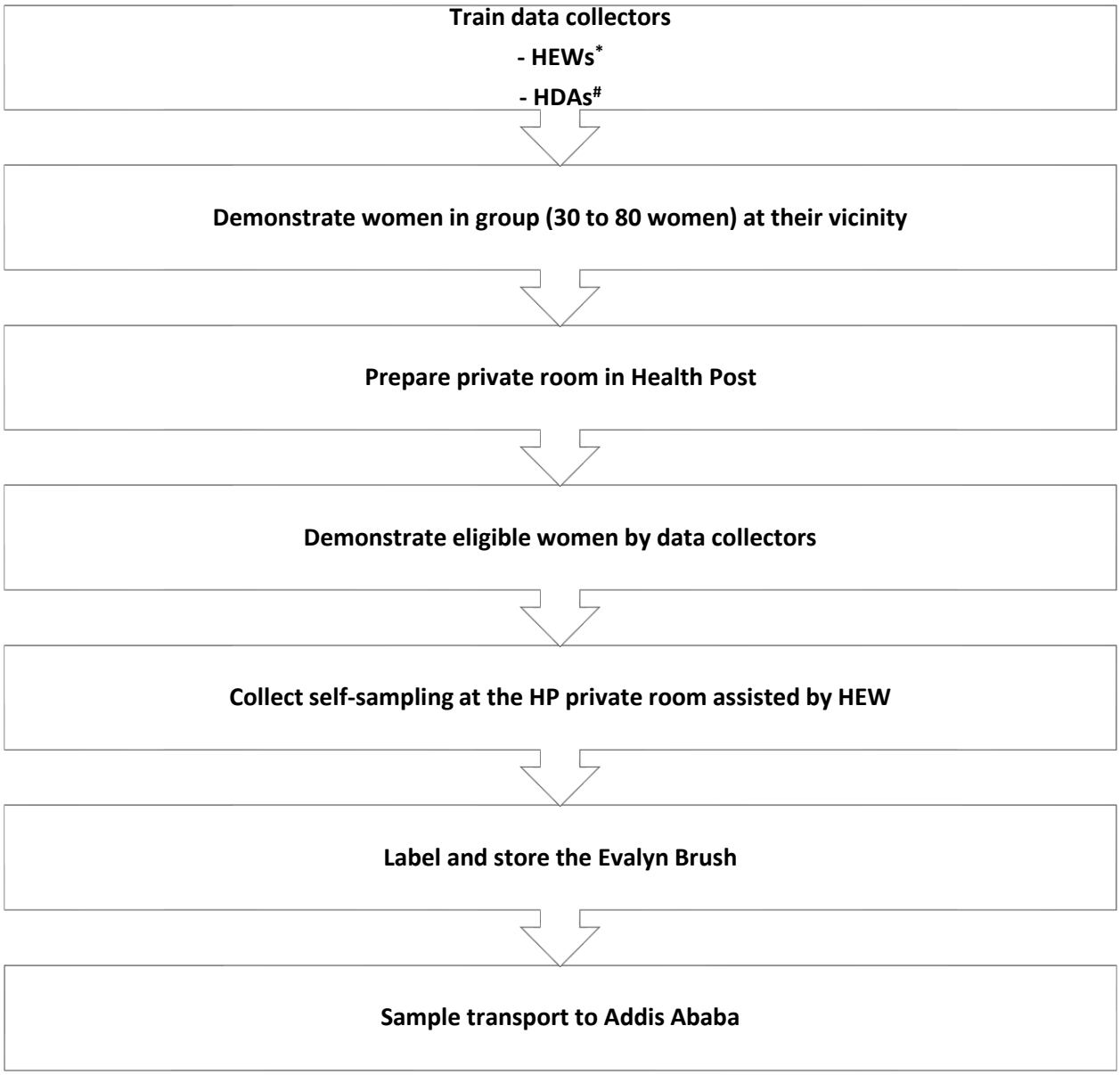
Figure 4.1: Components of The Evalyn Brush. It is about 20 cm in length consisting transparent case with wings. There is a pink stick inside the casing, and it has a pink plunger at the end and a white brush at the other end. The white brush can be pushed out of the casing and pulled back into the case using the pink plunger.

Since self- sampling devices including the Evalyn Brush® are new in their kind in our country and used for the first time in this study, a practical training on how to collect sample using the device and the advantage of the self-collection on cervical cancer screening was given to the health extension workers and health development armies (**Figure 4.2**). A local language (*Amharic*) translated video and a step- by-step demonstration on the self-collection device was used for the training. During and at the end of the training, a pilot was done on the self-sampling using the Evalyn Brush and 11 out of the 20 trainee gave samples after the training. The 9 trainees were excluded because of exclusion criteria. All the Health extension workers said that the collection device is user-friendly and easy to use. The acceptance of self-sampling in the rural Ethiopian women in our study area and the factors that might impede acceptance in the context of cultural tradition, religion and environment of our study were also explored and published (Muluken Gizaw *et al.*, 2019; Gizaw *et al.*, 2020). Regarding the sample quality, the 11 samples collected during the training gives us a clue that good quality samples can be collected in the Ethiopian context because all the collected samples were with enough cellularity for HPV DNA testing as measured by internal control values for ß-globin PCR.

Figure 4.2: Training of self-sampling using Evalyn Brush (Rovers, The Netherlands) for rural community health workers and eligible women.

4.1.8. Collection procedure

During the community screening campaign, women living in the pre-defined areas (Kebeles, lowest administrative unit) were sensitized and invited to undergo HPV self-sampling at nearby health posts with the Evalyn Brush®. At the health post, an explanation was given by the pre-trained HEW on how to collect the cervical self-sample. Then, the Evalyn brush was given to each eligible and willing woman and self-collection was done in a pre-set private room under supervision at the local health post. The rooms in each health post were selected in advance to ensure privacy, cleanliness and supporting facilities for the self-specimen collection. The overall procedure of the sample collection till transportation is summarized in **Figure 4.3.**



* Health extension workers
# Health Development Armies
HP: Health post

Figure 4.3: Summary of sample collection flowchart

4.1.9. Sample storage and transportation

After self-collection, a six- digit sample identification (ID) prepared in sticker was given for each sample, placed in plastic bag, and stored in dry and safe place in the health post until transported to Addis Ababa. The collected and packed sample was transported once a week from Butajira to the Molecular Biology Laboratory of Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University, to process the sample and DNA extraction according to the pre-defined protocol.

4.1.10. Sample processing and HPV DNA extraction

On day one, the Evalyn brush was removed from the plastic bag, the tip pulled off and put into a pre-labelled 2 ml Eppendorf tube and soaked in 1 ml PBS. Gloves were changed between each sample to prevent cross-contamination. The tubes with brushes were vortexed vigorously for 1min and left at room temperature overnight to wash the cells out from the dry Evalyn brush. The next day, after centrifugation for 5 min at 2500 rpm and vortexing vigorously for 1 min, an aliquot of 100 µl of the fluid was taken in 1.5 ml Eppendorf tube. The 100 µl fluid was used to produce crude DNA lysates using bacteria lysis buffer method (AID/GenID GmbH, Strassberg, Germany). To briefly describe the protocol for the crude DNA lysates using bacteria lysis buffer, 200µl of 5x BLP lysis buffer was added into each sample and incubated in Thermal Mixer at 65°C for 10min, 600rpm after short vortex. Then, lysis was stopped by adding 200µl of 5x Neutralizing Buffer.

Prior validation of this crude DNA lysates using bacteria lysis buffer method was done to compare crude DNA lysates and the DNA extracted using Maxwell 16 LEV Blood Kit (Promega GmbH, Mannheim, Germany) at Charité-Universitätsmedizin Berlin, Germany. The validation results showed that the crude DNA lysates have enough concentration and purity of DNA for the intended PCR use. Furthermore, for every batch of samples, a quality control (10% of the samples) for the DNA extraction was also done in Charité-Universitätsmedizin Berlin, Germany using Maxwell 16 LEV Blood Kit and the results were comparable with the crude DNA lysates. All the sample processing and isolation of DNA lysate were performed in the molecular biology laboratory of Department of Microbiology, Immunology and Parasitology School of Medicine, Addis Ababa University, Ethiopia. The DNA lysate was stored at -20^{o}C until shipment and further analyses.

4.1.11. HPV genotyping

For the HPV genotyping, a 20 µl aliquot of the DNA lysate was shipped to the collaborators Laboratory for Gynaecologic Tumour Immunology at Charité-Universitätsmedizin Berlin, Germany. HPV presence and genotype was determined using the L1 primer system BSGP5+/6+ PCR with MPG-Luminex Assay read out (Schmitt *et al.*, 2008). This assay detects 18 high risk genotypes (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 9 low risk genotypes (HPV6, 11, 42, 43, 54, 57, 70, 72 and 90).

4.1.11.1. Polymerase Chan Reaction (PCR)

The PCR and Luminex typing systems were performed following the methodology developed by Schmitt *et al* (2008). To briefly mention the protocol, broad spectrum GP5+/6+ (GP5+: 5'-TTT GTT ACT GTG GTA GAT ACT AC-3' and GP6+-5'-GAA AAA TAA ATT GTA AAT CAT ATT C-3') primers were used to amplify 150 nucleotide long conserved target gene flanking a highly variable type of specific sequence in the L1 ORF (Schmitt *et al.*, 2008).

The final PCR reaction mix was 25µL with 20 µL master mix and 5 µL DNA templates. The master mix was prepared from 25mMKCl, 0.4g/L Nonidet p40, 5mM Tris HCl (pH 8.8) (10xPCR buffer; MBI Fermentas GmbH, St. Leon Roth Germany), 100µM of deoxyribonucleoside triphosphate, 1.75mM $MgCl_2$ (Biozyme Scientific GmbH, Germany), 0.5U of DNA AmpliTaq polymerase (Roche Applied Biossystems, Germany) and 250nM each of the GP5+ and 5'-biotinylated GP6+ primers. The ß-globin-GP5+/6+ PCR, 50nM each of the ß-globin primers MS_3 and 5' biotinylated MS_{10} were added. Then it was stored at -20^{o}C for further use.

Then, the PCR mix was composed of 20µL PCR master mix and 5µL DNA sample and a negative control (NK) was loaded and carefully mixed. Then in the thermo cycler room a positive control (PK) was added to each plate and the PCR tubes were transferred into PCR rack. PCR conditions were as described elsewhere (Schmitt *et al.*, 2008). Briefly, the cycle time was 4-min denaturing step at 94^{o}C followed by 40cycles. Each cycle was composed of 94^{o}C denaturation for 20sec, annealing at 38^{o}C for 30sec and elongation at 71^{o}C for 80sec. The final elongation time was 4 minutes. Finally, the PCR products were stored (at -20^{o}C) until further analysis.

4.1.11.2. Hybridization and HPV genotyping

To detect HPV genotypes, the PCR products were denatured, and hybridized with bead-coupled probes in 96 well plate. Briefly in a hybridization plate (Millipore) 10 µL PCR product and 34.4µL bead mix were mixed. Then, it was tightly closed using sticky foil (HJ-Bioanalytic GmbH, Germany) and incubated at 95^{o}C for 10 minutes to denature the PCR products and hybridized with the bead-coupled mix. The plate was placed in a cooling rack (-20^{o}C) (Eppendorf) for a minute, then incubated at 41^{o}C, 650RPM for 35 minutes.

In the meantime, a filter plate filled with 100µL/well PBS was incubated for 15 minutes on vacuum pump (Millipore). Besides, stain buffer was prepared from 8µL stain buffer and 5 µL Streptavidin-PE (Invitrogen) (1:1600) in a 15mL tube and covered with Aluminium foil. The filter plate was sucked using vacuum pump to remove all the liquid, PBS. Then the sticky foil was removed carefully from the hybridization plate and the hybridized DNA-bead mix was transferred into the filter plate carefully. Then, the filter plate was pumped to remove the liquid, washed once using 100µL PBS/well and tapped on green papers to remove all the liquid.

Then, 75µL/well stain buffer (streptavidin-R-phycoerythrin conjugate) was added, covered with aluminium foil, and incubated at 650 RPM shaker for 30minutes. Then, the plate was pumped to remove the liquid and washed 3 times using 100µL/well PBS each time. Finally, the beads were re-suspended in 100µL/well PBS for Luminex (Luminex corp.) read-out.

The Luminex xMAP200 (Bio-Rad) instrument was used to read the signals using software, Bio-plex manager 6.1 (Bio-Rad). The result was expressed as the median fluorescence intensity (MFI) of at least 50 beads per set. Results were displayed in an Excel spread sheet with the HPV types.

4.1.12. Result reporting and triage of hr-HPV DNA positive women

A result notification format was prepared, and all the HPV negative women were notified at the nearby health post though the HEWs and HDAs with full information what this negative result meant in the context of cervical cancer screening. However, for those women who were hr-HPV positives, their result was notified by inviting them to come to the health post and discuss on the subsequent actions to be taken. During result notification, all hr-HPV DNA positive women were invited to visit Butajira Hospital for visual inspection using acetic acid (VIA) and cryotherapy treatment according to the national screening algorithm.

Briefly, the HPV DNA test result was notified as follows:

- "*Your test result based on the self-sample you provided earlier can be positive or negative. Furthermore, you may also need to repeat sampling.*
    - ***Positive:*** *A positive test for your sample means you* ***do*** *have a virus (called HPV) type that may be linked to cervical cancer. This does not mean you have cervical cancer right now. But it could be a warning. But this result indicates that there is a need for follow-up monitoring, further testing, or treatment of abnormal or precancerous cells (if there is any). Therefore, you need to have further testing, we already arranged for you in Butajira Hospital. I want to assure you again that until your result is further confirmed by the other test, there is no problem to be worried about.*
    - ***Negative:*** *A negative HPV test means you* ***do not*** *have an HPV type that is linked to cervical cancer. However, you are advised to undergo another screening after five years using this method or other methods.*
    - ***Repeat sampling****: The cervical self-sample you proved before did not show any result. This might be due to wrong sampling procedure. Therefore, you need to re-sample for repeat testing".*

4.1.13. Follow-up testing

The eligible women for the follow up study were those whose HPV DNA testing were hr-HPV positives and women whose HPV DNA testing were negative at baseline screening but randomly selected as controls. To determine the persistence, clearance, and re-infection rates, all women who were found to be hr-HPV positives at baseline were invited to attend two additional follow-up visits for HPV DNA testing at 6 and 24 months. At 6 months, all the hr-HPV-positive women were contacted by the local healthcare providers (health extension workers) through their house number and /or phone number and invited to a local district hospital at the Butajira city. Once at the hospital, a physician collected sample was taken for HPV testing.

The hr-HPV positive women also undertook a gynaecological examination using visual inspection with acetic acid (VIA) after the sample collection for HPV DNA re-testing. When VIA test result is positive by examining nurse and/or medical doctor Cryotherapy was given to the women according to the national guideline. The gynaecological exams including VIA and the physician-based samples were performed by experienced gynaecologists. During the 6^{th} month follow up visit, additional sample was taken for cytological examination using Pap smear.

Similarly, the 24 months follow up was performed by inviting all the baseline hr-HPV positive women to nearest health posts and a self-sampled specimen was collected for HPV DNA testing. For the sake of sensibly detecting precancerous lesions, all women who were found to have hr-HPV infections (type specific persistent and re-infected with new hr- HPV) were invited for colposcopy at Addis Ababa (about 150 Kms from the study area). Furthermore, at the 24 months follow up, approximately 13% of randomly selected from the hr-HPV negative women during the baseline screening were invited for HPV DNA testing as controls to compare the genotype specific hr-HPV re-infection rates with the baseline hr-HPV positives. In this study, those women who were found to have new infections in the control group will be retested 24 months after their test.

Generally, the follow-up contents at both visits included HPV genotype detection, cervical examination using VIA, cytology, colposcopy, and histological examination (if indicated). The outcomes of follow-up included changes of hr-HPV infection, clearance of hr- HPV infection, re-

infection by new hr-HPV type and cytological and histological changes. The overall flowchart of the follow-up study is shown in **Figure 4.4.**

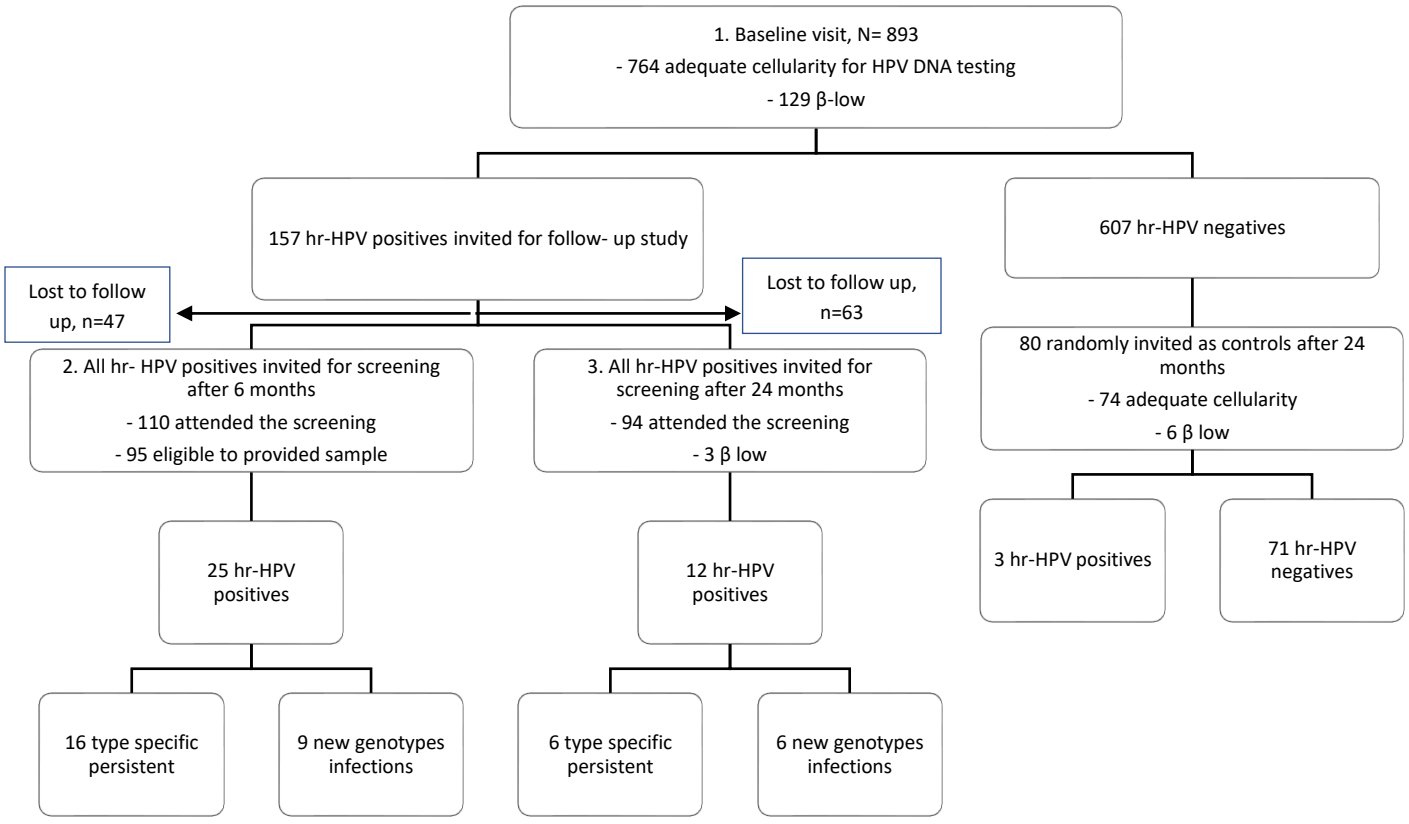


Figure 4.4: The follow-up study flow chart

4.1.14. Defining HPV persistence, clearance, and re-infection

In our study, persistence, clearance, and re-infections of HPV were referred to both any hr-HPV type and type-specific infections. The total hr-HPV persistence was defined as any hr-HPV positivity at the follow up visits which was hr-HPV positive at the baseline testing regardless of genotype similarity. Similarly, the total hr-HPV clearance was defined as if a women turned negative at the follow up visits for any hr-HPV who was positive at the baseline for any hr-HPV.

Type specific persistence was defined as the same HPV type being detected both at baseline and follow-up using the same HPV DNA detection method. Similarly, type specific clearance was defined as the proportion of women who were initially hr-HPV positive (first collection), but the same hr-HPV type was not found at the follow-up. That is the change from specific hr-HPV genotype positive to negative was considered as the clearance of hr-HPV. Re-infection was defined in this study as new hr-HPV genotype infection (other than the HPV genotype detected at baseline) after clearance from the base line infecting type.

4.1.15. Testing for sexually transmitted infections

In this study, all hr-HPV positives (in the first phase screening) were tested for other STIs. EURO Array STI – 11 (DNA microarray) (EUROIMMUN AG, Luebeck, Germany) was used to profile the STI pathogens. The assay enables simultaneous detection of the following eleven sexually transmitted pathogens in one test procedure.

- Chlamydia trachomatis
- Neisseria gonorrhoeae
- Herpes simplex virus 1
- Herpes simplex virus 2
- Haemophilus ducreyi
- Mycoplasma genitalium
- Mycoplasma hominis
- Treponema pallidum
- Trichomonas vaginalis
- Ureaplasma parvum
- Ureaplasma urealyticum

The EURO Array STI – 11 assays are automated microarray-based PCR system based on the amplification and fluorescently labelling of defined gene sections of the pathogens. The PCR products are then hybridized to BIOCHIP microarray slides containing immobilized complementary DNA probes and detected by their fluorescence signals. The evaluation, interpretation, and archiving of results is fully automated by EUROArrayScan software and is thus highly standardized and objective (**Figure 4.5).**

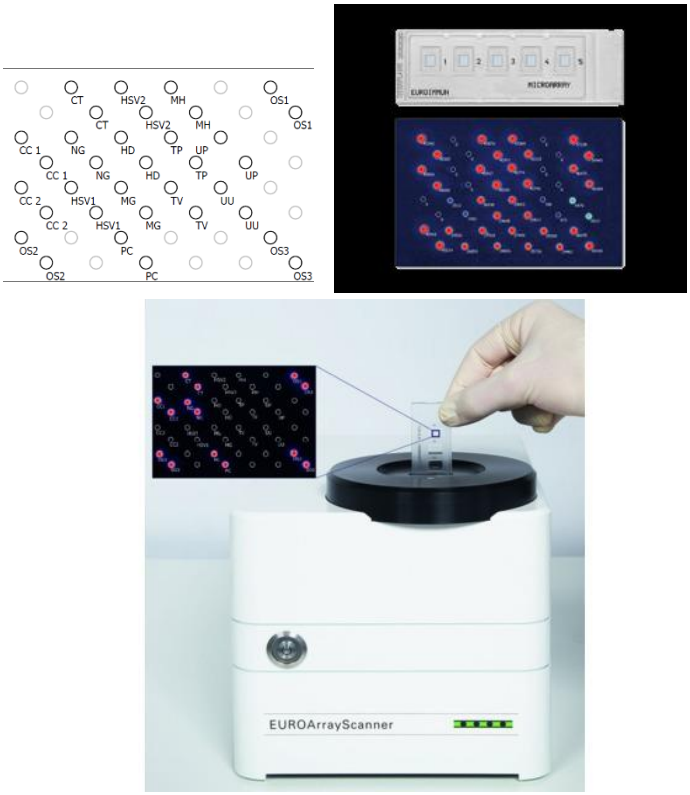


Figure 4.5: Automated EUROArrayScan PCR system

4.1.16. Cytology

In our follow up study, the Pap-smear test was performed from the PapCone® (Otto Bock, Germany) by an experienced pathologist at St. Paul Hospital pathology laboratory, Ethiopia to determine the stage of cervical cell abnormality based on the 2014 Bethesda System for reporting Cervical Cytology (Nayar R, 2015).

4.1.17. Visual inspection of cervix using acetic acid (VIA)

Visual inspection with acetic acid (VIA) was performed by Gynaecologists, and trained midwives following the standard protocol. VIA involves inserting a vaginal speculum and swabbing the cervix with 3% to 5% acetic acid solution before doing a cervical inspection. Application of acetic acid on cervical epithelium causes reversible intracellular dehydration and coagulation of the protein within the cervical cells and intensity of coagulation is dependent on amount of protein in the cell. As the dysplastic cells have more chromatin content, the coagulation is intense, and cells turn white after application of acetic acid. Butajira hospital has currently established the screen and treat service using VIA and cryotherapy with the help of partner organization, the Pathfinder International, and our collaborative projects.

4.1.18. Laboratory Quality control

Self-collected sample was placed in a dry dust free environment. HPV DNA testing services were provided in a dedicated laboratory area or facility. All areas were clean, well- lit and well ventilated. The laboratory procedures were performed in Addis Ababa, Germany and the United States of America following manufacturer's instructions. Standard operating procedures (SOPs) were in place for handling, storing, and transporting the cervical samples. For the procedures done in Addis Ababa, at least 20 % of the samples were tested in Germany and compared for quality control purposes. In every new experiment during this PhD, validation tests were done prior to the research sample testing and important considerations were put in place and contextualized not to compromise the quality of testing.

4.2. Performance evaluation and technical establishment of HPV genotyping assay in Ethiopia/Addis Ababa University, Department of Microbiology, Immunology and Parasitology research laboratory

Based on the recent years' recommendations, the current worldwide recommendation for cervical cancer screening is hr-HPV DNA testing using cervical self-sampling brushes (World Health Organisation, 2021). We strongly believed that this method would have especial importance in our country's context to alleviate the social and cultural barriers in cervical cancer screening and ultimately will significantly increase the low coverage of cervical cancer screening in Ethiopia.

Several studies conducted elsewhere supported that HPV testing is feasible in resource limited countries and appears to be the best strategy for cervical cancer screening in this context (Kamath Mulki and Withers, 2021). Currently, from the 2020 inventory of commercial molecular HPV tests, 254 distinct commercial HPV tests were identified in the global market (Poljak *et al.*, 2020). So far, the greatest challenges of HPV testing were the need for expensive laboratory infrastructure, robustness of the assay and the time to process the test. However, the innovation of rapid molecular methods for detecting hr-HPV DNA for screening is easing the challenges.

The Ethiopian MoH plan for cervical cancer screening has been a nationwide visual inspection and cryotherapy of the cervix as per the WHO recommendation for resource limited countries. But to assure precision of VIA and monitoring of the causative agent (HPV), HPV DNA testing is mandatory. This can be done by taking a vaginal self-sample and determining HPV types which we aimed to make available in our laboratory in the Ethiopian context.

4.2.1. Sample collection and Assay comparison

In this PhD project, we evaluated and compared the analytical performance of three HPV genotyping assays using samples collected from Butajira: MPG-Luminex, Anyplex hr-HPV Detection and EUROArray HPV genotyping assays. For the comparison, 110 samples were used which were collected by health care workers and compared the results from the three genotyping HPV assays. Samples were collected using the Cervix-Brush® (Rovers®, Oss, The Netherlands). It is a soft and flexible brush which enables dual collection of ectocervical and endocervical

samples, so all necessary cells can be collected in one movement. The Rovers Cervex-Brush device was rinsed immediately into PreservCyt® Solution filled ThinPrep vials (Hologic, Marlborough, USA) by pushing it onto the bottom of the vial 10 times, forcing the bristles apart followed by swirling the brush vigorously to further release material. The device was visually inspected to ensure that no material remained attached before it was discarded. The ThinPrep vial was labelled and stored at room temperature until shipment to the collaborator Laboratory for Gynecologic Tumour Immunology at Charité-Universitätsmedizin Berlin, Clinic for Gynaecology, Germany for DNA extraction and HPV genotyping.

The MPG-Luminex Assay was used as a gold standard to evaluate the analytical performances of the two assays because Hybrid Capture-2 or GP5+/6+ PCR-enzyme immunoassay hr-HPV DNA assays were validated in large, randomized trials and cohorts with a follow-up duration of 8 years or more. At the end of this PhD study, we have established an HPV DNA testing lab in the Department of Microbiology, Immunology and Parasitology, Addis Ababa University, as a reference centre for the purpose of population-based screening, epidemiological research, and vaccination efficacy surveillance. The study helped us to select the test with good performance in our context of a LMIC both for screening and monitoring HPV vaccine efficacy. Furthermore, we have shown from our study that the complexity and degree of automation for all steps like the hands-on time, risk of contamination, and user-friendliness of the HPV assay were essential components to consider during validation and customization of assays in an LMIC context.

4.2.2. EUROArray HPV

The EUROArray HPV assay (EUROIMMUN, Luebeck, Germany**)** is designed for the detection and genotyping of 30 human anogenital hr- and lr-HPV genotypes (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 70, 72, 73, 81, 82, 89 (CP6108)) from DNA preparations of cervical smear samples. The assay was performed according to the manufacturers' instructions (Cornall *et al.*, 2016). This assay combines multiplex polymerase chain reaction (PCR) amplification with oligonucleotide probe microarray for detection and genotyping of HPV DNA. HPV oncogenes E6 and E7 specific sequences are amplified and fluorescently labelled by means of a polymerase chain reaction (PCR) using a multiplex primer system. Fluorescently labelled amplicons bind to genotype-specific probes arranged on a

microarray. The specific binding (hybridization) of a fluorescent PCR product to the corresponding oligonucleotide probe is detected using a special microarray scanner (EUROIMMUN). The EUROArray Scan software evaluates all spots, measures fluorescence signals, and generates the test results. A region of ubiquitous human Hsp90 gene serves as an endogenous control to verify DNA extraction and amplification adequacy. Moreover, it has a fully automated standardized evaluation, interpretation and archiving of the results through the integrated software.

4.2.3. Anyplex™ II HPV HR Detection

The Anyplex™ II HPV HR Detection (Seegene, Seoul, Korea) is a multiplex PCR assay with reporter detection designed for HPV genotyping. It can detect 14 hr-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). It is a fully automated real-time PCR system. The PCR amplification is done using the CFX96 real-time thermocycler system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

The Anyplex assay utilizes TOCE (tagging oligonucleotide cleavage and extension) technology. The components of the assay are a dual priming oligonucleotide primer (DPO), Pitcher (a tagging oligonucleotide), and Catcher (a fluorescently labelled artificial template with a sequence complementary to the tagging portion of Pitcher). The DPO and Pitcher hybridize specifically on opposite sides of the target sequence of the HPV nucleic acid. The tagging portion of Pitcher is released during the DPO primer extension with *Taq* polymerase, which enables its hybridization to the capturing portion of Catcher. When “Duplex Catcher” (the tagging portion of Pitcher and the complementary Catcher sequence) is fully extended, it separates the reporter molecule from the quencher molecule, which results in a fluorescent signal. As an internal control (IC), the human housekeeping gene (beta-globin) is co-amplified simultaneously with the L1 gene sequences of the targeted HPV types in order to monitor nucleic acid isolation and check for possible PCR inhibition. The test result is generated automatically using Anyplex software (Hesselink *et al.*, 2016).

4.3. Cervicovaginal Microbiota Profiles in Precancerous Lesions, Cervical Cancer, and healthy controls

4.3.1. Study area

For the cervicovaginal microbiota profiling, samples were collected from Tikur Anbessa Specialized Hospital (TASH), Gynaecology out-patient department (Gyn-OPD) and from rural community of Butajira, South Central Ethiopia. TASH is a large referral teaching hospital, under the administration of Addis Ababa University, located in Addis Ababa, Ethiopia. The Gynaecology Department of the Hospital provides evaluation, examination, treatment, and screening of new and referral cases of cervical cancer, among other services.

4.3.2. Study participants and clinical characteristics

From TASH, all new eligible women aged 18 years old and above who had cervical complaint at the gynaecological clinics from October 2019 to February 2020 were screened and those women histologically confirmed for cervical cancer and pre-cancerous lesions were included in this study. From Butajira, Women screened cytologically positive for precancerous lesions and healthy women during cytologic examination were included. Women with pregnancy, who had history of hysterectomy, chemotherapy, and radiotherapy and who had cognitive or physical impairment that prevent them to give informed consent/participation were excluded from the study.

This comparative study comprised 120 women with three groups (60 cervical cancer patients who had not received any treatment, 25 women with dysplasia and 35 control women who were cytologically or histologically confirmed negative for cervical dysplasia).

4.3.3. Sample Collection and DNA extraction

The specimens for this cohort were collected using two devices: The Isohelix^{TM} DNA Buccal swab and the Evalyn Brush (Rovers Medical Devices, Oss, The Netherlands). The Isohelix cervical swabs were obtained by swabbing the surface of the cervix and placed into stabilization buffer then frozen within 30 minutes. Once suspended in stabilization buffer, DNA is stable for 2 weeks

at room temperature, a year at 4 degrees and longer when frozen. The collection and processing of the Evalyn Brush is as mentioned in cohort one above. DNA extraction then was carried out using the Qiagen DNA Extraction kit (QIAGEN, Germany). Both the Isohelix and Evalyn Brush Samples were shipped to the MDACC, US on dry ice for DNA extraction and 16S rRNA sequencing.

4.3.4. 16S rRNA Gene Sequencing and Sequence Data Processing

16S rRNA gene sequencing was performed by the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine. Sequencing was performed based on methods adapted from the Human Microbiome Project (Methé *et al.*, 2012). DNA was extracted using the MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories). The 16S V4 region is the most conserved and variable segment of the genome, which makes it a good target for phylogenetic analyses. The region was amplified by PCR using a 515F-806R primer pair. Sequencing was performed on the Illumina MiSeq platform using the 2x250bp paired-end protocol yielding pair-end reads.

16S rRNA sequence reads were processed using the QIIME2 microbiome bioinformatics platform (v2020.11) (Bolyen *et al.*, 2019). FASTQ sequences were imported and demultiplexed as QIIME2 artifacts. Denoising was performed using DADA2 with trim parameters set at 20-245 for forward strands and 8-230 for reverse strands. Trim parameters were set based on quality score plots generated on QIIME2 (Nearing *et al.*, 2018). Representative sequences were generated using DADA2 for phylogenetic tree construction and taxonomic classification. A pre-trained Naïve Bayes classifier was used for phylogenetic reference construction via the q2-feature-classifier plugin. We used a taxonomic classifier trained on the SILVA 138 database 515F/806R region of sequences trimmed to include 250 bases from the 16S V4 region (Pruesse *et al.*, 2007).

4.4. Data Management and Statistical analyses

Data were checked, validated, and entered IBM SPSS statistics v26 database system. A back up data was stored in a separate hard disc with similar folder and sub folder names. After validation, the data was cleaned and recoded. Descriptive statistics were used to analyse demographic and clinical characteristics of study participants and to compare results. Prevalence of HPV infection and individual genotype distribution were presented using bar and line graphs.

For the age-specific HPV prevalence, women participated in the study were stratified by age within 4 groups (30–34 years, 35–39 years, 40–44 years, and 45–49 years) and we reported HPV infections and age specific HPV prevalence. For the HPV-type-specific prevalence, the frequency of each hr-HPV and lr-HPV genotype was presented in hr-HPV positive samples and lr-HPV positive samples respectively.

For HPV assay comparisons, the agreement of genotype results from three HPV genotyping assays was evaluated using the Fleiss' kappa (κ) statistics. Agreement between tests was assessed according to κ –values, where values in the range 0.81–1.00 indicate almost perfect agreement, 0.61–0.80 substantial, 0.41–0.60 moderate, 0.21–0.40 fair, 0.00–0.20 slight, and <0.00 poor agreement. (Landis and Koch, 1977) Sensitivity and specificity were calculated using conventional contingency tables.

For the microbiome analysis, the rarefaction depth was set at 3248 reads. Alpha (within sample) diversity was examined using the Shannon diversity index, and beta (between sample) diversity was examined using UniFrac (weighted and unweighted) and Bray-Curtis distances in QIIME. The relative abundance of microbial taxa and genera were compared between samples; we then determined differentially abundant bacterial genera by case status using linear discriminant analysis effect size (Segata *et al.*, 2011), applying the 1-against-all strategy with a threshold of 4 on the logarithmic linear discriminant analysis score for discriminative features and an α of 0.05 for the factorial Kruskal-Wallis test among classes. linear discriminant analysis effect size was restricted to bacteria that were present in 20% or more of the study population. Observed differences were subjected to paired analysis using two sample Z test for proportions, or Student t test where appropriate. Statistical significance was considered at $P < 0.05$.

4.5. Ethical considerations

Ethical approval was obtained from the Institutional Review Board of the College of Health Sciences, Addis Ababa University (079/20/DMIP) and Martin Luther University, Halle Germany (2017-143). In addition, this study was also approved by the National Research Ethics Review Committee (NRERC) (SHE/RAAA/9.1/339/19/11) and a material transfer agreement was signed by both institutions to transfer samples to Germany and US. An official letter was taken from the University to the respective district health department to obtain permission. Informed consent was obtained from all study participants. The participants were fully informed of the objectives of the study, the potential harms and benefits of the study and requested for informed consent. Well trained health professionals were screen volunteer eligible women. The result was communicated only for the client. During sample collection, the sterile brush was given for volunteer women and samples were collected at the place where privacy is assured. The VIA was done in sterile, and privacy assured environment.

Women who found positive in the screening were linked for appropriate treatment and follow-up in Butajira Hospital and Tikur Anibessa Hospital, Addis Ababa if further investigation and treatment is required with their unique identifiers to insure their confidentiality. Moreover, any participant related data was collected in a way to ensure confidentiality of the respondent.

5. RESULTS

5.1. Population-based HPV infection and genotype distribution among women in rural areas of South-Central Ethiopia

5.1.1. Participant and sampling characteristics

In this population-based screening, 1020 women aged 30-49 years (mean=33 years) were attended HPV based screening after sensitization program at rural health posts. Out of the community sensitization attended women, 893 (87.5%) provided cervical self-sampled specimen and tested for HPV DNA. The rest 127 women (12.5%) were excluded due to pregnancy, refusal to participate, and being in active menses during sample collection time (**Figure 5.1**).

Among the 893 samples tested for HPV DNA, 717 (80.3%) had adequate cellularity to detect HPV in the sample while the remaining 176 (19.7%) were found with inadequate cellularity to detect HPV in the sample due to low internal control values for ß-globin PCR. The adequacy of the sample cellularity was measured by β-globin intensity in the Luminex xMAP200 readout. All the 176 women with low (inadequate) β-globin samples were invited for re-sampling. Only 62 (35.2%) of the women gave repeat self-sampling while 114 (64.8%) of the women refuse for repeat sampling. Of the repeat samples, 47 (75.8%) had enough cellularity for HPV DNA detection and included in the overall HPV analysis. However, 15 (24.2%) of the re-sampled were again with low β- globin. Overall, 764 (717 from the first phase self-sampling and 47 from the repeat sampling) were found to have sufficient β-globin positivity for HPV analysis and were included in this analysis (**Figure 5.1**).

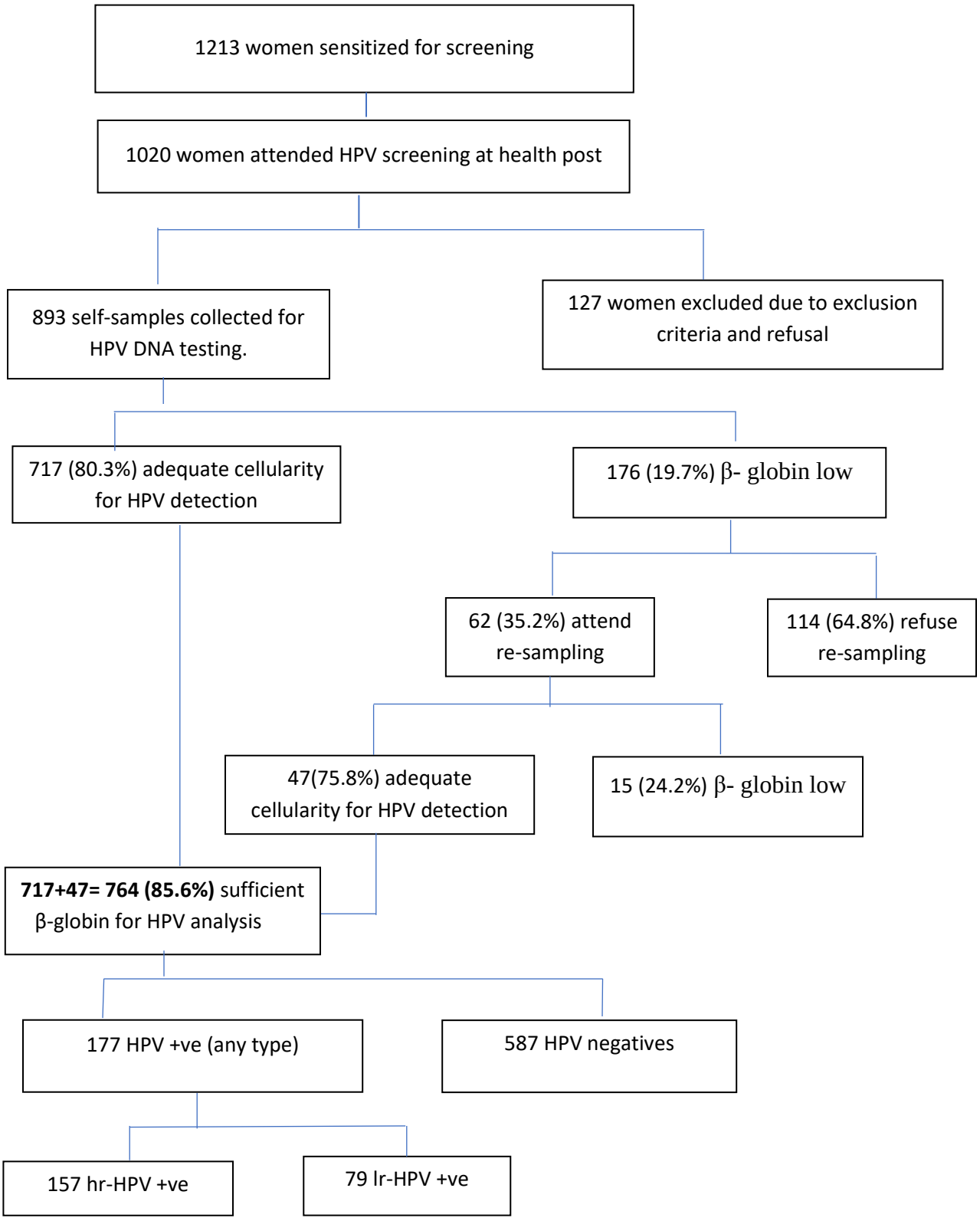


Figure 5.1: Participant and sampling characteristics of study participants at Butajira, South - central Ethiopia

5.1.2. Prevalence of HPV infection

Overall, 23.2% (95% CI: 23.54-22.86%) women were positive for HPV DNA. Of the tested women, 20.5% (95% CI=20.79-20.21) women were hr-HPV positives and 10.3% (95% CI=10.52-10.08) had lr-HPV DNA. High-risk HPV genotypes were detected more frequently than low-risk HPV genotypes. Among the women positive for hr-HPV, 13.4% had a single HPV infection and 7.2% had multiple HPV infections **(Table 5.1).** Among the 55 women with multiple hr-HPV infections, 41.8% had dual high-risk infections, 29.1% had triple high-risk infections and 29.1% had four or more hr-HPV infections. The hr- and lr-HPV co-infection in our study is 7.7% **(Table 5.1).** Furthermore, 3% of the tested women had non-typable lr-HPVs by the MPG-Luminex system used that genotype 9 lr-HPVs. The combined prevalence of HPV16 and 18 infections among the total screened women and among the hr-HPV positive women was 13.5% and 65.7%, respectively.

Table 5.1: Overall prevalence of HPV infection in Butajira women, south-central Ethiopia.

Variable	Frequency (n=764)	(%)
No HPV infection	587	76.8
Any HPV	177	23.2
hr* HPV	157	20.5
Single hr-HPV	102	13.4
Multiple hr-HPV	55	7.2
lr # HPV	79	10.3
hr & lr co-infection	59	7.7

* **hr includes**: HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82
# **lr includes:** HPV6, 11, 42, 43, 54, 57, 70, 72 and 90

5.1.3. HPV genotype distribution

In our study population, 18 hr-HPV genotypes (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) and 9 lr-HPV genotypes (HPV6, 11, 42, 43, 54, 57, 70, 72 and 90) were detected. The positivity rate of the detected genotypes in this study among all the tested samples and the distribution of each genotype in the HPV-positive women is shown in **Figure 5.2**. Of the identified HPV genotypes in this study population, HPV16 is the most prevalent genotype found (13.2%) followed by HPV11 (6.5%). From the hr-HPV genotypes identified in this study, HPV16, 35, 52, 31, and 45 were the five most prevalent genotypes in our study population, respectively. From the nine lr-HPV types detected in this study, HPV11 was found to be the most prevalent low-risk genotype followed by HPV42, 54, 43, and 6 (**Figure 5.2)**

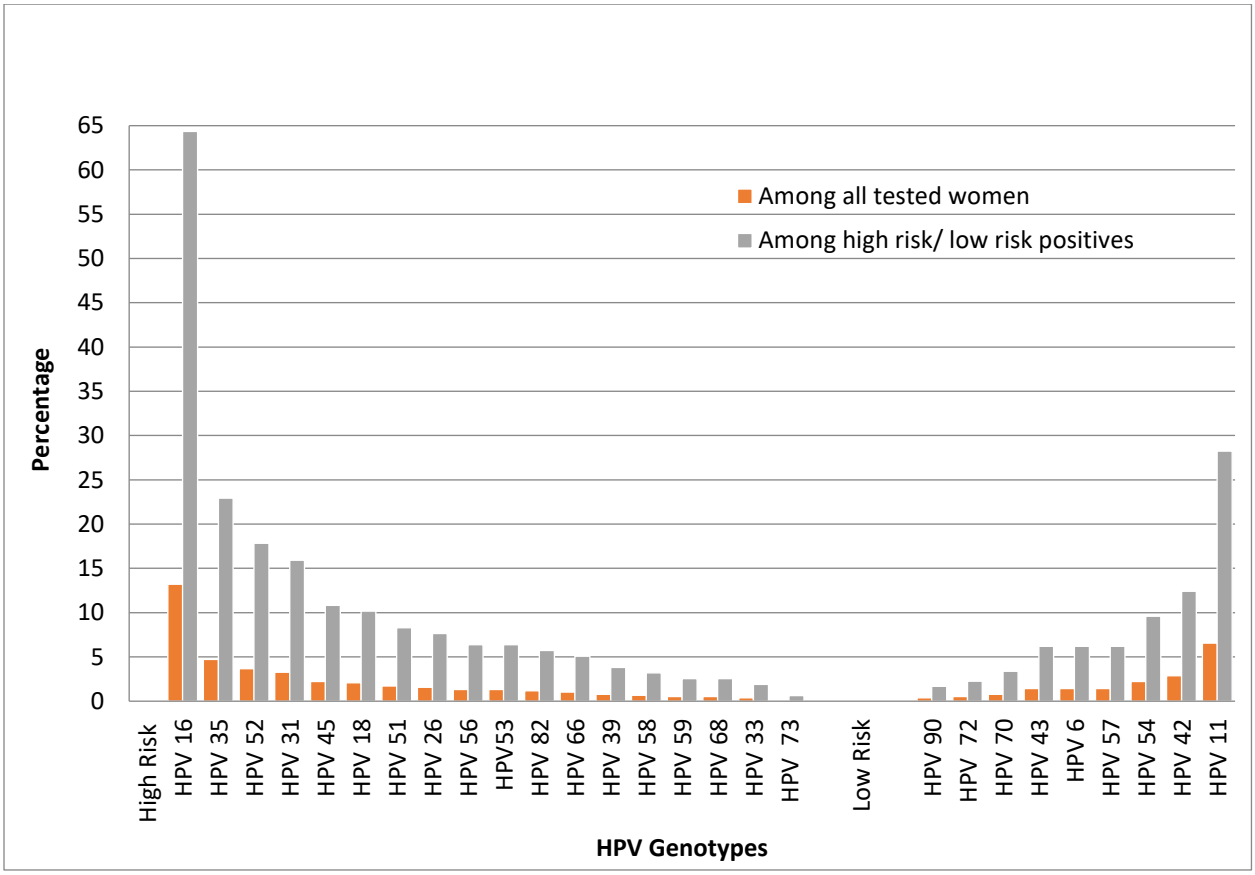


Figure 5.2: Prevalence and genotype distribution of hr- and lr-HPVs among Butajira women, south central Ethiopia

Among the HPV positive women, the proportion of the 9-valent vaccine preventable genotypes (HPVs 6, 11, 16, 18, 31, 33, 45, 52, and 58) was 6.2%, 28.2%, 57.1%, 9%, 14.1%, 1.7%, 9.6%, 15.8% and 2.8%, respectively. This amounts to 76.8% of all HPV infections detected from our study population. Thus, the seven hr-HPV types that are included in the 9-valent vaccine preventable HPV types contributes to 79% of the total hr-HPV infection in our study **(Figure 5. 3**).

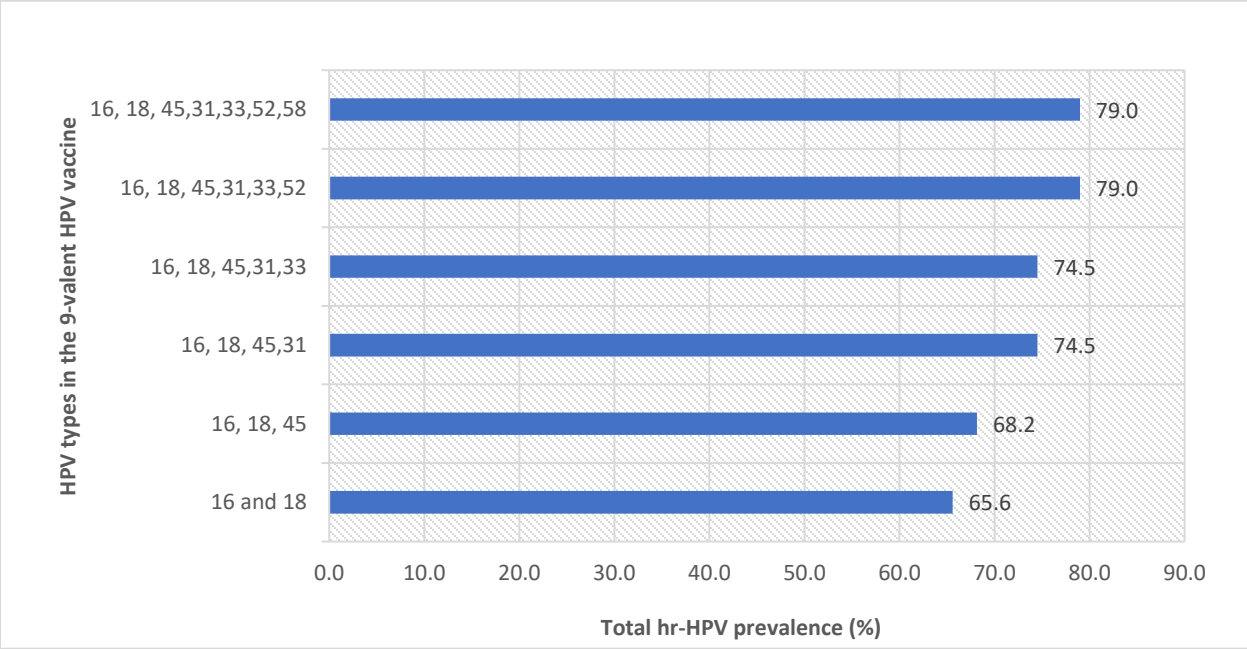


Figure 5.3: The contribution of the 9-valent vaccine preventable hr-HPV types to total hr-HPV prevalence in Butajira, south central Ethiopia

5.1.4. Age-specific prevalence of HPV infection

As HPV prevalence differs with age, we analysed the proportion of HPV infection in different age groups of our study population. For this age specific HPV infection analysis, the study women were divided into four age groups (30-34 years, 35-39 years, 40-44 years, and 45-49 years). The frequency of both any HPV infection and hr-HPV infection decreased with age (**Figure 5. 4**).

High-risk HPV infection was high (58.6%) at the younger age group (30-34 years) followed by 35-39 years (20.4%) and steadily decreased to 4.5% and 3.8 % in the age group 40-44 years and 45-49 years, respectively.

In the women from Butajira among the hr-HPV positive, the age specific prevalence of combined HPV 16 and 18 was higher (39.5%) in the age group 30-34 when compared to the other age groups. Only two women (33.3%) had HPV16 and 18 infections in the age group 45-49.

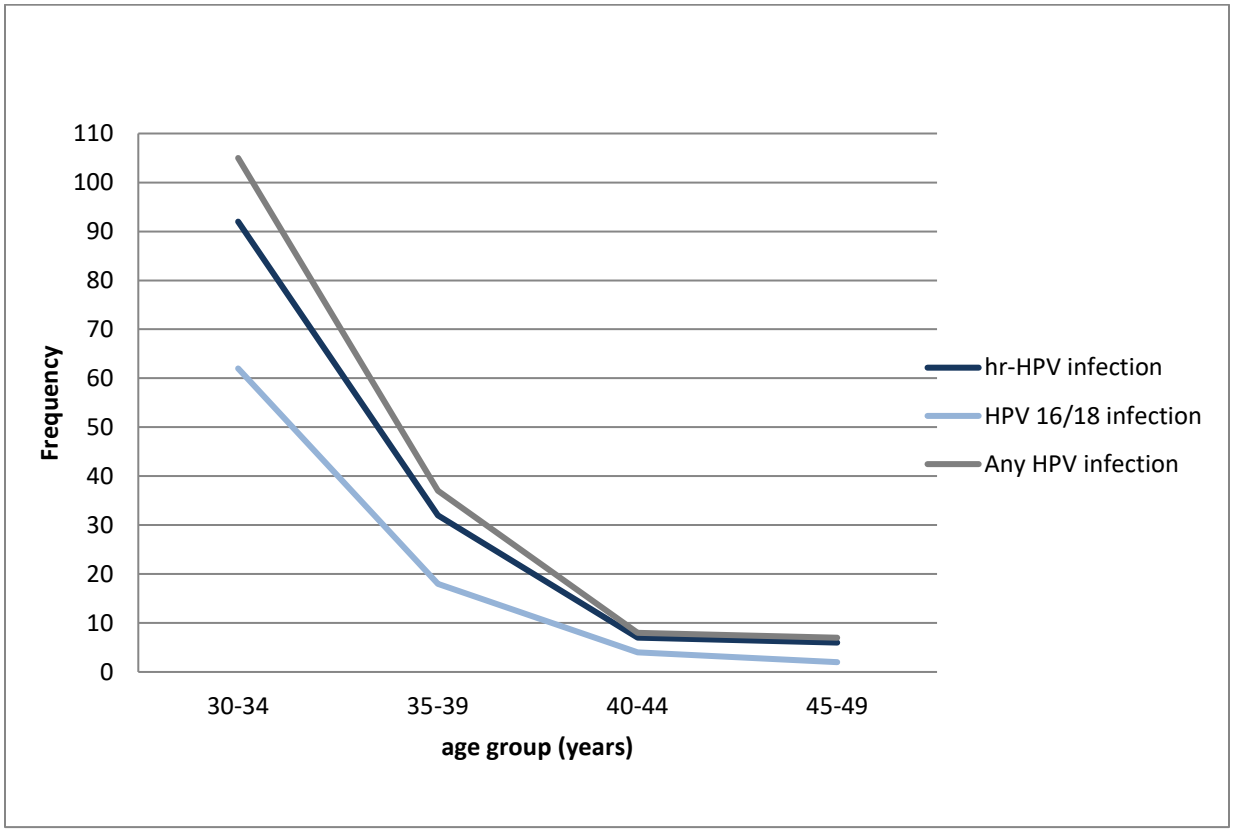


Figure 5.4: Age-specific prevalence of HPV infections among Butajira women, south central Ethiopia

5.2. A two-year follow-up of hr-HPV infection dynamics among Butajira women

5.2.1. Follow up characteristics of study participants

In this study, we followed those 30-49 years old women and screened hr-HPV positive in the initial screening. In addition, we also followed randomly selected control women who were screened negative for any hr-HPV genotype in the initial screening. Among the baseline hr-HPV positive women (n=157), 110 (70%) attended the 6 months follow-up and 95 (60.5%) of them provided cervical sample for HPV DNA testing while the 15 (9.6%) women were excluded from sample collection due to pregnancy and active menses during the sample collection time. On the other hand, 94 (59.9%) of the hr-HPV positive women at baseline screening have participated in the 24 months follow-up sample collection. Therefore, we reported a loss to follow-up of 47 (30%) participants at the 6 months follow-up and of 63 (40.1 %) participants at the 24 months follow-up visit.

Among the 94 samples collected at 24 months, 91 (96.8%) had adequate cellularity to detect HPV DNA in the sample while the remaining 3 (3.2%) were found with inadequate cellularity to detect HPV DNA in the sample due to low internal control values for ß-globin PCR. Seventy (44.6%) women attended (completed their follow up) both the 6- and 24-months sample collection. The major reasons identified for lost follow-up at both follow up times include husbands were not voluntary to send their wives to the health post for follow ups, women changed their residence after the primary screening, ethnic conflict at the study area during the time of follow up data collection, women not interested to repeat tests because they think that they feel healthy. In addition, some women did not also attend their follow up visits because of pregnancy.

5.2.2. Prevalence of HPV infections across the follow up testing

As mentioned in the above section, the hr- HPV prevalence at the baseline screening was 157 (20.5%). Among the women who were hr-HPV positive at base line and attended the 6 and 24 months follow- up, 25 (26.3%) and 14 (15.4%) were positives for any HPV-type respectively.

Furthermore, 25 (26.3%) and 12 (13.2%) of the women were hr-HPV positives after 6 months and 24 months respectively (**Table 5.2**).

Table 5.2: HPV positivity rates at baseline, 6 and 24 months follow up screening points at Butajira, south central Ethiopia

HPV infection	**Baseline screening (tested women=764)** **(n, %)**	**At 6 months, from the baseline hr-HPV positives, (tested women =95)** **(n, %)**	**At 24 months, from the baseline hr-HPV positives (tested women = 91)** **(n, %)**
Any HPV	177 (23.2)	25 (26.3)	14 (15.4)
hr-HPV	157 (20.5)	25 (26.3)	12 (13.2)
lr-HPV	79 (10.3)	5 (5.3)	4 (4.4)

Of the 607 hr- HPV negative women at baseline, we took randomly selected 80 (13%) women as controls of our follow-up study. Of these, 74 (92.5%) had adequate cellularity to detect HPV in the sample while the rest 6 (7.5%) were found with inadequate cellularity to detect HPV in the sample due to low internal control values for ß-globin PCR. Only 3 (4.05%) turned positive for hr-HPV and 71 (95.9%) remained negative for hr-HPV after 24 months follow-up testing. The positivity rates of any HPV and hr-HPV in the follow-up and control groups is shown in **figure 5. 5**.

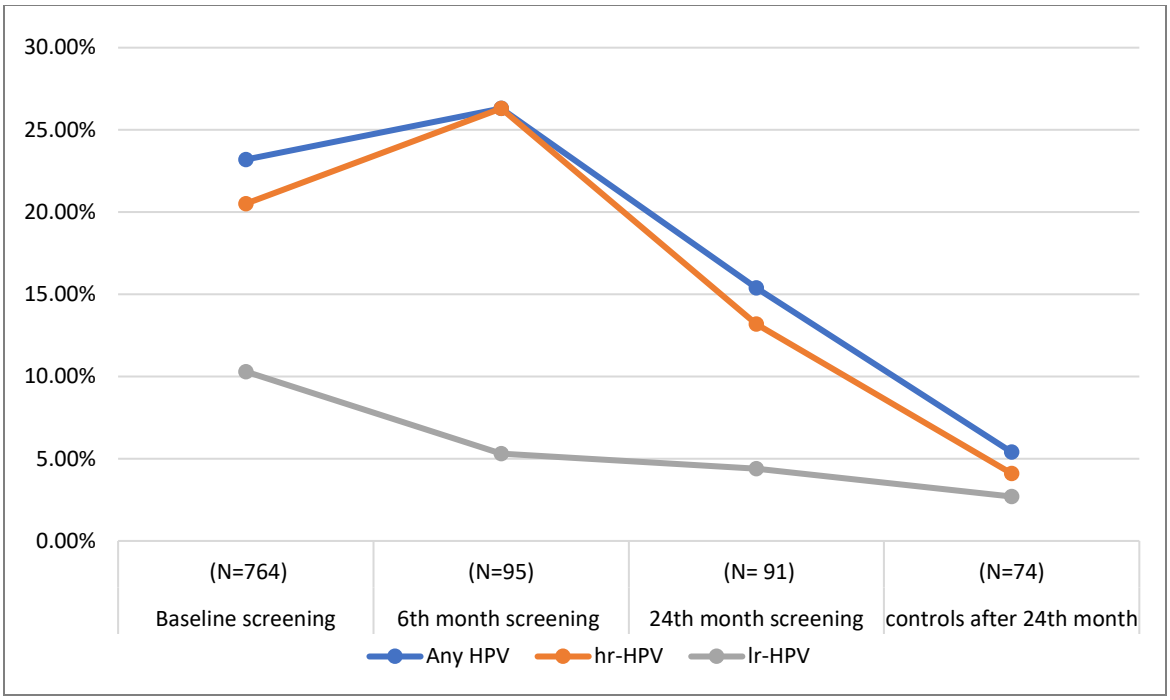


Figure 5.5: HPV positivity rates at baseline and different follow up screening points as compared to negative controls at Butajira, Ethiopia

5.2.3 hr-HPV type-specific point prevalence

The type-specific prevalence of HPV genotypes was determined in each follow-up visit separately. Eighteen hr-HPV genotypes (16,18,26,31,33,35,39,45,51,52,53,56,58,59,66,68,73,82) were detected at the baseline screening while 12 hr-HPVs (16,18,31,35,39,51,52,53,56,66,68,82) and 9 hr-HPVs (16, 31 ,39,52,56,59,66,68,82) were detected in the 6 months and 24 months follow-up screening points, respectively.

Despite HPV16 was the most frequent genotype across all-time points, HPV 52 after 6 months and HPVs 52, 66 and 59 after 24 months were also equally frequent genotypes (**Table 5.3**). Of the HPV DNA tested women (n=95) after 6 months, HPV 16 and HPV 52 were equally frequent (28%) followed by HPVs 82 (24%), 35 (16%), 53 (16%) and 68 (12%) while HPV16, 52, 66 and 59 (25%) were equally frequent in the women tested (n=91) after 24 months followed by HPV 56 (16.7%) (**Table 5.3**).

Table 5.3: Type-specific HPV point prevalence during Follow-up, Butajira, south central Ethiopia

		Follow- up no (%) of cases	
HPV	**Baseline n, %**	**6th months n, %**	**24th months n, %**
hr-HPV positive	157 (20.5)	25 (26.3)	12 (13.2)
hr-HPV negative	607 (79.5)	70 (73.7)	79 (86.8)
HPV genotype among the hr-HPV positive women			
HPV 16	101 (64.3)	7 (28)	3 (25)
HPV 18	16 (10.2)	1 (4)	0
HPV 26	12 (7.6)	0	0
HPV 31	25 (15.9)	1(4)	1(8.3)
HPV 33	3 (1.9)	0	0
HPV 35	36 (22.9)	4 (16)	0
HPV 39	6 (3.8)	1(4)	1(8.3)
HPV 45	17 (10.8)	0	0
HPV 51	13 (8.3)	2 (8)	0
HPV 52	28 (17.8)	7 (28)	3 (25)
HPV 53	10 (6.4)	4 (16)	0
HPV 56	10 (6.4)	2 (8)	2 (16.7)
HPV 58	5 (3.2)	0	0
HPV 59	4 (2.5)	0	3 (25)
HPV 66	8 (5.1)	1(4)	3 (25)
HPV 68	4 (2.5)	3 (12)	1 (8.3)
HPV 73	1 (0.6)	0	0
HPV 82	9 (5.7)	6 (24)	1(8.3)

5.2.4. Persistence and clearance of hr-HPV infections among Butajira women

Next, we assessed the persistence and clearance of HPV infections among single and multiple baseline hr-HPV infections. Of the 102 single hr-HPV infections at the baseline screening, 61 of them attended in the 6 months and 24 months follow up screening. Of those, 46 (75.4%) and 54 (88.5%) of them cleared their infections at 6 and 24 months respectively. In addition, from the 55 multiple hr-HPV infections at baseline, 34 of them attended 6 months follow-up and 33 attended 24 months follow-up. From the multiple hr-HPV infections, 24 (70.6%) and 28 (84.8%) cleared their infections at 6 and 24 months respectively (**Figure 5.6**).

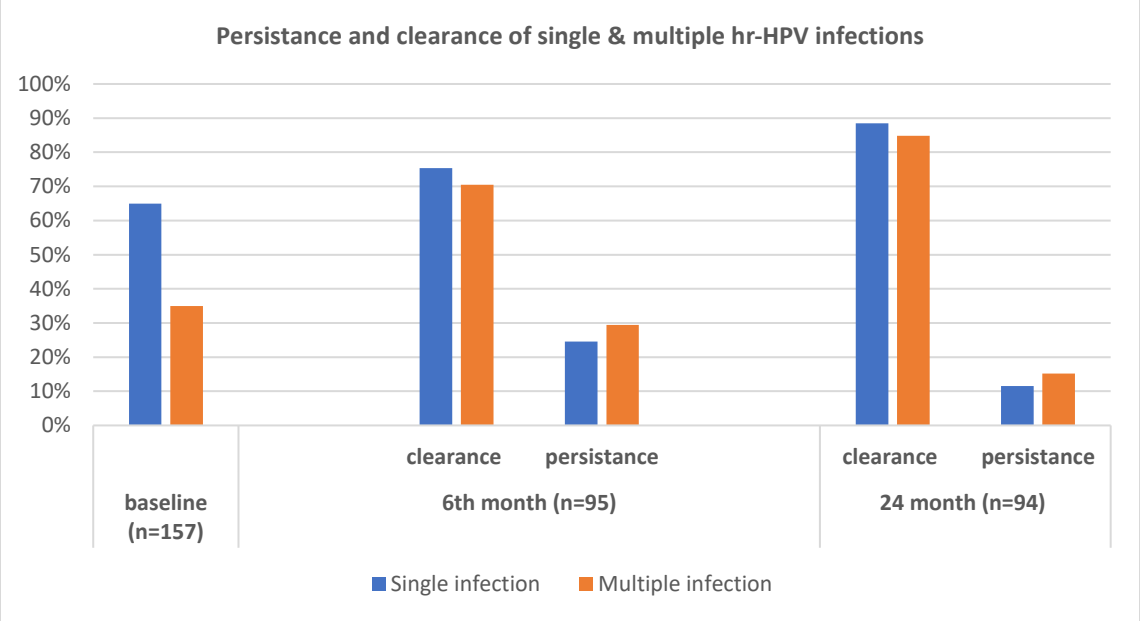


Figure 5.6: Percentage of persistence and clearance from single and multiple hr- HPV infections at Butajira, Ethiopia

5.2.5. Sociodemographic characteristics with hr-HPV persistence and clearance

Table 5.4 showed the persistence and clearance rates of hr-HPV infections and sociodemographic characteristics of women at different follow up points. The mean age of our study participants was 33 years. Of the 25 and 12 women who persisted their hr-HPV infection at 6 and 24 months respectively, half of them were in the age range 30-34 years. Out of the 79 women who cleared hr-HPV infections at the 24 months of follow up, 54 (68.4%) of them were 30-34 years old.

Table 5.4: Socio-demographic characteristics and hr-HPV persistence and clearance rates at Butajira, Ethiopia

Variable	HPV persistence				HPV clearance			
	6 month (n=25)	%	24 month (n =12)	%	6 month (n =70)	%	24 month (n = 79)	%
Age								
30-34	13	52	6	50	48	68.6	54	68.4
35-39	8	32	2	16.7	13	18.6	16	20.3
40-44	1	4	1	8.3	4	5.7	3	3.8
45-49	2	8	2	16.7	3	4.3	2	2.5
Do not know	1	4	1	8.3	2	2.9	4	5.1
Marital Status								
Single	0	0	0	0.0	0	0.0	1	1.3
Married	22	88	10	83.3	66	94.3	73	92.4
Divorced	3	12	1	8.3	2	2.9	3	3.8
Widowed	0	0	1	8.3	2	2.9	2	2.5

5.2.6. Genotype specific hr-HPV persistence and clearance during follow-up

At baseline screening, 177 (23.2%) of the 764 participants were HPV positive. After 6 months, 95 of them participated the follow-up testing and 70 (73.7%) cleared their infections while 25 (26.3%) had hr-HPV infections. Of the 25 hr-HPV infections found at 6 months, 9 (36%) had new infections while 16 (64%) of them had the same genotype specific persistent infections. Moreover, from the 91 followed women at 24 months, 77 (84.6%) of them cleared their infections and 14 (15.4%) of them had persisted for any HPV infection.

Among the hr-HPV positive women at the 6 months of follow-up, HPV68, 82, 53, 52, 56 were the most persisted genotypes with 100 %, 75%, 42.9%, 31%, 25% persistence rates respectively. HPV 18, 26, 33, 39, 45, 51, 58, and 59 were the least persisted HPV genotypes at 6^{th} months of follow up with 100% clearance rates each. The persistence of HPV 16 in our study participants was 11.3% (**Table 5.5**). After 24 months of follow-up testing, HPV 59, 68, 66, 52 and 16 were found to have persistence with 50%, 50%, 20%, 15.8% and 3.5% respectively **(Table 5.5).**

Table 5.5: Type specific persistence/clearance/new infection of hr-HPVs at 6 and 24 months of follow up.

	hr-HPV infection dynamics during the follow up											
	6th month	**24th month**	**Persistence**				**Clearance**				**New infection**	
HPV type	Prevalence at baseline from the follow up attended women (n=95)	Prevalence at baseline from the follow up attended women (n=91)	6th month		24th month		6th month		24th month		6th month	24th month
			n	%	n	%	n	%	n	%	n	n
HPV 16	62	57	7	11.3	2	3.5	55	88.7	55	96.5	0	0
HPV 18	12	13	0	0.0	0	0.0	12	100.0	13	100.0	1	0
HPV 26	10	10	0	0.0	0	0.0	10	100.0	10	100.0	0	0
HPV 31	12	12	1	8.3	0	0.0	11	91.7	12	100.0	0	1
HPV 33	2	3	0	0.0	0	0.0	2	100.0	3	100.0	0	0
HPV 35	24	21	3	12.5	0	0.0	21	87.5	21	100.0	1	0
HPV 39	6	4	0	0.0	0	0.0	6	100.0	4	100.0	1	1
HPV 45	13	14	0	0.0	0	0.0	13	100.0	14	100.0	0	0
HPV 51	7	10	0	0.0	0	0.0	7	100.0	10	100.0	2	0
HPV 52	19	19	6	31.6	3	15.8	13	68.4	16	84.2	1	0
HPV 53	7	6	3	42.9	0	0.0	4	57.1	6	100.0	1	0
HPV 56	8	7	2	25.0	0	0.0	6	75.0	7	100.0	0	2
HPV 58	3	5	0	0.0	0	0.0	3	100.0	5	100.0	0	0
HPV 59	3	2	0	0.0	1	50.0	3	100.0	1	50.0	0	2
HPV 66	7	5	1	14.3	1	20.0	6	85.7	4	80.0	0	2
HPV 68	2	2	2	100.0	1	50.0	0	0.0	1	50.0	1	0
HPV 82	4	4	3	75.0	0	0.0	1	25.0	4	100.0	3	1

5.2.7. Pap smear test Results

At the sixth months of follow up, cervical cytology by Papanicolaou (Pap) smears was performed in 97 women. Patients with HSIL constituted 10.3% of the tested women, followed by those with ASCUS (9.3%) and LSIL (8.2%) (**Table 5.6)**.

Table 5.6: Pap smear test results in Butajira follow up study

Cytology	n	%
NILM	59	60.8
Inadequate	9	9.3
ASCUS	9	9.3
ASC-H	2	2.1
LSIL	8	8.2
HSIL	10	10.3
Total	97	100

HSIL (High grade squamous intraepithelial lesion); ASC-H (Atypical squamous cells—cannot exclude HSIL); LSIL (Low-grade squamous intraepithelial lesion); ASCUS (Atypical squamous cells of undetermined significance) NILM (Negative for Intraepithelial Lesion or Malignancy); inadequate (sample was inadequate for cytological examination).

5.2.8. Visual inspection using acetic acid (VIA) in comparison with Pap smear

During the 6-month follow up, 90 women had both VIA and Pap smear. Of these, only 9 (10%) were positive for VIA. Among the VIA positive women, only 3 (33.3%) of them were identified with abnormal cytology while the rest (66.7%) were negative for intraepithelial lesion or malignancy **(Table 5.7).** Majority of the women (26/29; 89.7%) with abnormal cytology of any level were VIA negative.

Table 5.7: Comparison of VIA and Pap smear in Butajira follow up study

VIA		Cytology				
	n	NILM n (%)	ASCUS n (%)	ASC-H n (%)	LSIL n (%)	HSIL n (%)
Pos	9	6 (66.7)	1(11.1)	0	0	2 (22.2)
Neg	81	55 (67.9)	8 (9.9)	2 (2.5)	8 (9.9)	8 (9.9)

5.2.9. Cervical neoplasia in relation with hr-HPV infection at 6 months follow up

We determined the cytology of the followed-up women at the 6 months. **Figure 5.7** shows course of HPV infection in relation to cytological result at follow up. Of the 29 individuals with abnormal cytology including ASCUS during the 6 months of follow-up, only 8 (27.6%) of them had type specific persistent hr-HPV infections while 18 (62.1%) of them cleared their baseline hr- HPV infections. On the other hand, 3 (10.3%) of the cytologically abnormal results had new type hr-HPV infections other than their baseline infection.

Of the 10 women with high grade squamous intraepithelial lesion (HSIL) at 6 month follow up, 5 (50%) persisted their baseline type specific hr-HPV infections while 4 (40%) and 1 (10%) had cleared their hr-HPV infections and acquired new HPV infections respectively. On the contrary, of the 8 women with low grade squamous intraepithelial lesion (LSIL), 7 (87.5%) of them cleared their baseline hr- HPV infections while 1 (12.5%) had new hr-HPV other than the baseline **(Figure 5.7**). Furthermore, at the 6 month follow up, 9 (15.3%) of the women with negative for intraepithelial lesion or malignancy (NILM) had type specific hr-HPV infections.

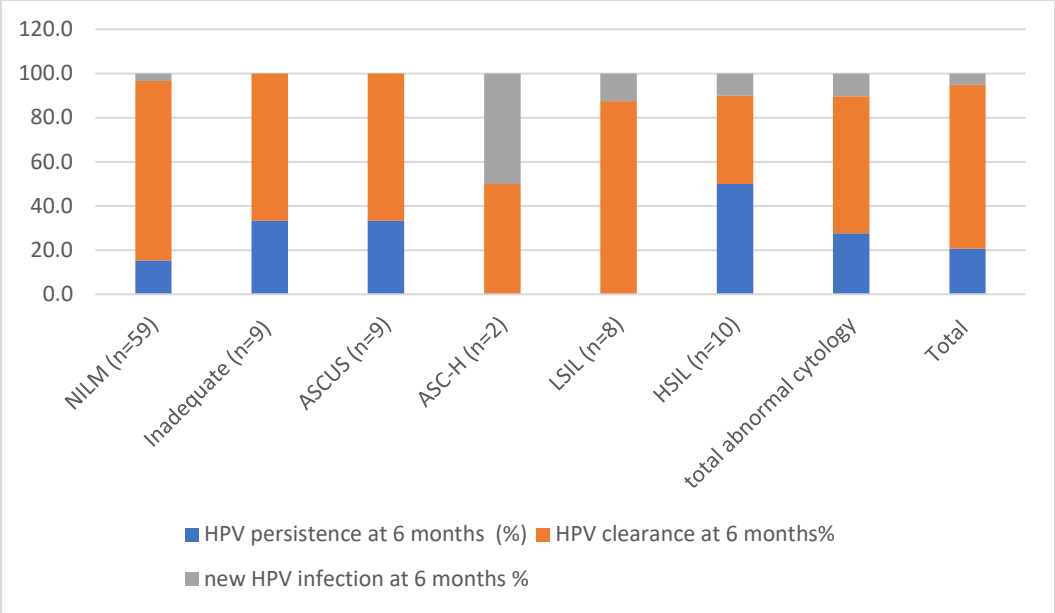


Figure 5.7: Persistence and clearance of hr-HPV infections in relation to evidence of cervical cytological results at 6 months follow-up

5.2.10. hr-HPV persistence and clearance related to cytology and VIA

At the 6 months follow up, all the hr-HPV positives at the baseline screening were also invited for VIA and Pap smear testing. The VIA and cytology results in relation to their hr-HPV persistence and clearance status of the women are presented in **Table 5.8**. Of the 25 women found hr-HPV positive at their 6 month follow up, none of them were VIA positives while 9 (36%) were with abnormal cytology including ASCUS. Four (16%) of the persistently hr-HPV positive at the 6 month follow up were found with HSIL. Three (18.8%) of the 16 women with a type of specific persistent hr-HPV infection were identified with HSIL. Seventeen (24.3%) of the 70 women who cleared their hr-HPV infection in the 6-month had abnormal cytology findings and 4 (5.8%) of them with HSIL. All the 9 women who found VIA positive were from the women who cleared their hr-HPV infections at 6 months. Among the 9 women who had new type specific hr-HPV infections at the 6 month follow up, only 1(11.1%) was found to be with HSIL (**Table 5.8).**

Table 5.8: VIA and cytology results in relation to HPV persistence and clearance at 6 month follow up, Butajira, South central Ethiopia

Study groups	VIA	CYTOLOGY (at the same time point)							
		n (%)	NILM n (%)	ASCUS n (%)	ASC-H n (%)	LSIL n (%)	HSIL n (%)	Inadequate n (%)	No Cytology n (%)
hr-HPV Positives At 6 Months (N=25)									
	Pos.	0	0	0	0	0	0	0	0
	Neg.	23(92)	12(48)	3(12)	1(4)	1(4)	4(16)	2(8)	
	No VIA	2(8)	0	0	0	0	0	0	2(8)
Type specific hr-HPV persistence (n =16)									
	Pos.	0	0	0	0	0	0	0	0
	Neg.	15(93.8)	7(43.8)	3(18.8)	0	0	3(18.8)	2(12.5)	
	No VIA	1(6.3)	0	0	0	0	0	0	1(6.3)
New type hr-HPV infections (n=9)									
	Pos.	0	0	0	0	0	0	0	0
	Neg.	8(88.9)	4(44.5)	0	1(11.1)	1(11.1)	1(11.1)	1(11.1)	
	No VIA	1(11.1)	0	0	0	0	0	0	1(11.1)
Cleared hr-HPV infections (n =70)									
	Pos.	9(12.9)	6(8.6)	1(1.4)	0	0	2(2.9)	0	0
	Neg.	58(82.9)	38(54.3)	4(5.7)	1(1.4)	7(10)	2(2.9)	3(4.3)	3(4.3)
	No VIA	3(4.3)	0	0	0	0	0	0	3(4.3)

5.2.11. Prevalence of sexually transmitted infections (STI) among hr-HPV positive women

During the 6 month follow up in our study, we have evaluated the women for sexually transmitted infections and 104 women gave sample for STI testing. Among the evaluated 11 STI pathogens (*Chlamydia trachomatis, Neisseria gonorrhoeae, Herpes simplex virus 1, Herpes simplex virus 2, Haemophilus ducreyi, Mycoplasma genitalium, Mycoplasma hominis, Treponema pallidum, Trichomonas vaginalis, Ureaplasma parvum, and Ureaplasma urealyticum),* 5 STI pathogens

were isolated (*Ureaplasma parvum, Ureaplasma urealyticum, Mycoplasma hominis, Herpes simplex virus 1 and Trichomonas vaginalis*).

The positivity for at least one STI was 52.9% (55/104). Among the STI-positive women, Ureaplasma parvum was the most prevalent pathogen (46/55, 84%), followed by Ureaplasma urealyticum (12/55, 22%) and Mycoplasma hominis (6/55, 11%). Trichomonas vaginalis and Herpes simplex virus 1 were identified in 1% (1/104) of the tested women (**Figure 5.8).**

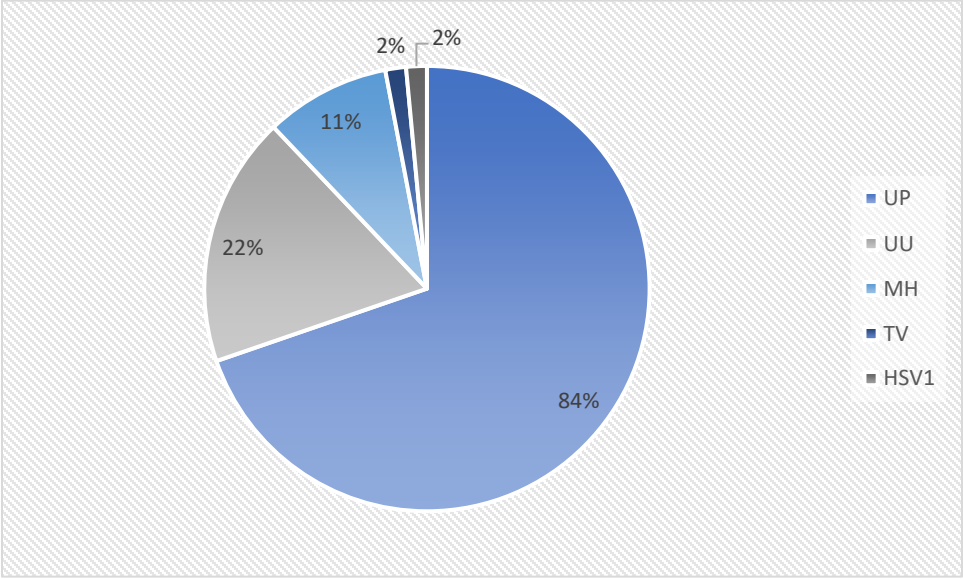


Figure 5.8: Proportion of STI pathogens in Butajira women

Most of the STI positive women were infected by a single pathogen (84%), whereas 13 % and 4 % were with two or three or more STIs, respectively **(Figure 5.9).**

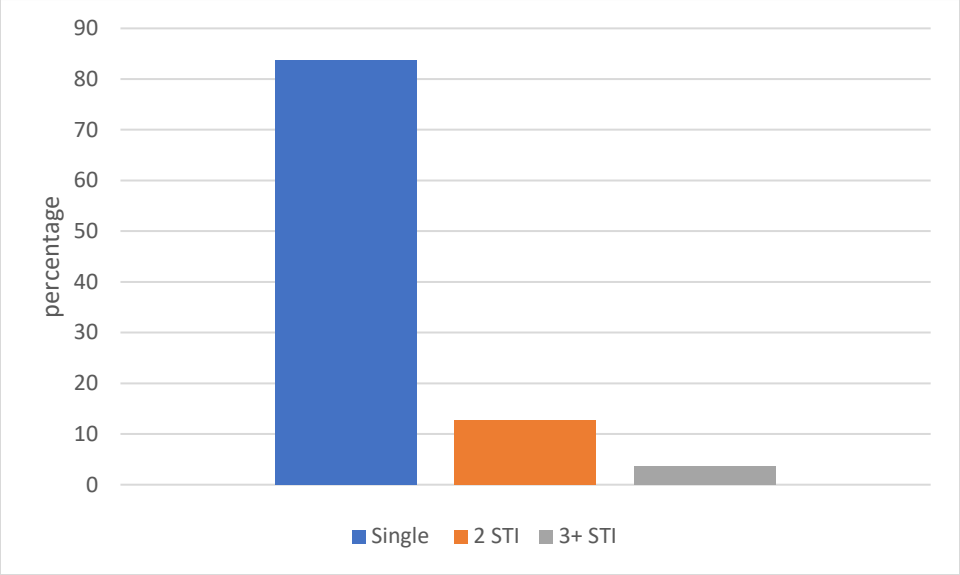


Figure 5.9: Single and multiple STI infections

5.2.12. Association of Cytology and STI

In the 6 month follow up, 36 (38.3%) women were positive for STI with no hr-HPV detected while 16 women (17%) were positive for both HPV and STIs (**Table 5.9)**. Of the 52 women positive for any STI, 13 (25%) had abnormal cytology including ASCUS, and 13 (30%) of the 42 negative STI women had abnormal cytology. Five (31.3%) of the women out of the 16 with both STI and HPV positive were with abnormal cytology while 4 (50%) of the 8 women with HPV positive and STI negative were with abnormal cytology results (**Table 5.9)**.

Table 5.9: Distribution of cytological findings by high risk-HPV types and STI status at the 6 month follow up testing.

STI & HPV Status	Total(n=94)	%	NILM	%	ASCUS/ ASC-H	%	LSIL	%	HSIL	%
STI (+)	52	55.3	39	41.5	6	6.4	3	3.2	4	4.3
STI (-)	42	44.7	29	30.9	4	4.3	5	5.3	4	4.3
STI (+) / HPV (+)	16	17.0	11	11.7	3	3.2	1	1.1	1	1.1
STI (+) / HPV (-)	36	38.3	28	29.8	3	3.2	1	1.1	3	3.2
STI (-)/ HPV (+)	8	8.5	4	4.3	1	1.1	0	0.0	3	3.2
STI (-)/ HPV (-)	34	36.2	25	26.6	3	3.2	5	5.3	1	1.1

5.3. Comparison of HPV genotyping assays

In this PhD study, one of the aims was to compare HPV genotyping assays and to gain experience and produce comparative data for assay characteristics and complexity. During the comparison, there was a difference in categorization of carcinogenicity between the three evaluated HPV assays. Therefore, the carcinogenicity categorization in this comparison study was according to the protocols of each assay. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 were classified as hr-HPV and HPV26, 53, 73, 82 as probable hr-HPV genotypes and the other genotypes were considered as lr-HPVs. The spectrum of HPV genotypes detected in the three HPV genotyping assays are summarized in **Table 5.10.** The highlighted genotypes were matching in the assays and represent the 14 hr-HPVs to be compared.

Table 5.10: Spectrum of HPV genotypes in three HPV genotyping assays

Assay	**Oncogenic potential**	
	High-risk HPV	Low-risk HPV
MPG-Luminex Assay	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	6, 11, 42, 43, 54, 57, 70, 72, 90
EUROArray HPV Assay	16, 18, 26, 31, 33, 35, 39,45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	6, 11, 40, 42, 43, 44, 54, 61, 72, 70, 81, 89
Anyplex™ II HPV HR Detection Assay	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Not included

The highlighting color designates the 14 hr-HPV genotypes common for all three genotyping assays.

Of the 110 samples, MPG-Luminex Assay, Anyplex™ II HPV HR Detection assay, and EUROArray HPV Assay detected 21.82%, 21.82%, and 16.36% hr-HPV genotype positive samples for all hr-HPV types, respectively (κ=0.734) **(Table 5.11**). For the lr-HPV genotypes the positivity rate was 5.45% in MPG-Luminex Assay and 7.27% in EUROArray HPV Assay that comprises 3 more genotypes **(**κ=0.237**).** The Anyplex™ II HPV HR Detection assay only includes hr-HPV genotypes (see Table 5.12).

Table 5.11: HPV positivity result for the 110 samples tested with MPG-Luminex Assay, EUROArray HPV Assay, and Anyplex™ II HPV HR Detection Assay

	MPG-Luminex Assay	**EUROArray HPV Assay**	**Anyplex™ II HPV HR Detection**	**κ–value**
	N (%)	N (%)	N (%)	
hr- and probable hr-HPV positive	24 (21.82)	18 (16.36)	24 (21.82)	**0.734**
lr-HPV positive	6 (5.45)	8 (7.27)	Not included	**0.237**
HPV negative	84 (76.36)	88 (80.00)	86 (78.2)	

Concerning HPV genotypes, both hr- and lr-HPVs were detected in MPG-Luminex assay and EuroArray assay. In contrast, Anyplex™ II HPV HR Detection only includes 14 hr-HPV types. In all the 3 assays, the genotypes most often detected were HPV16, 35 and 52. HPV16 was detected in 6.4%, 3.6%, and 3.6% of the tested samples using MPG-Luminex Assay, EUROArray HPV Assay, and Anyplex™ II HPV HR Detection, respectively, while HPV35 was detected in 3.6%, 3.6%, and 4.5%, respectively. The other dominant genotype (HPV52) was detected in 6.4%, 2.7%, and 6.4% using MPG-Luminex Assay, EUROArray HPV Assay, and Anyplex™ II HPV HR Detection, respectively. Considering HPV82 that was included only in the MPG-Luminex Assay and the EUROArray HPV Assay, this genotype was equally detected by EUROArray HPV Assay (4.5%) and MPG-Luminex Assay (4.5%). Among the lr- HPVs, only HPV6 and HPV42 were commonly detected in both assays and HPV43 only by EUROArray.

The genotype-specific agreements of the three evaluated HPV genotyping assays are summarized in **Table 5.12**. For most of the genotypes, the assays showed moderate or better agreement. However, the level of discordance between the three assays was considerably high in the detection of HPVs 18 (**κ**=-0.003), 33 (**κ**=-0.003), 45 (**κ**=-0.003), 51 (**κ**=-0.012), 58 (**κ**=-0.003), 59 (**κ**=-0.003), and 73 (**κ**=-0.005). However, this analysis of agreement was limited by a very restricted number of positive samples for each genotype (**Table 5.12)**. EUROArray HPV Assay did not detect any HPV51, and 68 infections compared with the other two assays. Anyplex™ II HPV HR Detection Assay detected HPVs 18, 33, 45, 58, and 59 which were not detected in MPG-Luminex and EUROArray HPV assays.

Table 5.12: Genotype-specific detection of HPV genotypes in 110 cervical samples, by HPV assay used

HPV Genotype		Number of positive samples			κ-value	Meaning
		MPG-Luminex Assay	**EUROArray HPV Assay**	**Anyplex™ II HPV HR Detection Assay**		
High-risk	16	7	4	4	0.790	Substantial
	18	0	0	1	-0.003	Poor[a)]
	31	1	1	3	0.594	Moderate
	33	0	0	1	-0.003	Poor
	35	4	4	5	0.920	Almost perfect
	39	1	1	2	0.747	Substantial
	45	0	0	1	-0.003	Poor
	51	2	0	2	-0.012	Poor
	52	7	3	7	0.628	Substantial
	56	2	1	2	0.594	Moderate
	58	0	0	1	-0.003	Poor
	59	0	0	1	-0.003	Poor
	66	1	1	1	1	Almost perfect
	68	3	0	3	0.491	Moderate
Probable high-risk	26	0	0	NI[c)]	CNC[b)]	
	53	4	3	NI	0.852	Almost perfect
	73	0	1	NI	-0.005	Poor
	82	5	5	NI	1	Almost perfect
Low risk	6	2	2	NI		
	11	0	0	NI		
	40	NI	0	NI		
	42	1	2	NI		
	43	0	4	NI		
	44	NI	0	NI		
	54	0	0	NI		
	57	1	NI	NI		
	61	NI	0	NI		
	70	0	0	NI		
	72	0	0	NI		
	81	NI	0	NI		
	89	NI	0	NI		
	90	2	NI	NI		

a) poor assay concordance results from too few samples positive for this genotype and generally a restricted power for this analysis.

b) CNC, cannot be computed since none of the test kits identified this genotype

c) NI= Not included

Next, we assessed the performance of the two commercial assays EUROArray HPV and Anyplex™ II HPV HR Detection Assay in the overall detection of the 14 hr-HPVs by using MPG-Luminex Assay as a reference method. This assay is based on the clinically validated GP5+/GP6+ PCR EIA that has been used as gold standard comparator test for clinical validation of other tests. The MPG uses the GP5+/GP6+ primer set with consecutive probe-based genotyping via Luminex bead-based technology read out. It has been shown to have equal (relative) sensitivity and specificity like the EIA assay format (Arbyn and Hillemanns, 2018).

The aggregated sensitivity in detecting the 14 hr-HPV infections of EUROArray HPV and Anyplex™ II HPV HR Detection assays was high, 70% and 100%, respectively, while the specificities of EUROArray HPV and Anyplex™ II HPV HR Detection kit in the detection of the 14 hr-HPV infections were 100% and 95.6%, respectively, when compared with MPG-Luminex Assay (**Table 5.13).**

Table 5.13: Detection performance for high-risk HPVs of EUROArray HPV and Anyplex™ II HPV HR Detection Assays

HPV Assay	**n**	**14 hr-HPV positive, n**	**14 hr-HPV negatives, n**	**Sensitivity**	**Specificity**
EUROArray HPV	110	14	96	70%	100%
Anyplex™ II HPV HR	110	24	86	100%	95.6%
MPG-Luminex Assay	110	20	90	Comparator assay	

After the analysis of the HPV status among the three methods (comparison of only 14 hr-types detected in all three test), there were 11 cases (10% of the whole study population) which showed a discordant result for at least one of the HPV assays, resulting in overall HPV positivity or negativity (**Table 5.14**). The Anyplex™ II HPV HR Detection assay showed a discordant result for 5 of the 11 discordant results and indicated positive HPV status which were negative in the

other two assays. HPV18, 31, 51, and 56 were detected in the 5 discordant results by Anyplex™ II HPV HR Detection assay.

For two samples, the MPG-Luminex assay displayed an HPV positivity (HPV51 and 52), whereas the EUROArray and Anyplex found no evidence of HPV positivity (see Table 5.14, sample ID 991374 and 991442). EUROArray HPV assay was discordantly negative for four samples while MPG-Luminex and Anyplex™ II HPV HR Detection assays were positive for those (**Table 5.14**). The four EUROArray HPV negative samples were positive for HPV16, 51, 52, 53, and 68 in MPG-Luminex assay and for HPV 31, 52 and 68 in Anyplex™ II HPV HR Detection assay.

Table 5.14: Discordant cases for hr-HPV genotypes in the comparison among the three HPV DNA genotyping assays

Sample lab. ID	**HPV DNA Assay**			
	MPG-Luminex Assay	**EUROArray HPV Assay**	**Anyplex™ II HPV HR Detection Assay**	**Discordant HPV genotype**
991530	negative	negative	positive	Pos for HPV18
991760	negative	negative	positive	Pos for HPV51
991365	positive	negative	positive	Neg for HPV68
991374	positive	negative	negative	Pos for HPV52
991376	positive	negative	positive	Neg for HPV51, 52
991429	negative	negative	positive	Pos for HPV56
991442	positive	negative	negative	Pos for HPV51
181506	negative	negative	positive	Pos for HPV51
181665	negative	negative	positive	Pos for HPV31
181719	positive	negative	positive	Neg for HPV31, 53
181735	positive	negative	Positive	Neg for HPV16, 68

The highlighting color designates discordant results between the different assays.

5.4. Cervicovaginal microbiota profiles in precancerous lesions, cervical cancer, and healthy controls

5.4.1. Participant characteristics

The general characteristics of the 120 participants are provided in **Table 5.15**. The study included 35 women with normal cytologic or histologic characteristics, 25 with low-grade or high-grade dysplasia, and 60 with cervical cancer (either squamous cell carcinoma or adenocarcinoma). Most participants (n=84, 70%) were younger than 50 years; 35 (29%) were aged 50 years or older. Twenty-seven women (23%) were HPV-negative and 93 (78%) were HPV-positive (any high-risk HPV genotype). Among those younger than 50 years (n=84), 28 had cervical cancer, 22 had dysplasia, and 34 had normal cytologic or histologic characteristics. Among those aged 50 years or older, 32 had cervical cancer, 2 had dysplasia, and 1 had normal cytologic or histologic characteristics (**Table 5.16**). No participants in our cohort had a low-risk HPV genotype. Cervical dysplasia was classified according to histologic grade of cervical intraepithelial neoplasia (CIN1–3) or according to cytologic grading of squamous intraepithelial lesions (high-grade or low-grade).

Table 5.15: Sociodemographic and clinical characteristics of participants (n = 120).

Characteristic	No. (%)
Age	
<50 years	84 (70)
≥50 years	35 (29)
Unknown	1 (1)
High-risk HPV infection	
Negative	27 (23)
Positive	93 (78)
HPV16 only	44 (37)
HPV18 only	2 (2)
Single HPV, not HPV16/18	20 (17)
Multiple HPV, including HPV16	20 (17)
Multiple HPV, including HPV18	3 (3)
Multiple HPV, not including HPV16/18	4 (3)
Histologic/cytologic characteristics	
ASCUS/NILM	35 (29)
Low-grade dysplasia (CIN1/2 or LGSIL)	13 (11)
High-grade dysplasia (CIN3 or HGSIL)	12 (10)
Cancer (SCC or ACC)	60 (50)

Abbreviations: HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesion or malignancy; CIN, cervical intraepithelial neoplasia; LGSIL, low-grade squamous epithelial lesion; HGSIL, high-grade squamous epithelial lesion; SCC, squamous cell carcinoma; ACC, adenocarcinoma.

Of the group under 50 years, cancer patients were 28, dysplasia patients were 22, and none were 34. The group of 50 and up years had 32 cancer, 2 dysplasia, and 1 none (**Table 5.16**).

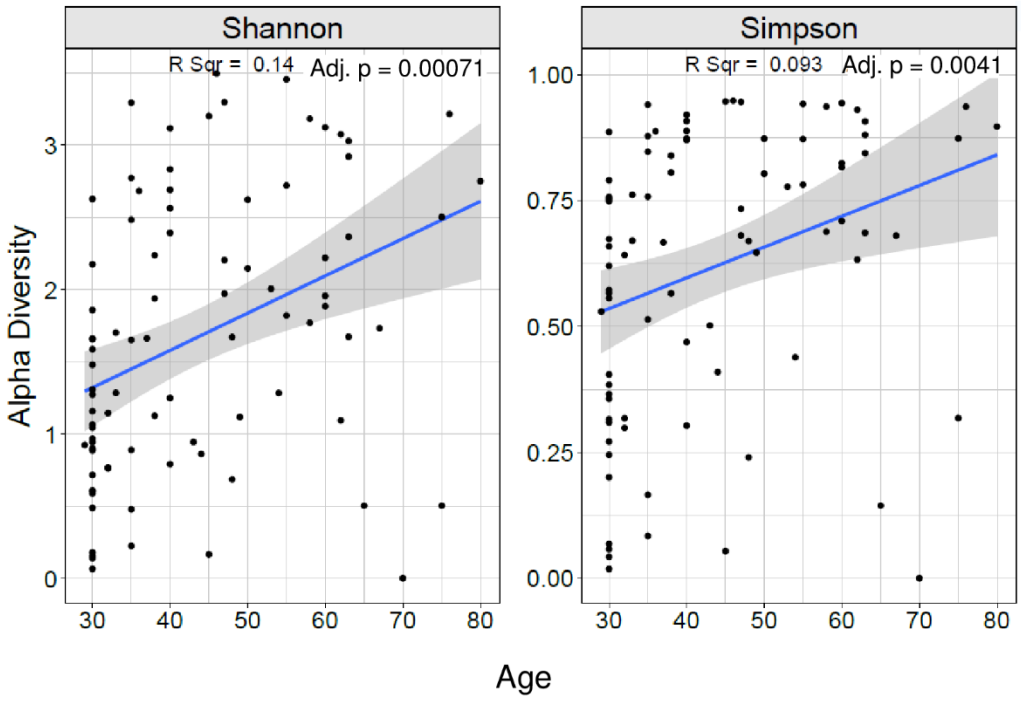
Table 5.16: Histological/Cytological status of study participants based on age category.

Histologic/cytologic findings	**Age <50 years**	**Age ≥50 years**
Cancer	28	32
Dysplasia	22	2
Negative	34	1

5.4.2. Age-related composition and diversity changes in the cervicovaginal microbiota

We observed age-associated alterations in cervical microbiota composition and diversity. Alpha diversity significantly increased with age (Shannon p=0.00071, Simpson p=0.0041; (**Figure 5.10 A**). Those younger than 50 years (n=84) had significantly increased levels of *Lactobacillus* (p=0.00003) and *Gardenella* (p=0.01), and those aged 50 years or older (n=35) had increased levels of *Porphyromonas* (p=0.00006), *Prevotella* (p=0.0041), *Bacteroids* (p=0.0023), and *Anaerococcus* (p=0.00097; (**Figure 5.10 B**). Because the two age groups had an uneven distribution of histologic or cytologic characteristics (**Table 5.16**), we compared only cancer patients from each age group (<50 years, n=28; and ≥50 years, n=32) (**Figure 5.11**).

A



B

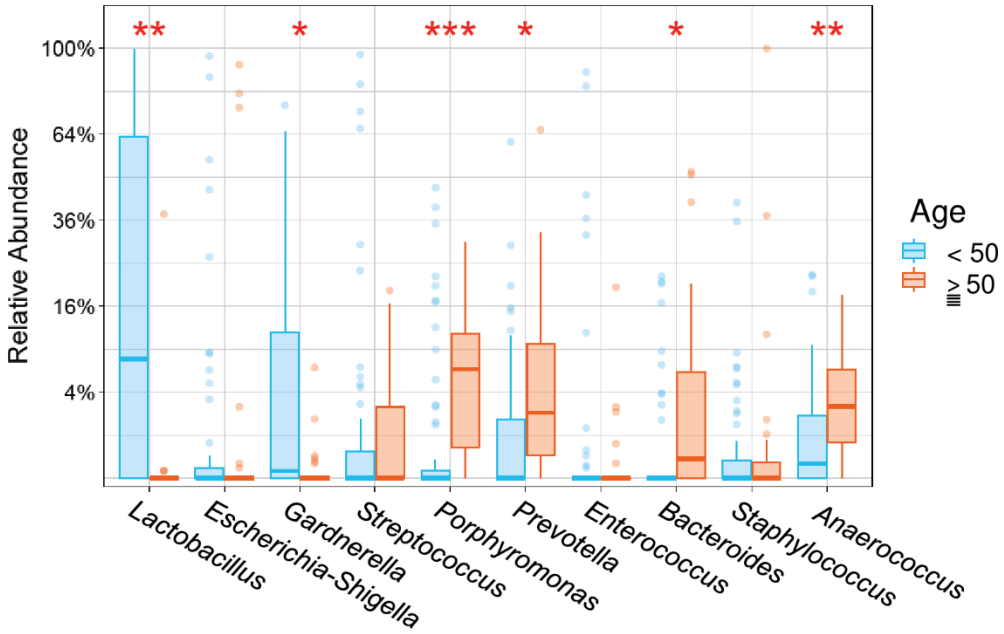


Figure 5.10: Correlation between alpha diversity (**A**) or relative abundance (**B**) and age in our study population. * Indicates statistically significant difference between age groups (*<0.05, **<0.005, ***<0.0005).

There were no differences in alpha (Shannon p=0.32, Simpson p= 0.39) or beta (p=0.46) diversities or microbiota composition among the women with cancer (**Figure 5.11**).

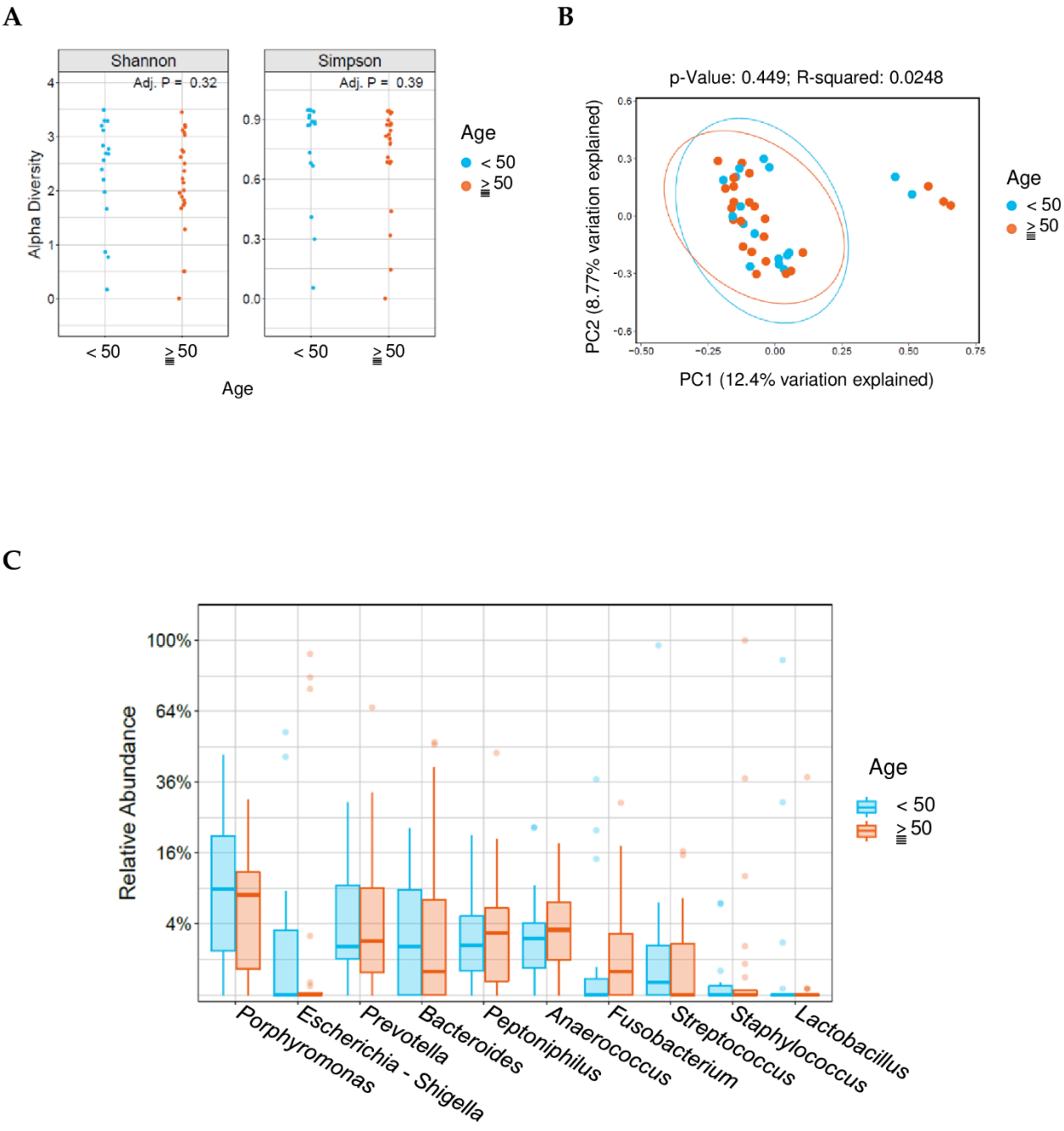


Figure 5.11. Alpha (A) and beta (B) diversity and microbial composition (C) among patients with cervical cancer who were younger than 50 years or aged 50 years or older.

5.4.3. Composition and diversity of cervicovaginal microbiota among HPV-positive and HPV-negative women

In our study population, we compared the alpha and beta diversity between patients who were HPV negative and those who were HPV positive. There was no significant difference in the alpha diversity between HPV positive and HPV negative women (Shannon P=0.21, Simpson P= 0.18) (**Figure 5.12 A**). Principal coordinate analysis (PCoA) (weighted UniFrac, Bray-Curtis) revealed significant differences in the microbial diversity between HPV positive and HPV negative women (P=0.02, **Figure 5.12B**).

A **B**

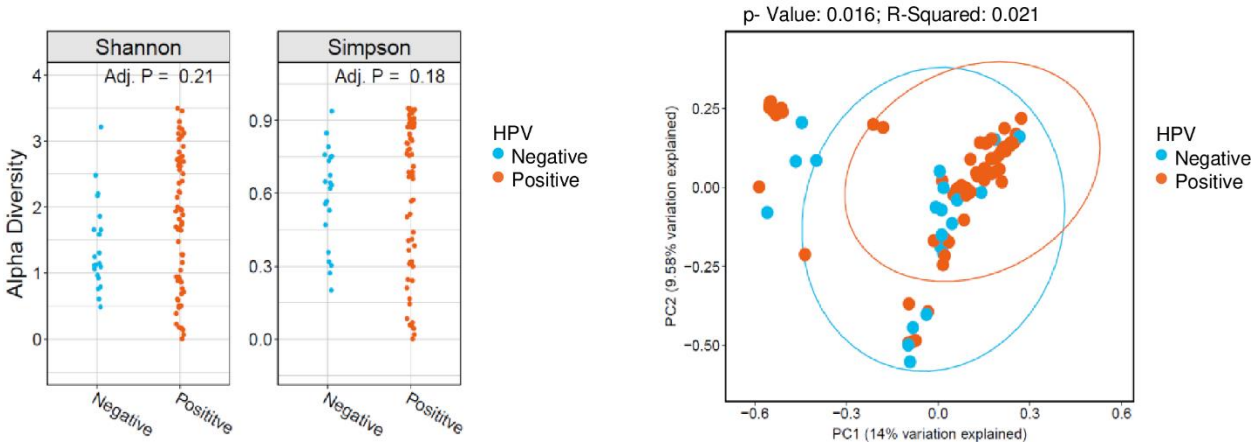
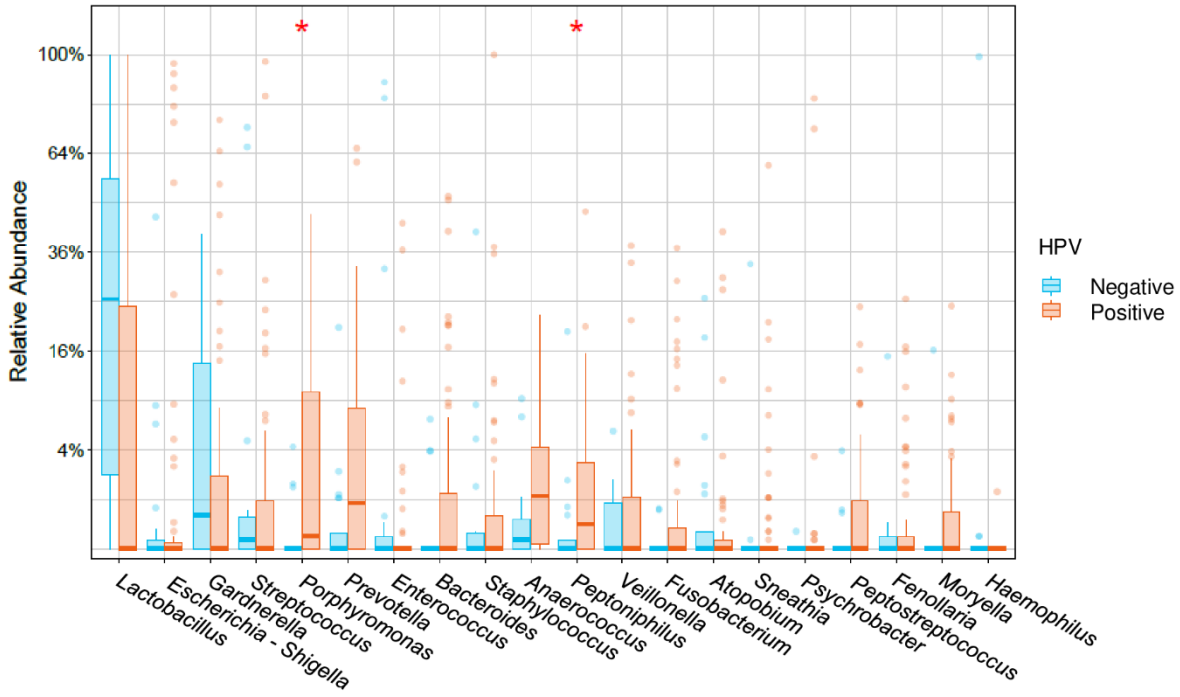


Figure 5.12. Alpha (A) and beta (B) diversity among HPV-positive and HPV-negative women in our study population.

Next, we compared the composition of the most abundant genera and species among the HPV positive and negative women. Looking at the top 10 most abundant genera, *Porphyromonas* (P=0.0204) and *Peptoniphilus* (P=0.0423) were significantly abundant in the HPV positive group (**Figure 5.13A**). However, we observed no significant difference in species among the HPV positive and HPV negative women (**Figure 5.13B**).

A



B

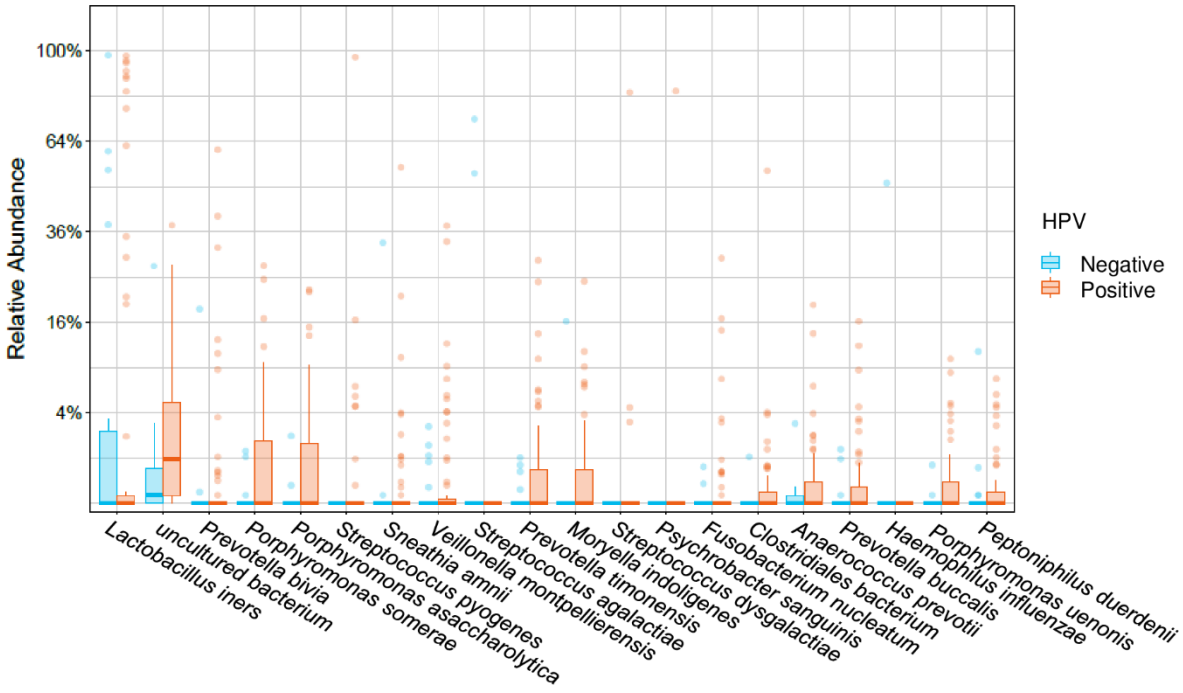
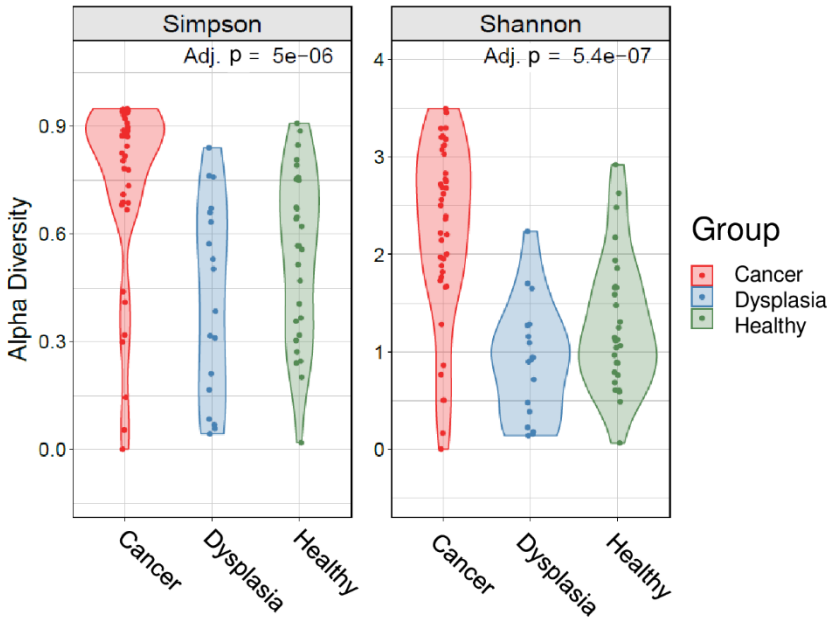


Figure 5.13. Microbial composition at the genus and species level among HPV-positive and HPV-negative women in our study population.

5.4.4. Cervicovaginal microbiota diversity and composition among healthy women, women with cervical dysplasia, and women with cervical cancer

We compared the cervical microbiome diversity among controls, women with dysplasia, and cervical cancer. We observed a significant difference in richness and evenness among the three groups (Shannon p= 0.00000054, Simpson p= 0.000005; **Figure 5.14 A)**. Using pairwise comparisons, all the indices showed that the cervical cancer group had the highest community diversity of cervical microbiota ($p < 0.05$, Kruskal-Wallis test). Principal coordinates analysis (PCoA) of the Bray-Curtis distances revealed differences in community structure in the cervical cancer samples when compared to dysplasia and normal women (Weighted Bray-Curtis UniFrac; p=0.001; **Figure 5.14 B)**.

A



B

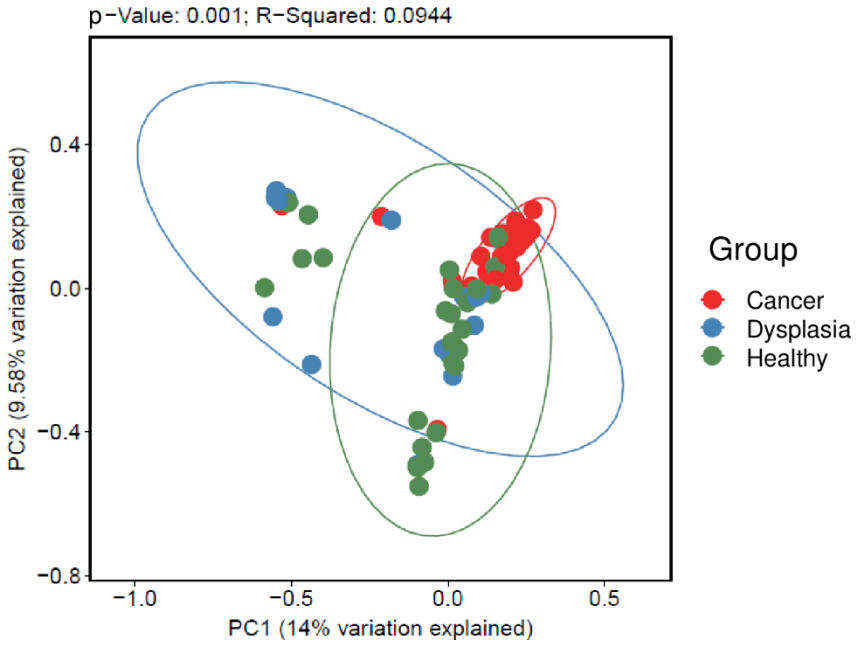


Figure 5.14: Alpha (A) and beta (B) diversity indices among women with cervical cancer, women with dysplasia, and healthy women. Beta diversity comparisons were calculated using the principal coordinate analysis discriminate. Abundance profiles in women with cervical cancer (n=60) were different from those of women with dysplasia (n=25) and healthy women (n=35).

Next, we characterized the microbial taxa abundances in our study population. The stacked bar plot (**Figure 5.15**) showed that there are distinct patterns of taxa in our study population. *Lactobacillus* was the dominant genera found in the study population.

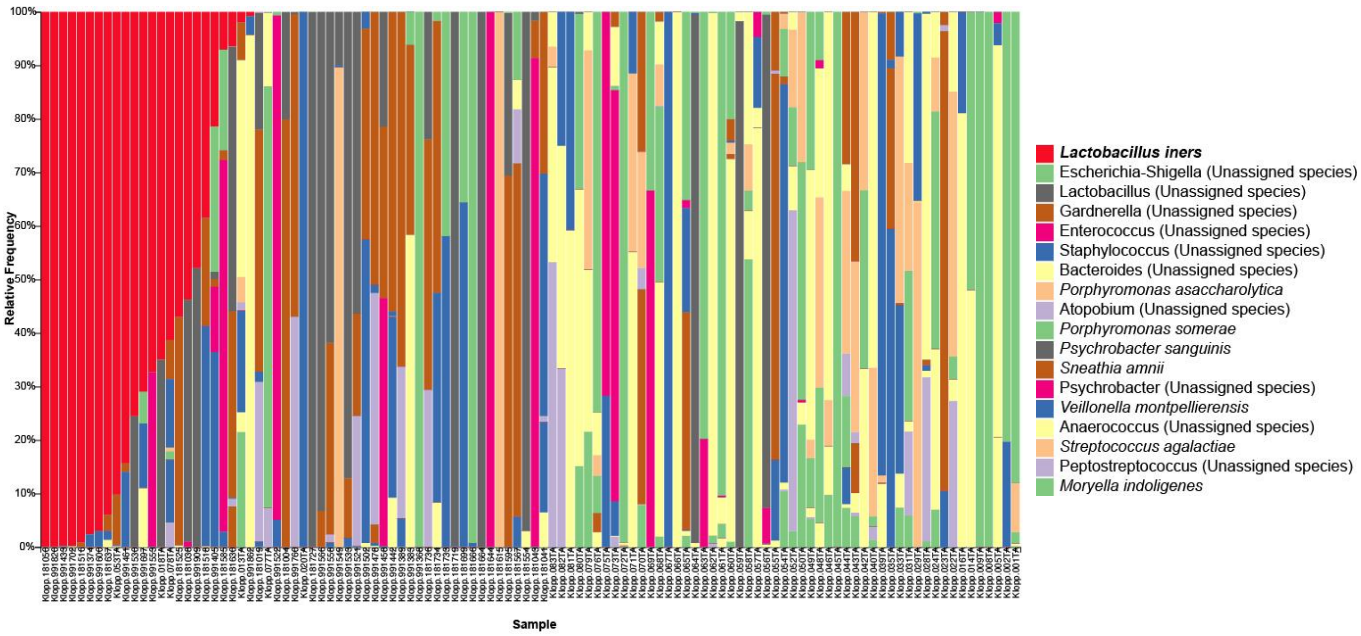


Figure 5.15: Stacked bar plot of the top 10 most abundant genus-level bacteria found in our study population. Each bar represents a single participant.

The relative abundance of cervicovaginal microbiota at the genus and species level was also characterized. The top 10 most abundant genera in the samples varied by group. A heat map (**Figure 5.16**; **Figure 5.17**) reflected the relative abundance of the most prevalent bacterial genera, showing that cervicovaginal microbiota composition changed from less diverse and more *Lactobacillus*-dominant in dysplasia to more diverse and less *Lactobacillus*-dominant in cervical cancer. Compared with healthy women and women with dysplasia, many of the women with cervical cancer had more *Porphyromonas* species.

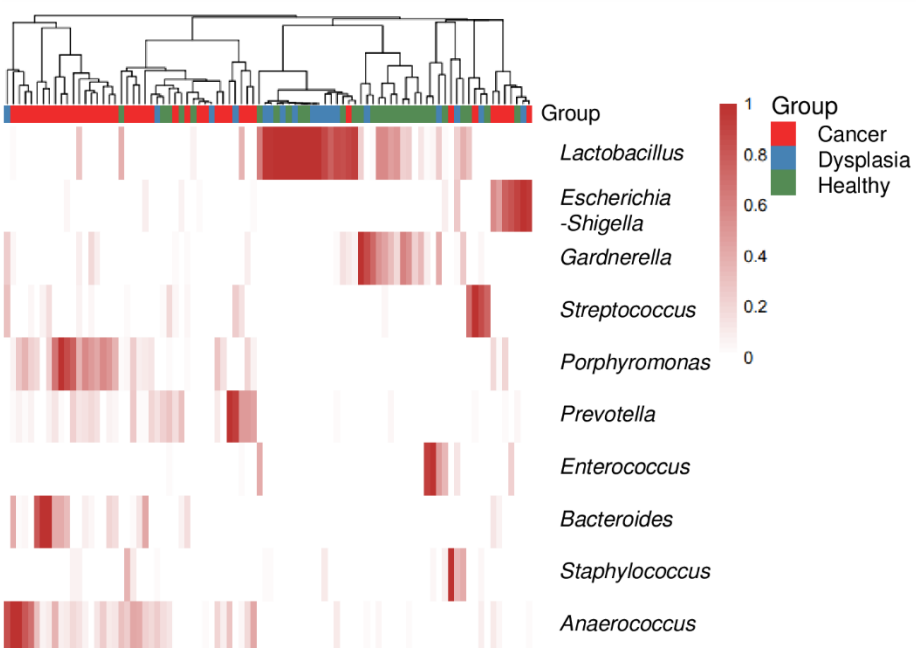


Figure 5.16: Heatmap of the top 10 most abundant genus-level bacteria in women with cervical cancer, women with dysplasia, and healthy women. Each bar represents a single participant.

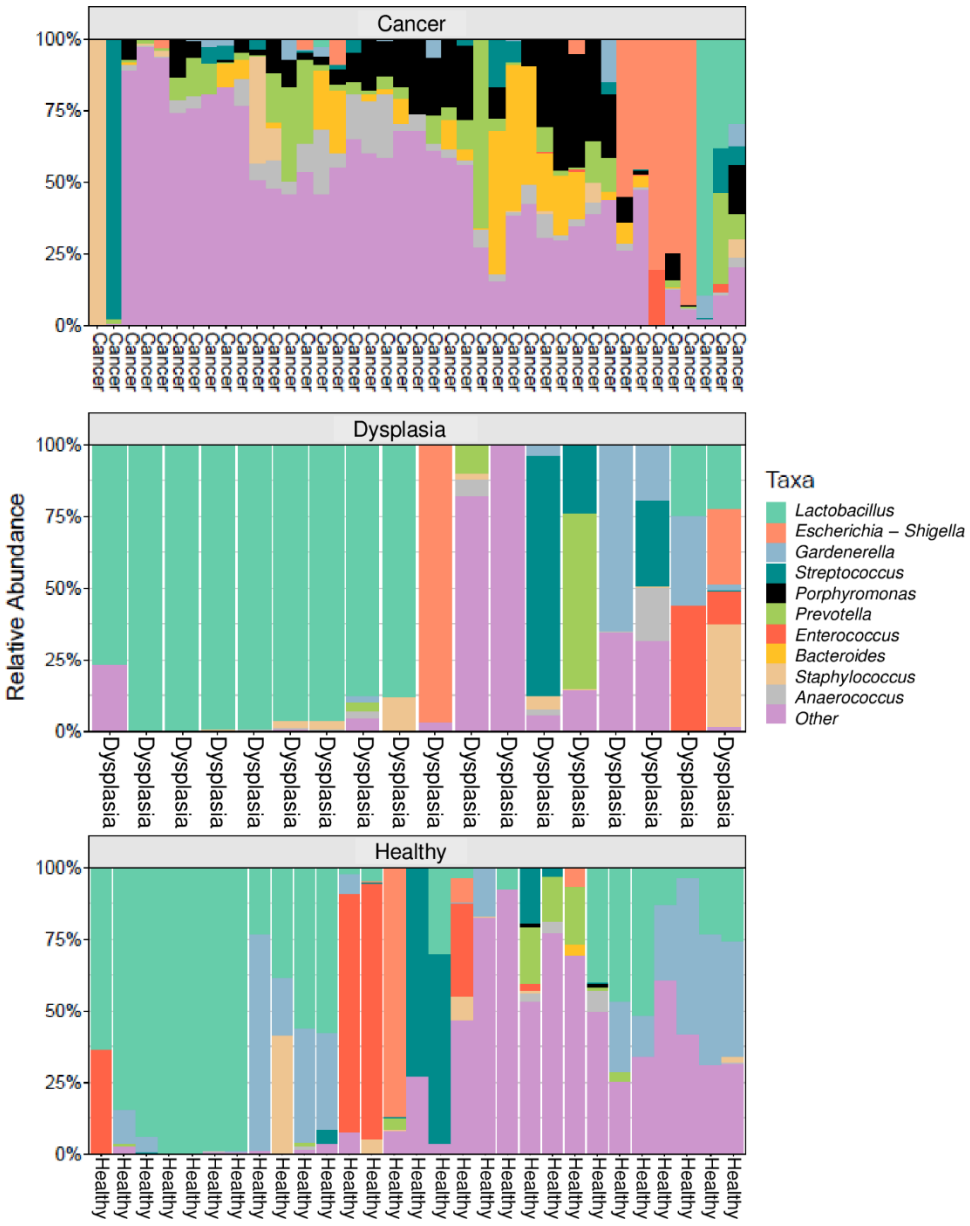
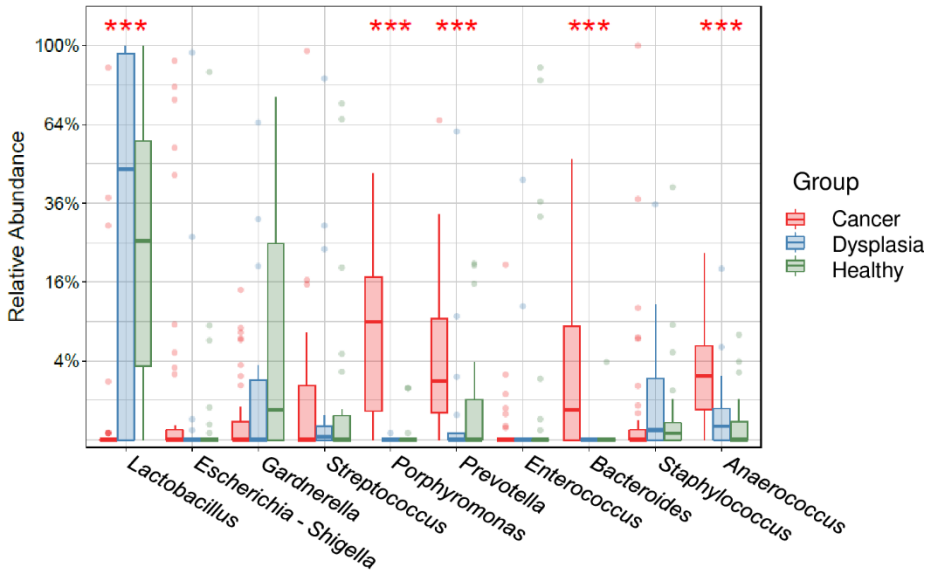


Figure 5.17. Stacked bar plot of the top 10 most abundant genus-level bacteria in women with cervical cancer, women with dysplasia, and healthy women. Each bar represents a single participant.

To identify significant differences in relative abundance at the species and genus level, we further analyzed taxa abundance using a box plot (**Figure 5.18**). *Lactobacillus* predominated in the dysplasia and healthy groups, and *Porphyromonas*, *Prevotella*, *Bacteroides*, and *Anaerococcus* predominated in the cervical cancer group ($p<0.05$, Kruskal-Wallis test). At the species level, *Lactobacillus iners* predominated in the dysplasia group ($p<0.05$) and *Porphyromonas somerae*, *Porphyromonas asaccharolytica*, and *Prevotella timonensis* were the most abundant species in the cervical cancer group ($p<0.05$; Figure 5.18).

A



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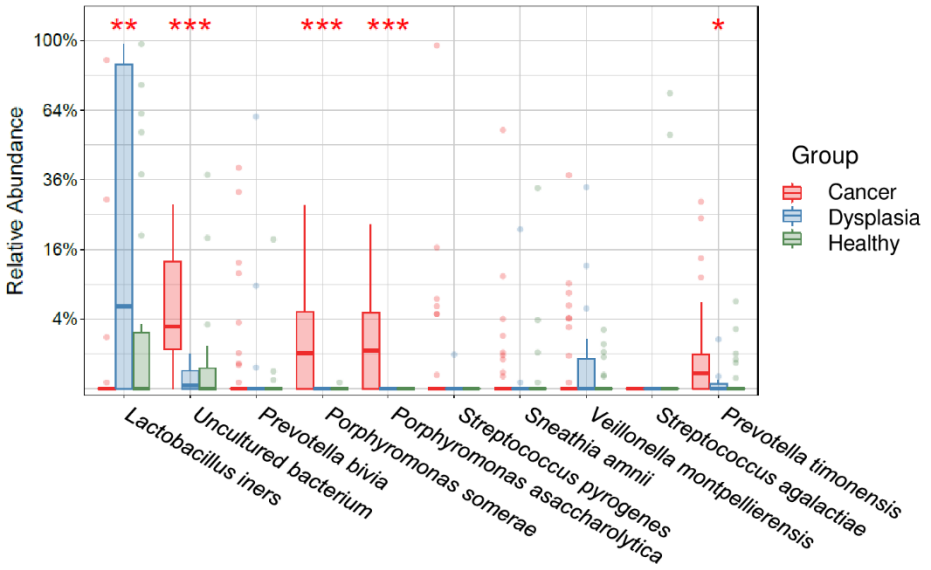

Figure 5.18: Relative abundance of the top 10 most abundant genera (A) and species (B) in women with cervical cancer, women with dysplasia, and healthy women.

6. Discussions

6.1. HPV prevalence and genotype distribution

One of the main objectives of this dissertation was to determine the prevalence of HPV and the circulating genotypes at the study area. As far as our knowledge is concerned, this is the first population-based study that determined overall HPV infection rates, genotypes of HPV circulating in the population, and age-specific prevalence of HPV by using self-sampling in rural Ethiopia. Previous few studies in the country have looked at HPV prevalence at hospital level among those women with gynaecological complaints, with cervical dysplasia or from Paraffin embedded cervical biopsy (Derbie *et al.*, 2022b).

The global strategy to eliminate cervical cancer as a public health problem calls for screening 70% of women by the age 35 and 45 years with a high-precision test and HPV testing is regarded as one of the most accurate and cost-effective method of primary screening (Cuschieri *et al.*, 2018). Therefore, establishment of laboratories that perform HPV DNA testing with the required quality and wider coverage is equally important in the effort to prevent cervical cancer.

In this first population-based study among rural women, self-sampling device to collect cervicovaginal specimen which in turn is new in its kind in the country was used and about 85.6% of the self-collected specimens had adequate DNA for HPV analysis according to our quality criteria while 14.4% inadequate DNA content as measured by the amount of β-globin. In our study, the problem of inadequate DNA content was high despite comprehensive demonstration of the self-sampling procedure and assistance offered by HEW upon request. This was in contrast to similar studies using a field approach and HEW supporting collection where less than 2% smears were with inadequate DNA (Krings *et al.*, 2019). Several factors could account for such relatively higher rate of poor-quality specimen. Cultural and religious issues associated with inserting foreign body and the fear of self-harm injuries may have contributed to the collection of not good samples as well as lack of experiences in self-sampling for other procedures will also account to some extent. We have reviewed literature to our capacity and very few had shown how much of the self-samples were analysable (Zehbe *et al.*, 2011). However, Zehbe *et al*., report was based on as few

as 49 samples. Otherwise, most of the studies we have reviewed did not disclose their quality of self-sample for the analysis of HPV DNA. Similar problems with the inadequacy of sample for HPV DNA test were observed in a study conducted in other part of Ethiopia (Jede *et al.*, 2020) at which significant number of the collected samples were with poor quality. The other possible reason why such amounts of samples were with inadequate DNA content might be due to most of the rural women in this study are illiterate (65.4%) and 30% only primary level education (Muluken Gizaw *et al.*, 2019) and could not manage to understand the demonstration and the self-collection so that the Evalyn brush self-sampler might not be inserted to the vagina as recommended. Our idea of associating illiteracy and being a rural woman with the quality of self-collected specimen is supported by other study our team is conducting in Addis Ababa (the capital city of Ethiopia) in which the proportion of invalid samples for HPV DNA testing was very low, less than 2% (unpublished data). Furthermore, the training given by the HEW might not be enough since the self-sampling device is used for the first time in the country. As far as what should be the case in Ethiopia in the future if self-sampling is the strategy for cervical cancer screening, we proposed to conduct a study to assess the challenges associated with this in the same and similar settings.

Any problem associated with transportation of the samples from the field to the lab also needs to be addressed. However, from this study, it was possible to proof that a self-sampling device can be used to increase participation in cervical cancer screening programme and employing HPV testing using self-sampling to screen for cervical (pre-) cancer in rural Ethiopia. Besides, HPV self–sampling increased the uptake of cervical cancer screening by approximately 20% in the study area, as compared to VIA invitation which is the current standard of care in Ethiopia, as we have shown in our previous publication (M. Gizaw *et al.*, 2019). Our result was supported by other similar studies indicating self-sampling might increase the number of people who are ready to take part in cervical cancer screening (Gupta *et al.*, 2018). This is primarily because it eliminates many of the obstacles that prevent women, particularly those with poor socioeconomic status, from taking part in routine screening programs.

Even if it is hard to compare and draw conclusions with previous few studies conducted in Ethiopia (Ruland *et al.*, 2006; R. Leyh-Bannurah, Prugger *et al.*, 2014) for a number of reasons, the observed population-based HPV prevalence in the study area was high (23.2%) and the hr-HPV infection was 20.5%. This prevalence was high not only compared with other studies in Ethiopia but also compared to the global HPV prevalence in women with normal cytology which is in the range of 11-12% (Forman *et al.*, 2012). However, the figure is comparable to the estimated prevalence of HPV burden in sub-Saharan African regions (24.0%) as stated in a meta-analysis of about 1 million women with normal cytology (Bruni *et al.*, 2010).

Because of the established fact that the higher the prevalence of HPV infection the higher the incidence of cervical cancer, determining how many of these hr-HPV positive women have persistent infection, progressive dysplasia, and/or invasive lesion is very crucial. A study from Ghana detected disease (CIN2+) predominantly in those women (6.7% of the investigated population) who had a genotype-specific persistence over >4 years (Krings *et al.*, 2019). In this study, the follow up results of the hr-HPV women is discussed in separate section in this cahpter. One of the inherent disadvantage of HPV testing is its low specificity and thus only defines women at risk of invasive disease. The implication of this is important in terms of what triage tests to employ in the region where there is limited resources and facilities if HPV-based screening is to be rolled out in national screening programs.

Determing the genotype of HPV circulating in the population is important to choose the HPV testing to implement in the national program of screening and assess the impact of the vaccination program in Ethiopia. In this first of its kind population-based study we found that HPV 16, 35, 52, 31, 45, 18 and 51 are the seven dominant high-risk genotypes in decreasing order that accounted for 87.9% of the infections. The association of the hr-HPV infection with invasive cancer and precancerous lesion is variable. In our study, of the total hr-HPV infected women, 35% had multiple infections. The relative contribution of such multiple infections in the development of invasive cancer and CIN3+ lesions require further investigation in our population. A study that involved a large number of women had shown multiple hr-HPV infection were present in 11.9% of ICC and 15.8% CIN3 lesions (Guan *et al.*, 2012).

In our study, HPV18 was less common than HPV31, 35, and 52. Compared to HPV31, 33, 52, and 58, the prevalence of HPV 18 in CIN3 lesion was less as described elsewhere (Guan *et al.*, 2012). In contrary, the absolute risk in causing CIN3+ and CIN2+ as well as the prevalence of HPV18 in cervical cancer is next to HPV16 (Castle *et al.*, 2010; Guan *et al.*, 2012; Denny *et al.*, 2014). However, unlike in different studies worldwide, HPV35 and HPV45 were also found as the most relevant hr-HPV types in HPV infected women. Most importantly, HPV35 (the second most dominantly detected in our study) is not included in the current 9-valent HPV vaccine (Gardasil 9). HPV35 is among the top 10 CC causing HPV types and reported in 3.9% of CC cases in Ethiopia (Bruni et al, 2019). Similarly, HPV35 was detected in 9.7% histologically confirmed ICC cases with single HPV infection in a study done in Ghana, Nigeria and South Africa (Denny *et al.*, 2014). Thus, the role of HPV35 in causing invasive cervical cancer in Ethiopia requires further studies.

In our study, the overall prevalence of hr-HPV infection declined sharply with age, the highest (12.0%) being in the age group (30-34 years). This is in agreement with the global trend of high infection rate at younger age (Jin *et al*., 2009; Baloch *et al.*, 2016; Khoo *et al.*, 2018). Even though the frequency of infection is lower, the infection identified in the older age group in our study could be a persistent infection and these women may require follow-ups more urgently. The prevalence of HPV infection at the younger age (30-34) is highest and one could conclude it will be more cost effective to implement HPV-based screening only as of the age of 34+ as an initial phase of HPV-based screening in Butajira setting.

6.2. hr-HPV persistence, clearance, and re-infection rates

This study also provided important data regarding longitudinal HPV infection dynamics at the population level in south central Ethiopia. This information is very crucial since cervical cancer is associated with persistent hr-HPV genotypes and there are no similar data in this population despite the high prevalence of HPV infection and cervical cancer in Ethiopia (Sung *et al.*, 2021; Teka *et al.*, 2021). Furthermore, our study is the first report concerning the natural history of hr-HPV infection among rural unscreened population. As indicated in the above-mentioned sections, the baseline prevalence of HPV infection in this study was 23.2% for any HPV and 20.5% for hr-HPV which determined the circulating genotypes at population level for the first time. The other objective of this study was, therefore, to follow all the hr- HPV positives for 2 years; HPV test was performed at 6 months and 24 months. Even though only approximately 60% of the invited women attended their 6 month and 24 months follow up, the prevalence of hr-HPV among the follow up women decreased to 26.3 % and 13.2% at 6 and 24 months respectively indicating high HPV clearance rates in our population.

Most of the hr-HPV infections were cleared during our follow up evaluation; with 73.3% and 86.8% clearance rates at 6 and 24 months respectively. Our results are consistent with viral clearance findings from previous reports (Ingabire *et al.*, 2018), which indicated that most (up to 90%) of hr-HPV infections are cleared within 2 years (Plummer *et al.*, 2007; Bosch *et al.*, 2008; Gravitt, 2011). However, this ratio of HPV viral clearance differs among countries due to several reasons. Our results were also supported by the justification that clearance of HPV is most frequent in the first six months of follow-up (de Sanjosé, Brotons and Pavón, 2018).

The natural history of HPV infection is influenced by different factors including environmental, host and viral factors. Therefore, it is very crucial to understand the specific population based factors that played key roles in the clearance and persistence of HPV infections since hr-HPV persistence for one or two years strongly predicts the development of cervical precancer and cancer (Kjær *et al.*, 2010; Castle *et al.*, 2011). Although our study did not assess and associate the possible factors for the persistence and clearance of hr-HPV, one of the possible reasons for this high clearance in our study population might be due to HPV antibodies acquired through natural

infection or cell mediated immunity. As we have seen from this study, baseline hr-HPV infection was high (20.5%) in the population, and this natural infection may provide protection against subsequent cervical HPV infections through high clearance rates. Our explanation was supported by other previous studies (Beachler *et al.*, 2016; Tian *et al.*, 2021; Yao *et al.*, 2021).

After clearance, however, there were also acquisition of new hr-HPV genotype in this follow up study. While the 70 and 77 of the women cleared their infections at 6 and 24 months respectively, 12.9% and 7.8% of them acquired new HPV infection as noted by the detection of a different genotype from the base line respectively. This indicated that the acquisition of new HPV infections after clearance is common when compared with previously negative HPV tests. This was supported in our report by determining the incidence of hr-HPV infection in the baseline negative women. That is from 80 tested women who were negative at baseline, only 3 (4.05%) acquired hr-HPV infections. What is important to note here is even though acquisition of new infection is common, the persistent infections would be the causes for a higher risk of malignant transformation compared to those with a type change after clearance (Elfgren *et al.*, 2017).

However, HPV persistency varies according to the target group and immune status of the women. When we look at the 24 months persistency, our study reported a relatively lower persistency compared to some results from previous studies; 13.2% and 26.3% at 24 months and 6 months respectively. The 2-year persistency were (19.2%) in Brazil (Rosa *et al.*, 2008), (49.1%) in Italy (Sammarco *et al.*, 2013), (31.4%) (Stensen *et al.*, 2016) and 26.9% (Nielsen *et al.*, 2010) in Denmark, (39%) (Ralston Howe *et al.*, 2009), in the United States and (44.1%) (Schmeink *et al.*, 2011) in the Netherlands. These differences might be due to differences in the target populations, who may have different potential risks for persistent infection and test intervals. The other co-factors for HPV persistence include smoking and hormonal exposure and smoking rate is very low in our study population (Schoenmaker, Hermanides and Davey, 2006; Wubegzier and Alemayehu, 2011) thus the low persistence rate can be explained by this. The other possible explanation of this relatively high clearance rate in our study was the age of the women, with mean age of 33 years, since studies have indicated that young women have the highest capacity for HPV clearance (Plummer *et al.*, 2007). Furthermore, the definition of persistence in different studies may also

contribute to the variation of persistence ratios since there is no consensus regarding the definition of persistent HPV infection.

The next action we asked was if HPV persistence rate is affected by the presence of multiple or single HPV infection at the baseline. However similar findings has been also reported by previous studies (Sammarco *et al.*, 2013; Stensen *et al.*, 2016; Sahin *et al.*, 2020). Previous reports suggested that carcinogenic HPV infections are essentially independent of each other, meaning the viral-viral level interactions are very minimal regarding persistence or clearance. However, other studies showed multiple HPV infections were more persistent as compared to single HPV infections when followed for 24 months (Philip E Castle *et al.*, 2009; Nielsen *et al.*, 2010; Miranda *et al.*, 2013).

From previous reports, different oncogenic HPV types have different duration until clearance and different carcinogenic potential. Our present study revealed that the most persistent HPV type at 6 months was HPV 68 (100%) which was followed by HPV 82 (75%) and HPV 53 (42.9%). At 2 years, the most persistent genotypes were HPV 68 and 59 (both with 50% persistence) followed by HPV 66 (20%) and HPV 52 (15.8%). But there were only 2 cases of HPV 68 and HPV 59 infections. Due to this small number of women with these HPV genotype, the type of specific persistence/clearance comparison may be difficult. The genotype of the most persistent HPV from previous studies were that HPV 16 and 18 (Rosa *et al.*, 2008; Sammarco *et al.*, 2013; Stensen *et al.*, 2016). Most studies of female genital HPV infections found that HPV 16 is the most persistent genotype. Another 6 year follow up Finnish Family HPV Study indicated that HPV16 was the most common HPV genotype during the entire follow-up (Louvanto *et al.*, 2010).

Despite the list is not yet complete, it is known that there are many co-factors that help HPV become a persistent infection and increase the risk of having cervical neoplasia and cervical cancer. STIs may facilitate hr-HPVs to enter the host and may also make it harder for the host to get rid of the HPV infection (Paba *et al.*, 2008a). Among the list of co-factors are infection with one or more other sexually transmitted infections. *Mycoplasma, T. vaginalis, C. trachomatis, N. gonorrhea,* and others are some of these sexually spread infectious agents and the association between these STIs and cervical abnormalities has been reported in many studies. In our follow-up study, we tested those women who were hr-HPV positives at the baseline screening for different

STIs at their 6th month follow up. The positivity rate for any STI was 52.9% (55/104). However, the prevalence of *T. vaginalis* and HSV-1 were identified in 1% of the tested women and this prevalence is lower than the reports from Africa. For example, *T. vaginalis* prevalence was estimated to be between 6% and 42% in Africa (Ramjee, Abbai and Naidoo, 2015). Although it has been shown in few studies that STI has no association with HPV, we are suggesting that further studies with larger sample sizes should be done to confirm the associations between STI pathogens and HPV in Ethiopia.

The other key aim of our study was to see the magnitude of abnormal cytological findings among those with persistently hr-HPV infected women. Because it has been stated that persistent infection with hr-HPV is the single best predictor of cervical cancer development (Muñoz *et al.*, 2003). In our study, the prevalence of abnormal cytology including ASCUS was 30% among the 97 women who attended the 6-month follow-up. This report was consistent with some previous studies that determined the prevalence of hr-HPV in women with abnormal cervical cytology. Song *et al* found that the overall prevalence of hr-HPV in women with abnormal cervical cytology was 32% (Song *et al.*, 2020). Regarding the prevalence of hr-HPV among HSIL, our report was supported by other studies. A study in Myanmar reported that 50% of HSIL patients were with hr-HPV DNA positive (Mu-Mu-Shwe *et al.*, 2014) which in agreement with our study that of the 10 women with HSIL, 50% of them were found to have hr-HPV infection.

However, studies have been indicating women who harboured persistent hr-HPV were significantly more likely to develop cervical neoplasia (Wallin *et al.*, 1999; Kjaer *et al.*, 2002; Cuschieri *et al.*, 2005). our study showed that only 27.6% of the women with abnormal cytology had type specific hr-HPV persistence. Furthermore, of the 10 women with HSIL, 50% of them were found to persist their type specific hr-HPV infection. This higher rate of hr-HPV clearance among women with abnormal cytology might be due the difference in quality of cytology reports and sample size in different studies. Furthermore, this might also be due to the age difference between our study and other studies so that the clearance rate is different with age groups. We are also suggesting that non-HPV cervical lesions needs to be studied in our population.

Despite its low coverage and uptake (Muluken Gizaw et al., 2019), the standard method of cervical cancer screening in Ethiopia is using VIA. The World Health Organization was advising low- and middle-income nations to use this visual examination and immediate treatment with cryotherapy.

In addition to the natural limitations of this method, many rural Ethiopian women find it challenging to obtain because the service was only available at the district hospital level in very few places.

In our study, we assessed the performance of VIA in cervical cancer screening by using it as a triage test for hr-HPV positive women. Of the 90 women who had both VIA and Pap smear, only 10% were positive for VIA while 32% of them were with abnormal cytology under Pap examination. In addition, among the VIA positive women 66.7% of them were negative for intraepithelial lesion or malignancy. This means that not only the specificity but also the sensitivity of VIA to detect cervical lesions is very low and it was discrepant with some studies (Chaman Ara; *et al.*, 218AD; Tayyeb, Khawaja and Malik, 2003; Naz and Hanif, 2014) showing that VIA had high sensitivity and acceptable specificity in pre-cancerous lesions and cervical cancer screening. However, due to the highly variable nature of the test, the sensitivity may be very low in some settings. Therefore, this different VIA performance in our study might be due to the subjective nature of the test and lack of training for the midwives who performed most of the test in our study because VIA demands rigorous training and periodic refresher courses for the providers. Accordingly, we are highly recommending to the ministry of health and other stakeholders in Ethiopia that efforts to improve the performance of VIA should be taken and an urgent need of replacing VIA with other alternative cervical cancer screening methods like the HPV DNA testing. Furthermore, additional large-scale studies should be done to assess the performance of VIA in Ethiopia.

6.3. Comparison of HPV genotyping assays

The current recommendation by WHO is to use HPV-based cervical cancer screening. Also it is required to assess the impact of the HPV vaccination worldwide (Wentzensen *et al.*, 2017), (World Health Organization, 2019). For both purposes, use of clinically validated tests is recommended in clinical practice (Arbyn *et al.*, 2016; Garland *et al.*, 2023). Extended HPV genotyping is used in some HPV based screening algorithm and the currently approved WHO guideline. Thus it is high time to compare the analytical sensitivity, genotype-specific specificity and the ease to use the different commercially available tests ability to compare results between timepoints and populations are important (Cornall *et al.*, 2017). Therefore, it has become very important to optimize HPV diagnostic workflow in different populations and settings. In this study, we investigated the analytical performance of three HPV genotyping assays using samples collected from selected women who previously tested hr-HPV positive in a population based follow up study in rural Ethiopia.

Although a good correlation of results was observed in both HPV positive and HPV negative samples, there were discordant results in positive samples among the three assays. The analytical performance of the different HPV assays can be affected by many independent and interdependent factors including the assay intrinsic sensitivity and specificity, storage condition of cervical swab samples and DNA extraction method (Donà *et al.*, 2011). However, in our case, because the samples were transported and processed under the same storage condition and the same DNA extraction method was used, the discrepancy of screening capacity of the three assays was solidly influenced by other factors such as inclusivity of HPV genotypes and type-specific sensitivity since the limits of detection inherent to an assay will determine how sensitively each genotype is detected.

Even though HPV16 and 18 are considered as the most important carcinogenic HPVs worldwide (Burd, 2003), there are also other HPV genotypes which are categorized as carcinogenic, probably carcinogenic, or possibly carcinogenic (Iarc, 2007). Therefore, due to their clinical significance, almost all available HPV genotyping assays can detect these high-risk group HPVs. For example, in our study, there were 14 overlapping hr-HPVs defined for detection between the three

genotyping assays. Two of the evaluated assays include 18 hr-HPV and potentially hr-HPV genotypes while the third is restricted to 14 high-risk genotypes. Hence, this inclusivity differs between HPV genotyping assays and non-uniformity in the classification of carcinogenicity. The comparisons of the tests were made based on the inclusivity of the tests.

For population screening, the analytical sensitivity of HPV testing assays needs considerable curiosity because high analytical sensitivity does not guarantee acceptable diagnostic i.e., clinical sensitivity (Trevethan, 2017) and specificity. Analytical sensitivity only represents the smallest amount of substance in a sample that can accurately be measured by an assay (Saah and Hoover, 1997). It is clear, that the analytical sensitivity differs among different HPV genotyping assays and may lead to controverted screening results between assays. Since the genotype-specific identification of HPVs with extended genotyping assays might be useful for test of cure, stratification of cancer risk, and to differentiate persistent from transient infections, genotype-specific validation of assays is crucial. Therefore, it is important to note that this study focused and evaluated/compared the analytical sensitivity of three different HPV assays, however, this was not further supported with pathologic findings. Thus, a highly analytically sensitive test could detect a large number of clinically insignificant positive results. Therefore, for population screening, analytical sensitivity for all HPV genotypes included in each assay should be adjusted to the result of cervical pathological findings, and both clinical sensitivity and specificity are important for patient safety and must be considered in the context of using current and future HPV DNA tests (Kinney, Stoler and Castle, 2010).

In this study, MPG-Luminex Assay was used as the reference genotyping test to determine the performance of the two assays as it has high and equal (relative) sensitivity and specificity like the EIA assay format (Arbyn and Hillemanns, 2018). The sensitivity of the EUROArray HPV assay to detect the 14 hr-HPV genotypes was 70% while Anyplex™ II HPV HR Detection Assay had an equal sensitivity (100%) with the reference assay. These differences can largely be explained by differences in the limits of detection of HPV genotypes in each assay. For example, among the less detected genotypes in EUROArray HPV assay in this study, HPV 16 and 51, have different detection limits in the two evaluated assays (150 vs 50 and 200 vs 50 copies/PCR for EUROArray HPV and Anyplex™ II HPV HR Detection, respectively) (Cornall *et al.*, 2017) . The decreased

detection of HPV 52 by EuroArray was unexpected since the detection limit of both assays for this specific genotype seems similar (50 copies/PCR). However, this might be due to the low copy number of HPV 52 so that it was missed in one 5µl sample taken for PCR in the EUROArray HPV detection. The low copy number of HPV 52 can be explained by the weak signal strength in Anyplex™ II HPV HR Detection. Out of the four missed HPV 52 HPV genotypes in EUROArray HPV detection, two of them were with weak signal strength (+) in the Anyplex™ II HPV HR Detection.

Regarding the genotype-specific agreement of the three assays in our study, moderate and above moderate agreements were observed for more than half of the HPV genotypes evaluated. Substantial agreement was perceived between the three evaluated assays for HPV 16, the most important carcinogenic genotype. HPV 18, the other important genotype, was detected only in one woman by Anyplex™ II HPV HR Detection Assay but not by the other two assays. This could be due to the low copy number of HPV 18 in the sample because it was detected with weak signal strength (+) in Anyplex™ II HPV HR Detection Assay. In our study, the other genotype detection difference was observed in HPV 68. HPV 68 was detected more frequently in Anyplex™ II HPV HR Detection Assay than EUROArray HPV when compared to the reference assay. This is likely due to variable efficiency of detection of HPV 68 subtypes a and b. EuroArray only being able to amplify subtype HPV 68A efficiently while Anyplex II amplifies both HPV68A and 68B subtypes (Cornall *et al.*, 2017) . Probes for both subtypes are also included in the MPG-Luminex assay.

Comparing the three assays with respect to the detection of Gardasil 9 included hr-HPV genotypes, four of the genotypes (HPV 18, 33, 45, and 58) out of the seven hr-HPV genotypes were only detected by Anyplex™ II HPV HR Detection Assay but not by the other two assays in the studied population. However, since the number of samples containing these types was too low it cannot be concluded yet whether Anyplex™ II HPV HR Detection Assay is more adequate to use for vaccine surveillance. The major limitation of this study is its small sample size of HPV positive samples that was in part due to an unexpectedly high clearing rate in the hr-HPV positive sampled screening population. Therefore, some HPV genotypes had too low prevalence for calculation of assay sensitivity and specificity. The other limitation of the study was the discrepancy of the genotyping assays might also be influenced by the viral load and the distribution of DNA in the

sample since the assays in this study were compared from single test run. However, this study enabled us to establish the technical competence and initial prevalence information for planning of future trials.

In different parts of the world, the access to different HPV assays is different. This mainly depends on the cost effectiveness, infrastructure, complexity of the assay, and whether the assays fit with existing processes and equipment within an individual laboratory. Since newer assays are being developed and released regularly, it is important to determine relative performance and levels of agreement before introducing them for screening or diagnostic use in different settings. After our comparison study, we observed that the Anyplex™ II HPV HR Detection Assay is 100% sensitive and 95.6% specific compared to the reference assay in detecting the 14 hr- HPV genotypes. Furthermore, from our experience and observation during the study, the Anyplex™ II HPV HR Detection Assay was with user-friendly workflow, requires less equipment, with few pipetting steps, no post-PCR handling and with short hands-on time which these in turn should be taken into account during HPV assays comparison and establishment in LMIC. Because most currently available molecular tests are too complex and/or costly for widespread use. Accordingly, we now established Anyplex™ II HPV HR Detection Assay in the HPV Reference Laboratory at Department of Microbiology, Immunology & Parasitology, Tikur Anbessa Specialized Hospital, Addis Ababa University.

6.4. Cervicovaginal Microbiota Profiles in Precancerous Lesions and Cervical Cancer

Although persistent infection with hr- HPV is a well-established risk factor and pre-requisite for cervical cancer, HPV infection is a heterogenous condition with varying outcomes, and the specific impact of other co-factors in cervical carcinogenesis is not yet well identified and characterized. Because the cervicovaginal microbiota differs with geography, ethnicity, and lifestyle, as well as infectious history, it is crucial to characterize the diversity and composition of the microbiota in different populations and the role of the microbiota in the progression of cervical cancer.

Recent literature has suggested that the cervicovaginal microbiota plays a mechanistic role in both HPV persistence and cervical cancer progression (Łaniewski *et al.*, 2018). In most healthy women of reproductive age, the cervicovaginal microbiota is dominated by *Lactobacillus species*, and a lack of this dominance is recognized as a cause of adverse reproductive health outcomes (Ravel *et al.*, 2011). Consistent with these findings, in the current study, we observed a positive relationship between alpha diversity and age, as well as *Lactobacillus* dominance in the reproductive years (i.e., women younger than 50 years). However, among the women with cervical cancer, there was no difference in the diversity or composition of the microbiota by age.

The current study was conducted among Ethiopian women with cervical cancer, histologically or cytologically confirmed dysplasia, or no evidence of cancer or dysplasia (healthy women) to characterize the diversity and composition of the cervicovaginal microbiota. To the best of our knowledge, the current study is the first to examine the relationship between the cervicovaginal microbiota and cervical cancer and/or HPV infection among women in Ethiopia. In our study, the cervicovaginal microbiota of most of the healthy women was dominated by *Lactobacillus*. Alpha and beta diversity was compared between HPV-positive and HPV-negative women regardless of their cervical cancer or dysplasia status, and we observed significant differences in beta diversity between HPV-positive and HPV-negative women, whereas alpha diversity analysis did not differ by HPV status.

Because our study participants with cervical cancer had different histologic diagnosis (i.e., squamous cell carcinoma or adenocarcinoma), microbial diversity was observed within the group, suggesting that microbiota diversity and composition may vary by specific cervical cancer diagnosis. Furthermore, genus and species diversity was higher in women with cervical cancer than in healthy women, which suggest that the stable composition of cervicovaginal flora, mainly *Lactobacillus* supplemented by other bacteria, is destroyed in carcinogenesis, resulting in increased microbial diversity. This finding is supported by other similar studies (Qingqing *et al.*, 2021; Wu *et al.*, 2021).

As indicated from previous studies (Borgdorff *et al.*, 2014; Aldunate *et al.*, 2015; Dareng *et al.*, 2020), depletion of *Lactobacillus* species from the cervicovaginal microbiota structure leads to proliferation of other pathogens and hence a change of the microbiota composition, including diversity and relative abundance of genera. This explains the higher alpha diversities in the women with cervical cancer in our study compared with those with dysplasia or healthy women.

Furthermore, among our study groups, relative abundance of some bacterial genera was significantly different. The predominant genus in the healthy group and dysplasia group was *Lactobacillus*. In the cervical cancer group, the genus *Lactobacillus* decreased in relative abundance, and other bacteria, such as *Porphyromonas*, *Prevotella*, and *Anaerococcus*, were the dominant genera. These results are consistent with those of other studies (Sims *et al.*, 2019; Chen *et al.*, 2020; Wu *et al.*, 2021). For example, Wu et al. demonstrated less *Lactobacillus* and higher diversity of microbiota were associated with more severe pathological status. Furthermore, *Porphyromonas* and *Prevotella* were identified as cervical cancer marker genera.

The role of *Porphyromonas* and *Prevotella* in carcinogenesis has been demonstrated in oral cancers (Karpiński, 2019), with three proposed mechanisms of action: chronic inflammation, anti-apoptotic activity, and carcinogenic metabolites released by these microbes. These bacteria produce inflammatory mediators that facilitate cell proliferation, mutagenesis, oncogene activation, and angiogenesis. *Porphyromonas gingivalis*, found in oral cavities, has been reported to induce lipopolysaccharides that lead to the production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) by macrophages and interleukin (IL)-1β and IL-6 by CD4+ T

helper cells (Amano *et al.*, 2004). Studies have also indicated that *Porphyromonas gingivalis* can mediate different signaling pathways that influence cell invasion, the cell cycle, anti-apoptosis, and inflammation (Karpiński, 2019).

Increased relative abundance of *Prevotella* in human mucosal sites has been shown to be related to various inflammatory disorders. This bacterium was indicated as a major modulator of host inflammatory responses in the female genital tract by increasing the amount of innate cytokines (IL-1α, IL-1β, IL-8, and TNFα) in cervicovaginal fluid, and production of cytokines related to Th17 (IL-23 and IL-17) and Th1 (IL-12p70 and interferon γ) (Anahtar *et al.*, 2016).

The increased relative abundance of *Porphyromonas*, *Prevotella*, and *Anaerococcus* in the cervical cancer group in our study indicates that these bacteria may play a substantial role in the development of cervical cancer, supporting the previously proposed mechanisms of chronic inflammation, anti-apoptotic activity, and production of carcinogenic substances. Future studies should assess the mechanistic relationship of a diverse cervicovaginal microbiota with cervical cancer in women in resource- limited settings, as well as the impacts of different intervention strategies. Currently, there are several treatment outlooks for cervical cancer: including the use of immunotherapy and target therapy, in combination with conventional chemotherapy or in combination with radiotherapy. Therefore, from such studies, cervicovaginal microbiota-derived bacterial markers can be used as a predictive model to predict the progression or regression of precancerous lesions and undertake further research with large sample size and possibility of identifying the right probiotics in women with persistent HPV infection or precancerous lesions of different stages, and invasive cancer that can affect chemotherapy and radiotherapy outcomes (D'oria *et al.*, 2022).

This study had both strengths and weaknesses. Among the strengths, it is the first study in the country that studied the natural history of HPV infections at population level. In addition, this was the first study which applied self-collection device and used a very sensitive HPV testing method in both the baseline and follow-up studies. Unfortunately, this study was limited by high number of lost follow up women. Despite repeated efforts to trace and invite all the women for follow up, only 60% of the women were available at 6 months and 59% at the 24 months follow up.

Furthermore, there were only few positive cases for some of the HPV genotypes and thus limits the analysis of persistence and clearance rates. The other limitation was two different sample collection devices were used in this study (the Evalyn Brush and Isohelix swab). Although studies revealed the Evalyn® self-sampling device performed equally well compared to samples taken by a clinician in Illumina MiSeq sequencing of the 16S rRNA gene (Virtanen *et al.*, 2017), the different sampling strategies may not yield comparable vaginal microbiota composition and diversity. In addition, we did not consider the detailed characteristics of the study participants, like sexual characteristics, behavioural characteristics, and clinical characteristics. Furthermore, we used cytology, not histology for the classification of dysplasia.

7. Conclusions

Our study provided new data on the overall prevalence of HPV infection and distribution of specific HPV types in rural Ethiopia. The overall prevalence of hr-HPV was high as expected for an unscreened population. This implies that the risk of developing cervical cancer could be high in Ethiopian women unless organized HPV-based screening and treatment programs are implemented in the country. In our study population, HPV16, HPV35, HPV52, HPV31 and HPV45 were the most prevalent genotypes. Since our study is the first population-based study in the country, this result serves as a baseline data and is essential for the development of public health policy for cervical cancer prevention.

The study provided information regarding changes in hr-HPV status over 6 months and a 2-year interval among Ethiopian women. Our results demonstrated that most infections resolved spontaneously, and only small fractions of the women experienced persistent infections or new HPV infections. The most persisted hr-HPV genotypes were different in our study. Further large-scale studies are needed to better understand the natural history of HPV infections and the factors that are associated with HPV type-specific change patterns in Ethiopia.

In our microbiome analysis, we found differences in cervical microbiota diversity, composition, and relative abundance between cervical cancer patients, women with dysplasia and controls. The diversity and composition of cervical microbiota changed from LSIL to HISL to cancer state and increased L. iners was found in women with LSIL. Other studies need to be carried out in Ethiopia or in any other regions in order to further validate the role of cervical microbiome in development of cervical cancer.

We also evaluated two L1-and one E6/E7-targeting PCR DNA tests for the detection and differentiation of HPV genotypes. The three evaluated assays showed similar analytical performance as a screening tool for the 14 hr-HPV infections proposed by WHO for cervical cancer screening and moderate or better concordance in HPV genotyping. Complexity of assays is profoundly different and can also impact on assay choice. The sensitivity of the HPV assays compared was high, however, this was not further supported with colposcopy (or other triage test)

or any histologic findings to confirm that the detected hr-HPV positivity had any clinical significance in this study. Further research is required to confirm the clinical benefit in a LMIC setting that can be gained from the full genotyping offered by these assays. Thus, we have planned to conduct another study that address this issue.

8. References

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9. List of publications and papers presented

9.1. List of publications in this Thesis

1. **Brhanu Teka** , Muluken Gizaw , Friederike Ruddies , Adamu Addissie , Zewditu Chanyalew , Anna Sophie Skof , Sarah Thies , Adane Mihret , Eva Johanna Kantelhardt , Andreas M Kaufmann , Tamrat Abebe . Population-based human papillomavirus infection and genotype distribution among women in rural areas of South-Central Ethiopia. *Int J Cancer*. 2021 Feb 1;148(3):723-730. doi: 10.1002/ijc.33278.

2. **Brhanu Teka**, Muluken Gizaw, Ededia Firdawoke, Adamu Addissie, Tesfamichael Awoke Sisay, Carola Schreckenberger, Anna Sophie Skof, Sarah Thies, Adane Mihret, Eva Johanna Kantelhardt, Tamrat Abebe, and Andreas M. Kaufmann. A Technical Comparison of Human Papillomavirus Genotyping Assays from a Population-Based Cervical Cancer Screening in South Central Ethiopia. *Cancer Management and Research.* 2022:14 1–11. https://doi.org/10.2147/CMAR.S360712

3. **Brhanu Teka**, Kyoko Yoshida-Court, Ededia Firdawoke, Zewditu Chanyalew, Muluken Gizaw, Adamu Addissie, Adane Mihret, Lauren E. Colbert, Tatiana Cisneros Napravnik, Molly B. El Alam, Erica J. Lynn, Melissa Mezzari, Jhingran Anuja, Eva Johanna Kantelhardt, Andreas M. Kaufmann, Ann H. Klopp, and Tamrat Abebe. Cervicovaginal Microbiota Profiles in Precancerous Lesions and Cervical Cancer among Ethiopian Women. *Microorganisms 2023, 11, 833. https://doi.org/10.3390/microorganisms11040833*

9.1.1. Co-authored publications (not included in this thesis)

1. Gizaw M, **Teka B**, Ruddies F, Abebe T, Kaufmann AM, Worku A, Wienke A, Jemal A, Addissie A, Kantelhardt EJ. Uptake of Cervical Cancer Screening in Ethiopia by Self-Sampling HPV DNA Compared to Visual Inspection with Acetic Acid: A Cluster Randomized Trial. Cancer Prev Res (Phila). 2019 Sep;12(9):609-616. doi: 10.1158/1940-6207.CAPR-19-0156. Epub 2019 Jul 23.

2. Gizaw M, **Teka B**, Ruddies F, Kassahun K, Worku D, Worku A, Wienke A, Mikolajczyk R, Jemal A, Kaufmann AM, Abebe T, Addissie A, Kantelhardt EJ. Reasons for Not Attending Cervical Cancer Screening and Associated Factors in Rural Ethiopia. Cancer Prev Res (Phila). 2020 Jul;13(7):593-600. doi: 10.1158/1940-6207.CAPR-19-0485. Epub 2020 May 5.

3. Ruddies F, Gizaw M, **Teka B**, Thies S, Wienke A, Kaufmann AM, Abebe T, Addissie A, Kantelhardt EJ. Cervical cancer screening in rural Ethiopia: a cross- sectional knowledge, attitude, and practice study. BMC Cancer. 2020 Jun 17;20(1):563. doi: 10.1186/s12885-020-07060-4.

4. Jede F, Brandt T, Gedefaw M, Wubneh SB, Abebe T, **Teka B**, Alemu K, Tilahun B, Azemeraw T, Gebeyehu A, Schmidt D, Pesic A, Kaufmann AM, Abebe B, Ayichew Z, Byczkowski M, Vaucher T, Sartor H, Andargie G, Bärnighausen T, von Knebel Doeberitz M, Bussmann H. Home-based HPV self-sampling assisted by a cloud-based electronic data system: Lessons learnt from a pilot community cervical cancer screening campaign in rural Ethiopia. Papillomavirus Res. 2020 Jun; 9:100198. doi: 10.1016/j.pvr.2020.100198. Epub 2020 May 8.

9.2. Conference presentations resulting from this Ph.D.

1. 32nd International Papillomavirus Conference (IPVC2018); October 2-6, 2018, Sydney, Australia.
    - **Brhanu Teka***, Muluken Gizaw, Adamu Addissie, Andreas M. Kaufmann, Eva Johanna Kantelhardt and Tamrat Abebe. Quality of samples collected with Evalyn self-sampling brush for HPV DNA testing in Ethiopia (**Poster presentation**).

2. African Immunobiology of Parasites, Pathogens, and Pathogenesis (AFRIBOP 2019), 26th – 30th August, KEMRI –Welcome Trust Research Programme, Kilifi, Kenya.
    - **Brhanu Teka***, Muluken Gizaw, Friederike Ruddies, Adamu Addissie, Adane Mihret, Anna Sophie, Sarah Thies , Andreas M. Kaufmann, Eva Johanna Kantelhardt, and Tamrat Abebe. Infection and genotype distribution of Human Papillomavirus in Ethiopia and the role of Vaginal Microbiome and Immune response in cervical carcinogenesis: A population- based follow up study (**Poster presentation**).

3. 33rd International Papillomavirus Conference (IPVC2020); July 20-24, 2020, Barcelona, Spain.
    - **Brhanu Teka***, Muluken Gizaw, Friederike Ruddies, Adamu Addissie, Adane Mihret, Anna Sophie, Sarah Thies, Andreas M. Kaufmann, Eva Johanna Kantelhardt and Tamrat Abebe. Population-based Human Papillomavirus infection and genotype distribution among women in rural areas of south-central Ethiopia (**ePoster presentation**, number **166).**
    - A. Kaufmann, **B.T. Endallew**, A. Skof, M. Gizaw, D. Worku, S. Thies, A. Addissie, T. Abebe, E. Kantelhardt. Quantigene-based HPV E7 and cellular biomarker mRNA detection increases specificity of HPV screening in Ethiopia (**ePoster** presentation, number **938).**
4. 12th International African Organisation for Research and Training in Cancer (AORTIC) Conference, November 5, 2019 - November 8, 2019, Maputo, Mozambique (**participation).**

5. 2nd Annual conference on HPV and Cervical Cancer Research Consortium in Ethiopia, February 20-21, 2020, in Addis Ababa, Ethiopia
    - **Brhanu Teka***, Muluken Gizaw, Friederike Ruddies, Adamu Addissie, Zewditu Chanyalew, Anna-Sophie Skof, Sarah Thies, Adane Mihret, Andreas M. Kaufmann, Eva Johanna Kantelhardt and Tamrat Abebe. Population-based Human Papilloma virus infection and genotype distribution among women in rural areas of South-Central Ethiopia (**Oral presentation**).

6. EUROGIN 2023 - International Multidisciplinary HPV Congress. February 8th to 11th, 2023 - BILBAO – SPAIN
    - **Brhanu Teka***, Muluken Gizaw, Ededia Firdawoke, Zewditu Chanyalew, Adamu Addissie, Adane Mihret, Eva Johanna Kantelhardt, Andreas M. Kaufmann, and Tamrat Abebe. A two-year community-based follow up study of HPV infection in Ethiopia: Molecular epidemiology, genotyping, persistence, clearance, and re-infection rates among rural women (**Oral presentation-** Session: FC17- Free communications- Epidemiology and natural history 3).
7. 35th International Papillomavirus Conference (IPVC 2023), April 17-21, 2023, in Washington DC, USA.
    - **Brhanu Teka***, Muluken Gizaw, Ededia Firdawoke, Zewditu Chanyalew, Adamu Addissie, Adane Mihret, Eva Johanna Kantelhardt, Andreas M. Kaufmann, and Tamrat Abebe. A two-year community-based follow up study of HPV infection in Ethiopia: Molecular epidemiology, genotyping, persistence, clearance, and re-infection rates among rural women (**Oral presentation –** Session: Public Health Oral: Screening for HPV-Related Disease 2) .
    - **Brhanu Teka**, Kyoko Yoshida-Court, Ededia Firdawoke, Zewditu Chanyalew, Muluken Gizaw, Adamu Addissie, Adane Mihret, Lauren E. Colbert, Tatiana Cisneros Napravnik, Molly B. El Alam, Erica J. Lynn, Melissa Mezzari, Jhingran Anuja, Eva Johanna Kantelhardt, Andreas M. Kaufmann, Ann H. Klopp, and Tamrat Abebe. Cervicovaginal Microbiota Profiles in Precancerous Lesions and Cervical Cancer among Ethiopian Women (**Poster Viewing** - Shift 01: Basic Science-01l. Microbiome)

10. Appendixes

10.1. Appendix 1: DRERC Ethical clearance

Department of Microbiology, Immunology andParasitology (DMIP)

Department Research Ethics Review Committee (DRERC)

Meeting No: DRERC/03/2020 Date: 29 May 2020

Protocol Title: Human Papilloma Virus genotype distribution, persistence and the role of cervicovaginal microbiome on rate of progression in cervical carcinogenesis: A population- based follow up study		
Principal Investigator	Brhanu Teka	
Institute/Department	CHS-AAU/DMIP	
Type of review	[x] Initial Review	[ ] Amendment [ ] Other (specify):_____
Elements Reviewed	[ ] Attached	[ ] Not attached
Decision of the meeting	[ ] Approved	[ ] Approved with Recommendation
	[ ] Revision requested	[ ] Disapproved
Action Required	[x] Send to IRB	[ ] Authorize Implementaion

Obligations of the PI:

i. Should comply with the standard international and national scientific and ethical guidelines
ii. All amendments and changes made in protocol and consent form needs DREC approval
iii. The PI should report Serious Adverse Events (SAE) within 10 days of the event
iv. End of the study, including thesis work and manuscript should be reported to the DREC

Follow up report expected in:

3 Months_____ 6 Months __X__ 9 Months _____ one year _____

Asrat Hailu (Prof)

Chair, DRERC

Signature

Date: 29/05/2020

Department Stamp:

10.2. Appendix 2: IRB Ethical clearance

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

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 08/2020 Meeting Date: August 26,2020

Protocol number: 079/20/DMIP

Protocol Title: Human Papilloma Virus genotype distribution, persistence and the role of cervicovaginal microbiome on rate of progression in cervical carcinogenesis: A population-based follow up study	
Principal Investigator:	BrhanuTeka
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: 02
2. Protocol Version Date:
3. Informed consent Version No: 02
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: October 06,2020 to, October 05,2021
Follow up report expected in: 3 Months___ 6 Months____ 9 Months _X_One year____

Co-Chairperson, IRB
Dr. Yimtubezinash W/Amanuel

Signature _____
Date: 06/10/2020

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

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature _____

Date Oct 7, 2020

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: 07/2017 Meeting Date: 07/09/2017

Protocol number: 057/17/SPH

Protocol Title: Optimizing Cervical cancer Screening modalities and identifying the Molecular Epidemiology of Human Papilloma Virus in Ethiopia: A cluster randomized Trial		
Principal Investigator:	Muluken Gizaw	
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: 02
2. Protocol Version Date:
3. Informed consent Version No: 02
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: February 26, 2020 to February 25, 2021
Follow up report expected in: 3 Months___ 6 Months__X__ 9 Months _ _one year____

Co-chairperson, IRB
Dr. Yimtubezinash W/Amanuel

Signature

Date: 26/02/2020

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

Director of Research & Technology Transfer, CHS
Dr. Wondwossen Amogne

Signature

Date

10.3. Appendix 3: MoSHE Ethical Approval

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Ministry of Science and Higher Education - Ethiopia

Ref.No. ሳ4/246/68/21
Date 25 MAY 2021

Addis Ababa University, College of Health Sciences (AAUCHS)
Addis Ababa

Subject: Letter of Approval

The Ministry of Science and Higher Education (MoSHE) via its National Research Ethics Review Committee has reviewed ***Human Papilloma Virus genotype distribution, persistence and the role of cervicovaginal microbiome on rate of progression in cervical carcinogenesis: A population- based follow up study***" Project protocol in an expedited manner. We are writing to advise you that MoSHE has granted full approval to the above named project, for a period of **one year (May 21, 2021- May 20, 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 MoSHE. Please ensure that you submit biannual progress report to MoSHE 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.

. Improving post-trial access arrangements in clinical trials in Sub-Saharan Africa

Sincerely

Daniel Taddesse Wolde
(PhD)
Research Ethics Director

Cc

- Office of the State Minister (Sector for Science and Community Service)
- Science and Research Affairs Directiorate General
- Research Ethics Directorate
  MoSHE
- Mr.Brhanu Teka (PI)
  AAUCHS

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10.4. Appendix 4: CDC sample transfer permit

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

Centers for Disease Control and Prevention
Division of Select Agents and Toxins
1600 Clifton Road NE, Mailstop H21-7
Atlanta, GA 30329
Telephone: 404-718-200; 404-488-7100 (after hours) Email: importpermit@cdc.gov

SAFER · HEALTHIER · PEOPLE

Permit to Import Infectious Biological Agents, Infectious Substances, and Vectors

In accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulators, cited on the bottom of this permit, permission is granted the permittee to import into any port under control of the United States, or to receive by transfer within the United States, the material described in Item 1 below.

PHS PERMIT NO. : 20210503-1755A	ISSUED DATE: 05/05/2021 EXPIRATION DATE: 05/05/2022
1. DESCRIPTION OF MATERIAL	HUMAN BODILY FLUIDS THAT MAY CONTAIN HUMAN IMMUNODEFICIENCY VIRUS.
2. PERMITTEE (NAME, ORGANIZATION, ADDRESS AND CONTACT INFORMATION)	**TRAVIS SOLLEY (409) 789-3962** **AUTHORIZED USER: KATHRYN AZIZ (832) 710-7754** **MD ANDERSON CANCER CENTER** **6565 MD ANDERSON BLVD UNIT 1052 Z6.4018** **HOUSTON TX 770304009**
3. SOURCE OF MATERIAL (NAME, ORGANIZATION, ADDRESS, COUNTRY)	TEKA ENDALLEW (251) 913-500065 ADDIS ABABA UNIVERSITY DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY, SCHOOL OF MEDICINE, COLLEGE OF HEALTH SCIENCES, ADDIS ABABA UNIVERSITY, TIKUR ANBESSA SPECIALIZED HOSPITAL ADDIS ABABA 9086 ETHIOPIA
4. TYPE OF PERMIT AND INSTRUCTIONS FOR USE	MULTIPLE IMPORTATIONS A. RECORD OF EACH IMPORTATION SHALL BE MAINTAINED ON PERMANENT FILE BY PERMITTEE. B. USDA/APHIS MAY REQUIRE ADDITIONAL PERMITS FOR MATERIALS FROM ANIMALS, MATERIALS EXPOSED TO ANIMAL PRODUCTS/BYPRODUCTS, AND AGENTS THAT ARE INFECTIOUS TO ANIMALS OR PLANTS.U.S.FISH AND WILDLIFE SERVICE MAY REQUIRE ADDITIONAL PERMITS FOR MATERIALS FROM ENDANGERED ANIMALS.
5. CONDITIONS OF ISSUANCE ITEMS APPLICABLE WHEN CHECKED	PACKAGING MUST CONFORM TO 49 CFR SECTIONS 171-180. WORK WITH THE AGENT(S) DESCRIBED SHALL BE RESTRICTED TO AREAS AND CONDITIONS MEETING REQUIREMENTS IN THE CDC/NIH PUBLICATION "BIOSAFETY IN MICROBIOLOGICAL AND BIOMEDICAL LABORATORIES. AS THE PERMITTEE, YOUR FACILITY WILL BE SUBJECT TO INSPECTION AT SOME TIME IN THE FUTURE TO CONFIRM THAT THE IMPORTERS BIOSAFETY MEASURES ARE COMMENSURATE WITH THE HAZARD POSED BY THE ITEMS TO BE IMPORTED AND THE LEVEL OF RISK GIVEN ITS INTENDED USE. ALL MATERIAL IS FOR LABORATORY USE ONLY - NOT FOR USE IN THE PRODUCTION OF BIOLOGICS FOR HUMANS OR ANIMALS.
6.SIGNATURE OF ISSUING OFFICER	Samuel S. Edwin SAMUEL S. EDWIN, PH.D. DIRECTOR, DIVISION OF SELECT AGENTS AND TOXINS

CDC 0728 (F 13.40) REV. 4-13

42 CFR 71.54. Permit to Import Biological Agents, Infections Substances, and Vectors
A person may not import into the United States any infectious biological agent, infectious substance, or vector unless: It is accompanied by a permit issued by the Centers for Disease Control and Prevention (CDC). The possession of a permit issued by the CDC does not satisfy permitting requirements placed on materials by the U.S. Department of Agriculture that may pose hazards to agriculture or agricultural production in addition to hazards to human health.

10.5. Appendix 5: Material Transfer Agreements

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Addis Ababa University, School of Medicine, Department of Microbiology, Immunology and Parasitology, Ethiopia ("**Provider**") and The University of Texas M. D. Anderson Cancer Center, located at 1515 Holcombe Boulevard, Houston, Texas 77030, USA ("**Recipient**"), a member institution of The University of Texas System and an agency of the State of Texas, in the transfer of Provider research material (samples, derivatives, and specimens) to the Recipient related to the protocol "*Molecular and microbial drivers of cervical cancer progression and response.*"

"**Provider Scientist**": Brhanu Teka, Department of Microbiology, School of Medicine, Immunology and Parasitology,Addis Ababa University, Ethiopia

"**Recipient Scientist**": Dr. Ann Klopp, Department of Radiation Oncology, The M. D. Anderson Cancer Center, Houston, Texas, USA.

Provider agrees to transfer to Recipient's the following research materials /specimen:

Cervical Swab before and after therapy in stabilization buffer for microbiome analysis, TCR sequencing, HPV genotyping and 5-10ml blood for the analysis of serum markers, ("**Material**").

1. The Material will only be used for research purposes as described in the protocol by Recipient Scientist in the Recipient Scientist's laboratory for the research project described below, under suitable containment conditions. This 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 applicable rules and regulations related to the Research Project and the handling of the 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 Health Research Ethics Review Committee (NERC) and Addis Ababa University faculty of Medicine Ethics Review Committee recommendations and their approval?

   Yes [√] No [ ]

2. This Material will be used by Recipient Scientist solely in connection with the following research project ("**Research Project**") described with specificity as follows "*Vaginal Microbiome in cervical cancer: the driver of malignant progression and treatment resistance.*"

3. In all presentations or written publications concerning the Research Project, Recipient shall include and acknowledge Provider's contribution of the Material as academically and scientifically appropriate, based on provision of the Material or other direct contribution to the Research Project..

4. The Material represents a significant contribution on the part of Provider and is considered proprietary to Provider. Recipient therefore agrees to retain control over the Material and further agrees not to transfer the Material to other people not under her/his direct supervision without advance written approval of Provider.

5. The Provider does not take any responsibility for loss, damage, wastage or spoilage of the 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 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 Material will not infringe any patent or proprietary right of third parties. Notwithstanding the foregoing, Provider represents that to the best of its knowledge, Provider has not received any claims that the use of the Material infringes any patent, patent application, trade secret, or other property or proprietary rights.

6. The Recipient shall notify the Provider in confidence and in witting of any improvement or discovery conceived and first actually reduced to practice during the conduct of the Research Project and arising from the performance of the Research Project under this Agreement, here in after referred to as "**Inventions**". 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, other than as explicitly provided herein.

7. The under- signed Provider and Recipient expressly certify and represent that the contents of any statements made herein are truthful and accurate.

8. The Provider maintains, ownership right of the Material unless stated otherwise.

9. The Provider will retain a copy of every sample sent abroad as much as possible for local research needs.

10. This MTA is subject to cancellation by providing 30 (thirty) days' notice to the other Party.

11. Notice of State Agency: Recipient is an agency of the State of Texas and under the Constitution and the laws of the State of Texas possesses certain rights and privileges, is subject to certain limitations and restrictions, and only has such authority as is granted to it under the Constitution and laws of the State of Texas. Notwithstanding any provision hereof, nothing in this Agreement is intended to be, nor will it be construed to be, a waiver of the sovereign immunity of the State of Texas or a prospective waiver or restriction of any of the rights, remedies, claims, and privileges of the State of Texas. Moreover, notwithstanding the generality or specificity of any provision hereof, the provisions of this Agreement as they pertain to Recipient are enforceable only to the extent authorized by the Constitution and laws of the State of Texas; accordingly, to

the extent any provision hereof conflicts with the Constitution or laws of the State of Texas or exceeds the right, power or authority of Recipient to agree to such provision, then that provision will not be enforceable against Recipient or the State of Texas.

Material Transfer Agreement

Signature page

For Recipient:

Duly Authorized
DocuSigned by:
Nyma Shah
Name: Nyma Shah
Title: Director Research Funding
Date: 7/12/2019

Read and Acknowledged by Recipient Scientist:
DocuSigned by:
Ann Klopp, M.D., Ph.D.

Mailing Address for Material:
Dr. Ann Klopp
Radiation Oncology Department
The University of Texas M. D. Anderson Cancer Center
1515 Holcombe Boulevard - Unit 1422
Houston, Texas, 77030 USA
Tel: (713) 563-2444
aklopp@mdanderson.org

Notice should be given to:
The University of Texas M. D. Anderson Cancer Center
Legal Services
7007 Bertner Ave., 1MC11.3433
Houston, TX 77030
Attn: Chief Legal Officer
Phone: (713) 745-6633; Facsimile: (713) 745-6029

A copy of the notice should be given to:
The University of Texas M. D. Anderson Cancer Center
Office of Research Administration
7007 Bertner Avenue, Unit 1676
Houston, TX 77030
Attn: Associate VP, Research Administration
Phone: (713) 792-3672; Facsimile: (713) 792-7455

Reviewed and Approved by
UTMDACC Legal Services for
UTMDACC Signature:
#21705 06/10/2019

For Provider:

Provider's Investigator
Brhanu Teka
Signature

Date: July 19, 2019
Mailing Address:
Addis Ababa University, College of Health Sciences, School of Medicine, Department of Microbiology, Immunology and Parasitology, Tikur Anbessa Specialized Hospital
CHS building 6th floor room number 610,
P.o.Box 9086, Addis Ababa, Ethiopia
Tel: +251913500065

Duly Authorized:
Prof. Daniel Asrat
Signature/ Stamp
July 19, 2019

Date: _____
Mailing Address for Notices:
Addis Ababa University, College of Health Sciences, School of Medicine, Department of Microbiology, Immunology and Parasitology, Tikur Anbessa Specialized Hospital (TASH)
TASH building 2nd floor room number 69,
P.o.Box 9086, Addis Ababa, Ethiopia
Tel: +251911 223019

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 Martine Luther University, Halle, Germany in all transfer of research material (samples, derivatives, and specimens) related to the protocol "*Optimizing cervical cancer screening modalities and identifying the molecular epidemiology of Human Papilloma Virus in Ethiopia: A cluster randomized trial*".
Provider: Brhanu Teka, Addis Ababa University, Department of Microbiology, Immunology and Parasitology, Ethiopia

Recipient: Dr. Eva Kantelhardt, Institute of Epidemiology, Biometry and Informatics Martine Luther University, Halle-Wittenberg, Germany. Provider agrees to transfer to recipient's designated (provider) the following research materials /specimen. Cervical swab and DNA extracted from the cervical swab collected from women attended in cervical cancer screening.

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 '' *Optimizing cervical cancer screening modalities and identifying the molecular epidemiology of Human Papilloma Virus in Ethiopia: A cluster randomized trial* ''.
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

Dr. Eva Kantelhardt

Senior scientist
Signature ----------
Date ---------- 9.1.18

Mailing Address for Material:

Magdeburger Street 8
Halle, Germany
Tel: +49-(0)345-557 4166
+49-(0)1511 692 4147
Fax: +49 (0)345 557-3580

Martin-Luther-Universität Halle-Wittenberg, Institut für Medizinische Epidemiologie, Biometrie und Informatik 06097 Halle (Saale)

Duly Authorized

Prf. Dr. Rafael Mikolajczyk
Director, Institute of Epidemiology, Biometry and Informatics, Martine Luther University, Halle Germany

Signature/ Stamp ----------
Date ----------

Mailing Address for Notices:

Magdeburger Street 8
Halle, Germany
Tel: +
Fax: +49 (0)345 557-3580

Martin-Luther-Universität Halle-Wittenberg, Institut für Medizinische Epidemiologie, Biometrie und Informatik 06097 Halle (Saale)

For Provider:

Provider's Investigator
Brhanu Teka
Signature----------
Date: 10/01/2018
Mailing Address:
Addis Ababa University, Department of Microbiology, Immunology and Parasitology, College of Health Sciences, School of Medicine, CHS building 6th floor room number 610,
P.O.Box 9086, Addis Ababa, Ethiopia
Tel: +251913500065

Duly Authorized

Dr. Tamrat Abebe

Signature/ Stamp ----------
Date: 10/01/2018

Mailing Address for Notices:

Addis Ababa University, Department of Microbiology, Immunology and Parasitology, CHS building, 6th floor room number 608,
P.O.Box 9086, Addis Ababa, Ethiopia
Tel: +251911447227

10.6. Appendix 6: Standard Operating procedures (SOPs)

EVALYN BRUSH PREPARATION
Document type: Standard Protocol of Operations (SOP)
Written by: Brhanu Teka
Date: 30.01.2018

DAY 1

1. Clean the work surfaces
   Use alcohol to clean the working bench and place a new green working paper.
2. Prepare the materials needed
   Make sure you have enough 2ml Eppendorf tubes and gloves.
3. Take out the Evalyn brushes
4. Label the 2ml Eppendorf tubes according to numbers of Evalyn brushes
5. Using the 2ml Eppendorf tube remove the Evalyn brush
   Place the brush inside the tube, close the cap of the tube and pull
6. **Change gloves between each brush!!!**

- Especially crucial step to prevent cross-contamination

7. Fill each tube with 1ml of PBS
   Change tips between samples
8. Vortex the tubes with brushes vigorously for 1min
9. Keep the tubes at room temperature overnight (or if longer at 2-8C)

DAY 2

10. The next day: Prepare new 2ml and 1.6ml Eppendorf tubes for each brush

- Label according to brush numbering

11. Vortex the tubes with brushes vigorously for 1min
12. Centrifuge for 5min at 2500rpm
13. By using the 1000µl pipette tips (non-filter) remove the brush from the tube careful not to spill around

Place the tip of the 1000µl pipette tip inside the top of the brush, and by tipping carefully place in a new 2ml Eppendorf tube you already prepared

14. Save the tubes with brushes at -20C
15. After removing the brush, under the hood **thoroughly mix the left-over PBS (re-suspend the pallet)** and aliquote 100ul into a 1.6ml Eppendorf tube you already prepared.

- Note: Make sure the Hood has been sterilized with UV light before use. If not turn UV on for 10min

16. The leftover of PBS is to be stored at -20C
17. The tubes with 100ul are now ready to be extracted.
18. Continue with SOP "DNA Extraction" or store at 2-8C for shorter period. For longer storage of aliquots keep them at -20C.

DNA EXTRACTION WITH BACTERIA LYSIS BUFFER
Document type: Standard Protocol of Operations (SOP)
Written by: Aleksandra
Date: 30.01.2018

1. Turn on the Thermal Mixer - should be set to 65°C and let warm up
2. Clean the hood and surfaces around

   Use alcohol to clean the working bench and Sagrotan to clean the hood, pipettes, markers that are in the hood.
3. Turn the UV light on for 10 – 15min
4. Change gloves
5. Take samples from the freezer/refrigerator

   Leave to thaw.
6. Prepare the materials needed for extraction

   Make sure you have enough 1000µl pipette filter-tips, Bacteria Lysis Buffer (BLP) and Neutralizing Buffer.

Add one more tube for Negative control. Label it as NC DNA and with date of DNA extraction

7. Add 200µl of 5x BLP into each sample
   Mix well.
8. Vortex shortly
9. Incubate samples in Thermal Mixer at 65°C for 10min, 600rpm
   Before starting, make sure the mixer is warmed up to 65°C. Samples should be incubated under continuous shaking.
10. Centrifuge for 30sec at 2500rpm to remove the water droplets that are on the cap of the tube
11. Stop lysis by adding 200µl of 5x Neutralizing Buffer.
    Mix well.
12. Vortex shortly
13. Centrifuge for 30sec at 2500rpm to remove the water droplets that are on the cap of the tube
14. Samples are to be stored at -20C for longer, or at 2-8C for shorter period of time (few days).
15. Clean the hood and surfaces
16. Turn on the UV light

Make sure to place the racks in the hood for cleaning too.

DNA Purification from Blood or Body Fluids (Spin Protocol) using QIAamp DNA Mini kit
Document type: **Standard Protocol of Operations (SOP)**
Written by**: Brhanu Teka**
Date: **20/02/2019**

- This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from whole blood, plasma, serum, buffy coat, lymphocytes, and body fluids using a ***microcentrifuge***.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- 200 µl of whole blood yields 3–12 µg of DNA. Preparation of buffy coat is recommended if a higher yield is required.

Things to do before starting

- Clean the bench, pipettes and equipments (use alcohol first and then Sagrotan for cleaning)
- Wear Gloves at all times of the extraction process
- Check all equipments and consumables are in place
- Equilibrate samples to room temperature (15–25°C).
- Heat a water bath or heating block to 56°C for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions.
- If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.

Procedure

1. Label 1.5 ml micro tubes with sample Id.
2. Mix the sample in order to have homogenous sample
3. Vortex the proteinase K briefly
4. Quickly centrifuge the proteinase K approximately 10 seconds (optional)
5. Pipet 20 µl proteinase K into the bottom of a each 1.5 ml microcentrifuge tube
6. Vortex the sample briefly and add 200 µl of sample to each microcentrifuge tube.
   Note: If the sample volume is less than 200 µl, add the appropriate volume of PBS.
7. Add 200 µl Buffer AL to each sample. Mix by pulse-vortexing for 15 seconds.
   Note: Do not add proteinase K directly to Buffer AL
8. Incubate the microtubes at heating block which is pre heated at 56°C for 10 min.

9. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
10. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s.
11. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

    Note: If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally.
12. Prepare the QIAamp Mini spin column for each sample by labelling the same number
13. Carefully apply the mixture from step 11 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min.

    Note: Close each spin column to avoid aerosol formation during centrifugation
14. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided) and discard the tube containing the filtrate.
15. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided) and discard the collection tube containing the filtrate.
16. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min
17. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min

    Note: This step helps to eliminate the chance of possible Buffer AW2 carryover
18. Label 1.5 ml microcentrifuge tubes (not provided)
19. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate.
20. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min
21. Discard the spin column and store the 1.5 ml microcentrifuge tubes containing DNA at -20 ^{o}C.

Polymerase chain reaction (PCR)

SOPS Loading of PCR

Rule: Make sure you have not entered any other laboratory room before you enter the Pre-PCR Master Mix room. Clean everything before beginning (refrigerator, cupboard doors, edges, and handles)

- UV eradiate equipment for 5min

  Calculate the required amount of MM: 20µL/PCR tube (ready as 1mL) + 5µl template DNA = final solution 25 µL
- Takeout from freezer to defreeze, quickly spin down in small table centrifuge and put inside the Pre-PCR station
- Takeout PCR tubes from cabinet, close and put them in racks. Pipet 20 µLMM into each carefully
- Transfer the PCR tubes into transfer racks to take them to >DNA room
- Then clean everything, put used station equipment to radiate UV for 30min

  Leave them room and lock it

DNA loading

- Clean everything to be used
- Takeout the DNA from freezer for loading to defreeze
- Put the DNA tubes in racks in logical order leaving one space between rows for easy pipetting
- Transfer the MM tubes from the transport rack to the loading rack for more stability while pipetting.
- Label each MM tube with the number on respective DNA tube
- Put DNA rack on left side and MM rack on right side (this depends on the people's preferences)
- For pipetting, use the dedicated pipet in the paper box "for PCR loading only"
- Open DNA carefully using left hand and MM right hand (It depends on the person's preference)
- Pipet 5 µL to each MM with exact the same number (mix a little by pipetting up and down once)

- Always put used DNA sample one row away to monitor pipetting steps, you can use a new pipet tip box and use the tips in the same order as pipetting the samples to keep track of the order (people in the lab use different techniques to avoid mistakes in this regard).

PCR room

- Take lab coat
- Put PCR tubes in the machine rack
- Switch ON the thermocyclers
- Put the tubes in thermocycler
- Take the Positive Control out of the Freezer and add to PK tube
- Choose program: Dana >TypisirungAmpliTaq> press START

  The machine will ask you if you use tubes or plates, select "tubes" and press "Start" again.
- Wait for machine to reach 100C and make a sound
- Come back in 3h or next day. Store at 4C-8C in PCR room fridge (use purple racks), for long time storage transfer the tubes on to the transport racks and store the PCR product at -20°C.

Hybridization of PCR products for genotyping using Luminex

Aim of the method:

Hybridization of PCR products generated with the GP5+/6+ general primer system, with HPV-type specific probes for the detection and genotyping of HPV.

Test procedure:

1. Make sure to first create a plan with your sample numbers and the plate set up. You can find an empty form in the following folder: P:\HPV-Typisierung\Luminex\Proben. Save it with the respective date in your folder.
2. Prepare the hybridization:
   - Take the reagents from the fridge in the S1 lab (Beadmix, Stain buffer, PBS, if needed the calibration kit)

- Switch on the thermal shaker on the S2 lab hood, set the temperature at 42 °C, 650 rpm, > 35 minutes. Switch it on for a few seconds; otherwise, it will not start heating up. The thermomixer needs about 30 minutes to warm up, so make sure you switch in on in time to avoid stringency problems.
- Switch on the Luminex first and then the respective computer and open the Luminex program for the laser to warm up.
- Label your hybridization and the filter plate with your name and the date
- Make sure you have enough yellow and grey filtered pipette tips as well as non-filtered yellow tips

3. Aliquot 7 µl TE-Buffer in each well needed
4. Pipet 10 µl of the respective PCR product into each well of the 96-well plate, according to your plate set-up.
5. Vortex the beadmix for 30 seconds.
6. Calculate how much beadmix you will need for your samples. For this calculate the following: number of samples x 33µl + 10%
7. Pipet 33 µl beadmix into each sample well using the multi-channel pipette. Mix the sample, TE-buffer and beadmix by pipetting up and down once. Avoid creating bubbles though. Make sure to change the tips after each well.
8. Place the sticky foil on the 96-well plate to close all the wells.
9. Place the plate into the thermocycler in the PCR room, select the following program: „tina/dana/hyb 10min95“ and press start.
10. Press “Abbrechen” about 3 seconds before the cycler starts cooling the samples down and take them out of the cycler. Quickly press the hot plate on the ice tube rack.
11. Cool the samples down on the ice tube rack for about 1 minute, while carrying them back into the S2 lab. Then place them in the pre-heated thermoshaker and incubate at 42°C for 35 minutes.
12. During this incubation time prepare the filter plate, with which you will work from now on. Place it on the vacuum pump and fill every row you will use for your samples with 100µl PBS/well and let it rest like this until the incubation time is over (at least 15 min).

13. Also, during the incubation time prepare the stain buffer. For this add 8ml stain buffer with 5µl Streptavidin-PE (1:1600) for a full plate or 4ml stain buffer + 2,5 µl Streptavidin-PE for half a plate in a 15ml falcon tube. Keep the tube in aluminum foil to keep it dark.
14. And finally, you can already set-up the Luminex during this incubation step. Make sure you use the right needle for your work with the Luminex and if needed change it. Also, fill the sheeth container up to the mark with sheeths and close the lid well.
15. After the incubation time is over, remove the PBS from the filter plate by switching on the vacuum pump and pressing the plate with the lid closed on it. Look closely if all PBS was sucked out of the plate. The vacuum pressure should not rise above 5 bar.
16. Take the hybridization plate and spin it down in the plate centrifuge for 10 seconds.
17. Carefully remove the sticky plastic foil and make sure to not spill into any other well.
18. Transfer the samples with a multi-channel pipet and filtered pipet tips into the respective well of the filter plate. Make sure to pipet the sample up and down 5 times carefully to swirl up the beads and avoid clumps before transferring. Always change tips between the different samples.
19. Remove the liquid with the vacuum pump as descrived above (step 15).
20. You can leave the plate on the vacuum pump during the following washing steps.
21. 1x washing: pipet 100µl PBS into all rows that you use of the 96-well plate and then remove it again with the vacuum pump.
22. Place the filter plate on a green paper towel to remove all potential liquid drops from the bottom of the plate. Also clap on the plate to make sure all liquid is removed.
23. Add 75µl of the earlier prepared stain buffer into each well and incubate for 30 min on the horizontal shaker while shaking at approx. 650 rpm.
24. During this incubation time either run the “calibration” program or “wash between plates” program with the luminex. The program will guide you through what is needed.
25. Remove the stain buffer and wash 3x with 100µl PBS per well.
26. Remove all liquid as described in step 22 and then resuspend the beads in 100µl PBS for the Luminex read-out. The plate is now ready for measurement.
27. After your measurements are finished press “shut down” within the Luminex program and follow the steps as indicated. Clean your workbench and return all reagents and materials to their respective places.

HPV genotyping using EURO Array HPV

Test principle: The test is based on the amplification of defined gene sections of 30 human anogenital high-risk and low-risk papilloma viruses (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 70, 72, 73, 81, 82, 89 (CP6108) and subsequent detection via a hybridisation reaction with immobilised DNA probes in a microarray system. In the first reaction step, regions of the viral oncogenes E6 and E7 from the HPV that are present in the sample are amplified and fluorescently labelled by means of a polymerase chain reaction (PCR) using a multiplex primer system. In the second reaction step the products are detected using an oligonucleotide microarray. The specific binding (hybridisation) of a fluorescing PCR product to the corresponding oligonucleotide probe is detected using a special microarray scanner (EUROIMMUN). The EUROArrayScan software evaluates all spot signals and generates the test results.

Test Protocol

EUROIMMUN
a PerkinElmer company

Medizinische
Labordiagnostika
AG

EUROArray Short Instruction
Molecular Infection Diagnostics

1. Pre-PCR area

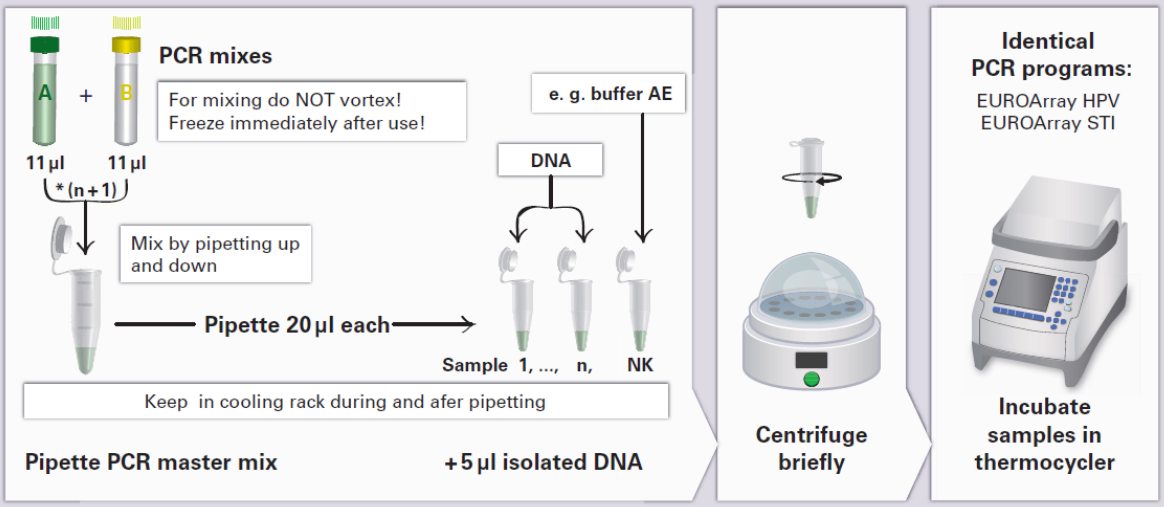
Sample preparation

- **DNA isolation from smear material (swab, swab in NAT preservative solution, samples in solution for thin-layer cytology):**
  see short instruction for DNA isolation from smear material (EUROArray HPV + STI)
- **DNA isolation from formalin-fixed, paraffin-embedded (FFPE) samples:**
  see short instruction for DNA isolation from FFPE samples (EUROArray HPV)
- **DNA isolation from urine:**
  see short instruction for DNA isolation from urine (EUROArray STI)
- **DNA isolation from skin scales, nail shavings or hair stubs:**
  see short instruction for DNA isolation from skin scales, nail shavings or hair stubs (EUROArray Dermatomycosis)

Approx. 15 min before PCR preparation

- **Remove cooling rack for 0.2 ml reaction vessels from freezer**
- **Thaw PCR mixes at +2 °C to +8 °C in the cooling rack for 1.5-ml reaction vessels; thaw samples if required**
- **Create new protocol (EUROArrayScan program); switch on thermocycler (preheat lid)**

Polymerase chain reaction (PCR)



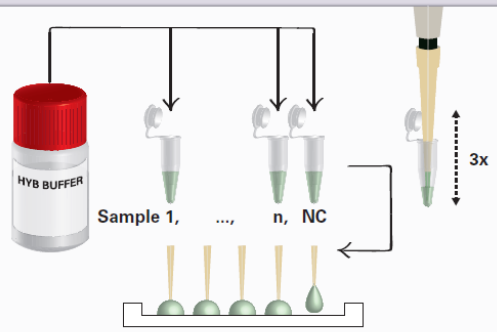
FOR INFORMATION PURPOSES ONLY! Always refer to the test instruction for performing the analysis!
This short instruction applies to the following products: MN 2540, MN 2830 and MN 2850

EUROIMMUN AG · Seekamp 31 · 23560 Lübeck (Germany) · Tel +49 451/5855-0 · Fax 5855-591 · info@euroimmun.de · www.euroimmun.com

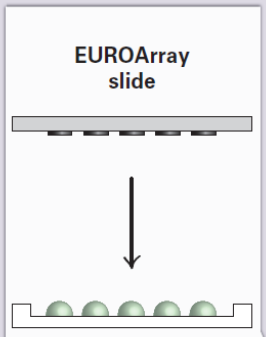
2. Post-PCR area

Hybridisation of amplified DNA on the EUROArray

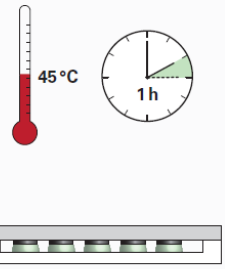
- Approx. 45 min before starting: Set hybridisation station to 45 °C, insert TITERPLANE reagent tray
- Approx. 15 min before starting: Remove slides from the protective pouch after they have reached room temperature, label them and place them onto the reagent tray (omitting position 1)



+ 65 µl of hybridisation buffer to each PCR reaction vessel, mix by pipetting up and down three times, directly apply 65 µl onto reagent tray



Place slide on top (after pipetting three to five samples)



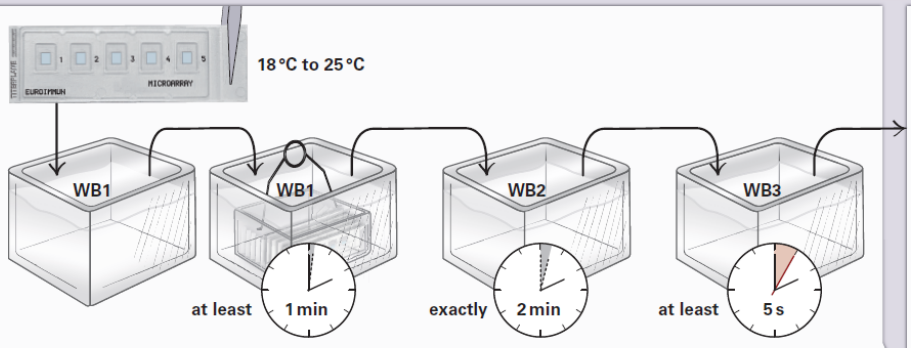
Incubate on hybridisation station

EUROArray Dermatomycosis: 1 h, 55 °C

Washing and drying of slides

- Option 1: Preparation of wash buffers with EUROArray wash buffer set (according to instructions)
- Option 2: Preparation of wash buffers with wash reagents 1 and 2 (for two wash cycles):

Wash buffer 1 (WB1)		Wash buffer 2 (WB2)		Wash buffer 3 (WP3)	
Wash reagent 1	50 ml	Wash reagent 1	2,5 ml	Wash reagent 1	25 ml
Distilled water	~800 ml	Distilled water	~400 ml	Distilled water	ad 500 ml
Wash reagent 2	10 ml	Wash reagent 2	5 ml		
Distilled water	ad 1 l	Distilled water	ad 500 ml		



Briefly immerse slide in WB1 and collect up to ten slides in second cuvette, incubate after transfer of last slide → Transfer glass frame + slides into WB2 and incubate → Transfer glass frame + slides into WB3 and incubate

Dry slide: Dry front with compressed air

Wipe edges + rear side with lint free tissue

FOR INFORMATION PURPOSES ONLY! Always refer to the test instruction for performing the analysis!
This short instruction applies to the following products: MN 2540, MN 2830 and MN 2850

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