# DNA Methylation Changes, Prevalence and Risk Factors Associated with HPV Genotypes among Ethiopian Women Diagnosed for Cervical Cancer

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# DECLARATION

I, the undersigned, declare that the dissertation hereby submitted for the Degree of Doctor of Philosophy (PhD) to the Addis Ababa University is my own work and has not previously been submitted at any other university. The materials obtained from other sources have been duly acknowledged.

**Name:** Birhanu Kumbi Jufara **Signature:** _____ **Date:**_____

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## Table of Contents

# List of Figures

Page

# List of Tables

Page

# Abbreviations

| | |
|---|---|
| AAFSI | Age At First Sexual Intercourse |
| AIS | Adenocarcinoma In Situ |
| AUC | Area Under ROC curve |
| CCDB | Cervical Cancer Gene Database |
| CIN | Cervical Interaepithelial Neoplasia |
| CKC | Cold Knife Conization |
| DAL-1 | differentially expressed in adenocarcinoma of the lung |
| *EPB41L3* | Erythrocyte membrane protein band 4.1 like 3 |
| FGAE | Family Guidance Association of Ethiopia |
| HCU | Horminal contraceptive use |
| HPV | Human Papillomaviruse |
| hrHPV | high risk Human Papillomavirus |
| IARC | International Agency for Research on Cancer |
| LBC | liquid based cytology |
| LCR/URR | Long control region /upstream regulatory region |
| LEEP | loop electrosurgical excision procedure |
| LLETZ | large loop excision of the transformation zone |
| NPV | negative predictive value |
| ORF | Open Reading Frame |
| PPV | positive predictive value |
| qPCR | Quantitative Polymerase Chain Reaction |
| ROC | receiver operating characteristic |
| SCC | squamous cell carcinoma |
| VIA | visual inspection with acetic acid |
| VILI | visual inspection with Lugol’s Iodine |

# Abstract

Cervical cancer is the second most common among cancers in Ethiopia with an incidence of more than 6000 cases and around 5000 deaths per year. Infection by high risk human papillomavirus (hrHPV) is the major risk factor for cervical cancer with almost all cases being infected. HPV infection is a very common sexually transmitted infection that majority of sexually active women acquire. Most of the infected women however clear the infection spontaneously in short time while it persists and causes cervical cancer in only small portion of the infected women. Various factors are known to determine the outcome of the infection but with scarce information on the mechanisms. DNA methylation changes in both human genes and HPV genes are among the biological events associated with cervical cancer progression. This study was aimed at determining prevalence of hrHPV, socio-demographic risk factors for cervical cancer and epigenetic changes associated with cervical cancer and evaluate their potential as diagnostic markers. The study was conducted as an observational case-control study by taking records of various risk factors and clinical information along with cervical cell samples for hrHPV testing and DNA methylation analysis. The human *EPB41L3* gene promoter region and HPV L1 and L2 regions were PCR amplified from bisulphite converted DNA. The PCR amplicons were then pyrosequenced and proportion of converted cytosine is measured and means of the targeted CpG sites methylation were recorded. Prevalence of both hrHPV and demographic risk factors were compared among women with cervical lesions (cases) and women with normal cytology (controls) to determine associations with various stages of cervical cancer. The DNA methylation assays were evaluated and compared for their performance using Reciever Operating Characteristics (ROC) curve analysis. From a total of 135 women, 96 had cervical cell lesions ranging from CIN1 to invasive cancer (cases) and 39 had no lesion (controls). Parity was higher in cases 5.44(±3.01) than in controls 2.03(±2.04). Cases started sexual intercourse earlier, at mean age of 16.57(±4.04) years, than the controls 20.18(±4.24) years. Hormonal contraceptive use showed no significant difference among cases and controls. HPV DNA was detected in 79(82.3%) of cases and 7(17.9%) of controls from which 77(80.2%) of cases and 6(15.4%) of controls were hrHPV. HPV16 was the most prevalent virus constituting 84% of all hrHPV positive cases and 33.3% of hrHPV positive controls. HPV45, HPV18 and HPV31 were detected in 17.7%, 5.2% and 3.8% respectively of the hrHPV positive cases. Level of methylation in both human and hrHPV DNA was found to be higher in higher grade lesions than in low grade lesions (CIN1) and normal cervical cells. Methylation assays, both *EPB41L3* promoter methylation and S5 score discriminated normal and CIN1 from CIN3 or worse lesions with sensitivity and specificity of greater than 95%. In conclusion, higher parity and earlier age at first sexual intercourse are among the factors that put women at higher risk of cervical cancer in addition to hrHPV infection. HPV16 is the most prevalent (69.8%) hrHPV type followed by HPV45 (14.6%) in Ethiopian women with cervical lesions. Methylation levels of the human *EPB41L3* promoter region and HPV L1 and L2 regions are potential biomarker to improve precision of diagnosing the cancer and targeting for therapy. EBP41L3 methylation alone discriminated normal and CIN1 cells from CIN3 or worse lesions with 95% sensitivity and 96% specificity while S5 detected with 96% sensitivity and 95% specificity.

Key words: Cervical cancer, CIN, DNA methylation, HPV genotype, hrHPV, Risk factor

# Chapter 1

# 1. Introduction

## 1.1. Background

Cervical cancer is the third most common cancer in women with an estimated incidence of 569,847 cases globally in 2018 and the second most common in women aged 15-44 years (Bruni *et al.,* 2019). The majority of cervical cancer incidence and mortality occur in less developed countries (de Martel *et al.*, 2017). GLOBOCAN (2018) reported cervical cancer had claimed lives of more than 311,000 women globally in 2018 alone. According to the report, the majority (more than 60%) of cervical cancer deaths occur in developing countries. Cervical cancer ranks the second most frequent cancer among the general female population as well as among women between 15 and 44 years of age in Africa (Bruni *et al.,* 2019). Africa with age standardized incidence rate of 27.6 per 100,000 is the world's leading continent in 2018. From all world regions, three highest ranking regions by cervical cancer incidence and four by mortality are in Africa. Eastern Africa is the second hardest hit region of Africa and the world by cervical cancer next to southern Africa and the first by mortality caused by cervical cancer (Bruni *et al.*, 2019). The 2019 International Agency for Research on Cancer (IARC)/WHO report on HPV and related diseases indicates that 119,284 African women are diagnosed with cervical cancer and 81,687 died from the disease in a year. With 6,294 new cases per year, cervical cancer stands the second most common cancer in Ethiopia (GLOBOCAN, 2018).

Infection by oncogenic human papillomavirus (HPV) also known as high risk HPV (hrHPV) types is a necessary condition for almost all cervical cancer cases all over the world (Walboomers *et al.,* 1999). Prevalence of HPV cervical infection in the general global population of women is 18.6% on average with the highest prevalence (20.3%) being in eastern Africa followed by western Africa (19.5%) (GLOBOCAN, 2018). Although no statistics of HPV prevalence in the general population is available for Ethiopia, it is more likely higher as Ethiopia is located in a region with the world's highest HPV prevalence. Global prevalence of cervical

HPV infection among women with normal cytology is highest among women younger than 25 years with prevalence of 28.4% (95% CI =28.0-28.9%) with gradual decline with increasing age (Bruni *et al.,* 2019). Women in the age group older than 65 years are exception having higher prevalence (6.3%, 95% CI = 6.1-6.5%) than the younger age group. Prevalence of the hrHPV types among women with normal cytology throughout the world is also considerable with HPV types 16 and 18 being the leader with prevalence of 2.8% (95% CI = 2.8-2.9%) and 1.1% (95% CI = 1.1-1.1%) respectively (https://hpvcentre.net/datastatistics.php).

So far prevalence of HPV infection in Ethiopia has been studied very little. Only six HPV prevalence studies with 1535 Ethiopian women participants published in peer reviewed journals were found. Four of the six studied HPV prevalence in 668 women with cervical abnormality (Fanta, 2005; Ebba Abate *et al.,* 2013; Dawit Wolday *et al.,* 2018) or gynecological problems (Bekele *et al.,* 2010). Only one study (Dawit Wolday *et al.,* 2018) compared HPV prevalence between women with normal and abnormal cervical tissue. The other two studies (Ruland *et al.,* 2006; Leyh-Bannurah *et al.,* 2014) were conducted with 726 women from general female population visiting a hospital for various medical reasons including cervical complaints but clinical and pathological information is not available.

Incidence of and mortality due to cervical cancer is higher in developing countries than in developed countries. This is mainly due to lack of well organized prevention and control programs in developing countries. Early diagnoses of precancerous lesions and vaccination against HPV infection (the major causative agent) are the major cervical cancer prevention strategies. Introduction of cytological screening (Pap smear) alone had reduced age standardized incidence rate of cervical cancer by at least 25% in most of the countries (Gustafsson *et al.,* 1997). Incidence of cervical intraepithelial neoplasia grade 2 (CIN2) and worse had declined by up to 56% in USA after introduction of HPV vaccination (Gargano *et al.,* 2019). Both robust screening and vaccination programs are lacking in developing countries and this contributed for disproportionately higher incidence and mortality rates (Cohen *et al.,* 2019).

Though almost all cervical cancer cases are associated with hrHPV infection, not all hrHPV infections cause cervical cancer (Moscicki *et al.,* 1998). Only a small fraction of women infected

with hrHPV develops cervical cancer while the vast majority of infection regress (Evander *et al.*, 1995; Moscicki *et al.*, 1998; Elfgren *et al.*, 2000; Moscicki *et al.*, 2004) by the individuals' immune response even without medical intervention (zur Hausen, 1991; Moscicki *et al.*, 2004). This led to research efforts in search for other factors which may contribute for development of cervical cancer since hrHPV infection alone is not sufficient to cause cervical cancer. Among other factors that contribute to development of cervical cancer are environmental factors and the host genetic and epigenetic factors (Wolf *et al.*, 2003). A number of environmental and biological (both host as well as HPV) factors may play some role in the carcinogenesis. Among the biological factors, immune competence (Moscicki *et al.*, 2004; Rintala *et al.*, 2005; Reusser *et al.*, 2015), host genetic variations (Agarwal *et al.*, 2011) and epigenetic changes (Kabekkodu *et al.*, 2017) are widely studied. Epigenetic changes such as gene expression suppression through DNA methylation at promoter regions of tumor suppressor genes is common phenomenon in cancers. *EPB41L3* is a human gene that encodes for Erythrocyte membrane protein band 4.1 like 3, also known as differentially expressed in adenocarcinoma of the lung (DAL-1) or protein 4.1B. *EPB41L3* is a tumor suppressor gene first discovered in adenocarcinoma of the lung for its differential expression (Tran *et al.*, 1999). Studies on cervical cancer identified *EPB41L3* promoter methylation as one of the most consistently happening epigenetic alteration in the carcinogenesis process (Eijsink *et al.*, 2011a; Sen *et al.*, 2018; Kelly *et al.*, 2018). One major event repeatedly associated with carcinogenicity of hrHPV is the viral gene expression deregulation following its genome integration into the cellular genome (McBride and Warburton, 2017; Torres-Rojas *et al.*, 2018). This HPV gene expression deregulation and genome integration often associate with epigenetic changes; which presumably is the cause of altered gene expression (Yang-chun *et al.*, 2017; Liu *et al.*, 2018).

Cervical cancer has latent or asymptomatic premalignant stages which may regress spontaneously at younger ages. Detection of cancer at early curable stage, which enables early treatment and increase positive treatment outcomes, is one of the primary goals of cancer prevention programs. Cervical cancer screening to detect the various stages of premalignant lesions is recommended by WHO as one of cervical cancer prevention program (WHO, 2013). Effective prediction of the fate of premalignant stages of cervical lesions is important to treat the lesions without both missing the progressive lesions as well as over-treating the lesions that

could regress spontaneously (Basu *et al.*, 2018). Diagnostic and screening techniques currently in use include visual inspection of the cervix for presence of lesions (Belinson *et al.*, 2001), cytological Pap smear examination (Flanagan, 2018) and detection of HPV DNA (Burd, 2016; Flanagan, 2018). Visual inspection tests have lower sensitivity of around 71% (Belinson *et al.*, 2001) and work for women in the pre-menopause age as detection is affected by changes associated with menopause (Cremer *et al.*, 2011). Pap smear has lower sensitivity (30-87%) and lower reproducibility but considerably higher specificity (Chrysostomou *et al.*, 2018; Basu *et al.*, 2018; Shah *et al.*, 2016). HPV tests have significantly higher sensitivityof close to 95% but lower specificity (~94%) than cytology (~97%) (Mayrand *et al.*, 2007). Alternative screening tests that effectively discriminate between healthy and pathological stages of cervical cancer are important in the battle against the cancer. Differential methylation of various human genes that are involved in carcinogenesis were evaluated and promising results were also obtained in differentiating between healthy and cancerous cells as well as low grade and high grade cervical lesions (Wisman *et al.*, 2006; Shivapurkar *et al.*, 2007; Kim *et al.*, 2010; Hesselink *et al.*, 2011; Pun *et al.*, 2015; Eijsink *et al.*, 2011a; Lai *et al.*, 2010; Eijsink *et al.*, 2011b; Kim *et al.*, 2010; Overmeer *et al.*, 2011; Bierkens *et al.*, 2013).

Understanding the DNA methylation pattern of hrHPVs may lead to understanding the mechanism of viral carcinogenesis. In addition, determining association between the viral genome modification and cancer progression may help in predicting what is to happen in the cells. Knowledge about the different molecular phenomena in hrHPV infected cervical cells would be of paramount importance in prevention, diagnosing and treating cervical cancer. One way of getting information about the changes that are associated with the cellular transformation is by comparing what is happening in healthy and malignant cells (Hanahan and Weinberge, 2011).

Knowledge of the environmental risk factors is also very crucial to devise preventive actions such as avoiding exposure to potential carcinogens and developing or selecting prophylactic vaccination. Identifying the risk factors also helps to focus prevention measures on sections of population at higher risk. Risk stratified intervention programs are more effective both in clinical outcomes as well as wise use of resources.

Good diagnostic tests that precisely detect the problems are also very important to plan timely and effective treatment options. It is very important for diagnostic tests to more precisely discriminate the diseased from healthy. Because erroneously identifying diseased women as healthy may deny the patients proper treatment and erroneously identifying healthy as diseased may expose subjects to unnecessary treatment side effects and emotional problems and costs. To develop efficient diagnostic tests, understanding the biological mechanisms involved in the development of a disease condition is very important.

## 1.2. Statement of the problem

Cervical cancer is potentially preventable through various interventions, but still it is one of the major causes of mortality and morbidity to women especially of the less developed nations. The success of preventing the losses caused by cervical cancer is heavily dependent on early detection of the cancer. Though vaccination against HPV is expected to play a great role in protecting against cervical cancer, it is still far from sufficient especially in developing countries, because of various economical, social and biological factors such as prevalent health conditions.

Information on HPV prevalence in Ethiopia is scarce, with only 1535 individuals in only six studies, for a country with more than 50 million women. Determining the prevalence of HPV genotypes in Ethiopian population is so important both in devising preventive strategies like vaccination as well as predicting progression of infection. Currently available HPV vaccines are type specific, targeting two, four or nine HPV types (WHO, 2017). HPV types 16 and 18 are associated with the majority (~70%) of cervical cancer (Saadeh *et al.,* 2019) and HPV genotypes behave differently in their course of infection and progression to cervical cancer (Jaisamrarn *et al.,* 2013). However, very little of the epidemiology of cervical cancer and it etiologic agent hrHPV have been studied in Ethiopia (Ruland *et al.,* 2006; Fanta, 2005; Bekele *et al.,* 2010; Ebba Abate *et al.,* 2013; Leyh-Bannurah *et al.,* 2014; Dawit Wolday *et al.,* 2018).

Despite high protection efficiency of vaccination against HPV infection, the gap is still wide to efficiently address the challenge posed by cervical cancer. Understanding the mechanism of cancer development and progression is the most fundamental in devising protective, diagnostic

and therapeutic strategies. The fact that most women infected by hrHPV do not develop cervical cancer indicates presence of other determinants. Very few studies are there on what environmental factors contribute for the progression of these infections in to cervical cancer especially in Ethiopia. Understanding risk factors is very important in understanding the disease mechanism as well as designing preventive methods. Screening tests that detect infection by hrHPV types fail to predict the consequence of the infection as majority of the infection is cleared in short period of time. Since the ultimate target of cancers is the cellular environments which are mainly tailored by their molecular compositions and functions, various genetic phenomena are believed to be the major players. Studies have revealed a lot of genetic alteration, from mutations to gene expressions regulations, associate with cervical cancers. Both human genes and HPV genes have showed various types of deregulation that associate with the carcinogenesis. DNA methylation of promoter regions of various human genes and different CpG sites in HPV genes are reported to be good at predicting progression of cervical lesions. These methylation markers need to be evaluated for their reproducibility and diagnostic performance and/or utility if they improve the sensitivity and specificity of the available screening tests.

## 1.3. Significance of the study

Explicit understanding of the nature, etiology, risk factors and mechanisms of certain disease is the basis for all sorts of intervention including, prevention, diagnosis and treatment, at all levels from patients, health service providers to policy makers. This study provides information on the prevalence of hrHPV types which is helpful in selecting the most appropriate prophylactic HPV vaccine for Ethiopia. It also determines the risk factors which can be used to identify the most vulnerable members of the population so that vaccination and screening priorities are given to the most at risk women. Determining methylation patterns of HPV and human genes are also important in understanding the mechanism of carcinogenesis which in turn serves to design diagnostic tests and treatment targets. Finally, evaluating diagnostic potential of DNA methylation assays is an important step in recruitment of more specific and sensitive markers which is important imput in the provision of the necessary medical service to those who deserve

it. DNA methylation based markers are promising to be utilized to improve sensitivity and specificity of the existing screening and triage tests.

## 1.4. Hypotheses

I. CpG islands in the L1 and L2 genes of hrHPV are differentially methylated among healthy cervical tissue and various stages of lesions and invasive cervical cancer among Ethiopian women.
II. There is differential methylation of the human *EPB41L3* gene promoter regions in cervical tissue of various stages of pathology among Ethiopian women.
III. DNA methylation assays can clearly discriminate between healthy cervical tissue, precancerous lesions and different stages of invasive cervical cancer among Ethiopian women.

## 1.5. Objectives of the study

### 1.5.1.General objective

To determine the prevalence of hrHPV, socio-demographic risk factors for cervical cancer and epigenetic changes associated with cervical cancer and evaluate their potential as diagnostic markers in Ethiopian cervical cancer patients.

### 1.5.2. Specific objectives

Specific objectives of this study were to;

- identify socio-demographic risk factors prevailing in Ethiopian women population with cervical lesions of various levels.
- determine the prevalence of hrHPV and genotype distributions among Ethiopian women with normal cytology and cervical lesions.
- determine methylation pattern of HPV *L1* and *L2* genes in Ethiopian cervical cancer patients.

- determine methylation pattern of *EPB41L3* promoter region in Ethiopian cervical cancer patients
- evaluate the potential of DNA methylation assays to differentiate between healthy cervical cells and various stages of cervical malignancies

# Chapter 2

# 2. Literature Review

## 2.1. Cervical cancer

Cervical cancer (carcinoma of uterine cervix) is one of the most common malignancies of women worldwide with more than half a million new cases and more than 300,000 deaths a year (Bruni *et al.,* 2019). It is a carcinoma that arises from the epithelium of the uterine cervix. Persistent infection by human papillomavirus (HPV) was found in almost all cervical cancer cases, implying its causative role (Ho *et al.,* 1995; Walboomers *et al*., 1999). Though infection by carcinogenic HPV -also known as high risk HPV (hrHPV)- is the major etiologic factor, several other factors ranging from socio-demographic (Muñoz *et al.,* 2002; Thulaseedharan *et al.,* 2012; Raychaudhuri and Mandal, 2012) and other clinical factors (Dawit Wolday *et al.,* 2018; Kashyap *et al.,* 2019) to genetic susceptibility (Czene *et al.,* 2002; Hemminki and Chen 2006; Raychaudhuri and Mandal, 2012) play important role in the cervical cancer development. Great deal is done and achieved in prevention and management of cervical cancer especially in the developed western countries. Early detection of the case through screening programs (Martín, 2007; Dijkstra *et al.,* 2014), treatment of low grade precursor lesions (WHO, 2014b) and vaccination against the causative HPV types (Gargano *et al.,* 2019) have brought about considerable success in the fight against cervical cancer.

## 2.2. Epidemiology of cervical cancer

Cervical cancer is the third common type of cancer and cause of cancer related deaths among women worldwide next to breast and colorectal cancers and ranks seventh from cancers in the general population (Bruni *et al.,* 2019). It constitutes nearly 6.4% of all cancers in women (GLOBOCAN, 2018) with incidence of 569,847 and 311,365 mortality in 2018 alone (Bruni *et al.,* 2019). Less developed nations are the most affected having the highest incidence and mortality rates.

With 119,214 new cases per year Africa had the second largest incidence of cervical cancer in the world in 2018 next to Asia (Fig. 2-1A). Although Asia is the leading by the total number of incident cases and mortality, the rate per 100,000 women is the highest in Africa with 27.6 new cases and 20 deaths. Cervical cancer is the second most common cancer of the female population but the leading cause of cancer related death with 81,687 deaths per year among African general female population. It is the second incident and second cause of cancer deaths in females aged 15 to 44 years of age both worldwide and in Africa (Bruni *et al.*, 2019).

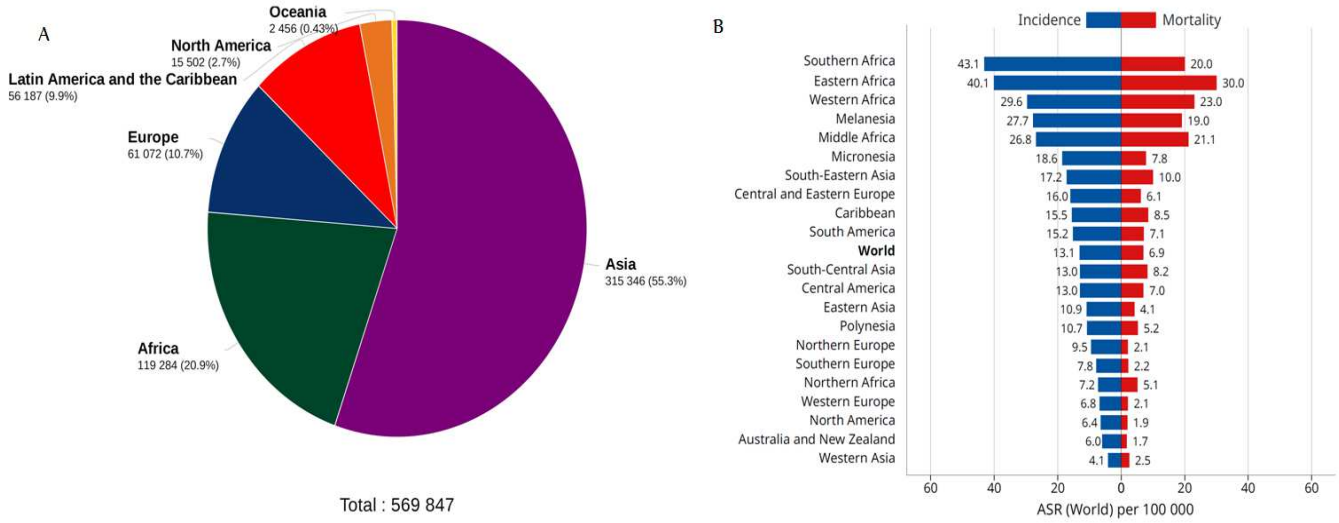


**Figure 2-1:Global cervical cancer incidence and mortality of the year 2018 (A) cervical cancer incidence rate by continents (B) incidence and mortality rates by world region (http://gco.iarc.fr/today/data/factsheets/cancers/23-Cervix-uteri-fact-sheet.pdf).**

According to GLOBOCAN 2018 report the world's highest cervical cancer incidence and mortality rate are from Africa. The eastern Africa region has the second highest incidence but the largest mortality rate of all the world regions (Fig. 2-1B). HPV prevalence in African women with normal cytology is highest (>60%) in females younger than 25 years in the eastern Africa region followed by the same age group in southern Africa (>40%) and women older than 65 years in northern Africa. Prevalence of hrHPV is 14.9% in women with normal cervical cytology the highest being HPV16 (2.4%). HPV16 is the leading HPV type with prevalence of 49% in cervical cancer followed by HPV18 (18.2%) in Africa (Bruni *et al.*, 2019).

In Ethiopia cervical cancer is the second most common cancer with incidence rate of nearly 6,300 cases per year and constituting 9.3% of all cancer cases in the nation and 13.6% of cancers in female population (http://gco.iarc.fr/today/fact-sheets-populations). The GLOBOCAN (IARC) (2018) report also shows mortality rate per year due to cervical cancer of nearly 5,000 in Ethiopia. The age standardized incidence rate of cervical cancer and mortality due to cervical cancer is reported to be 18.9 and 15.3 per 100000 respectively in Ethiopia (Fig. 2-2). Eastern Africa, a region to which Ethiopia belongs has the second highest cervical cancer incidence rate and the largest mortality due to cervical cancer in the world (Fig. 2-1B).

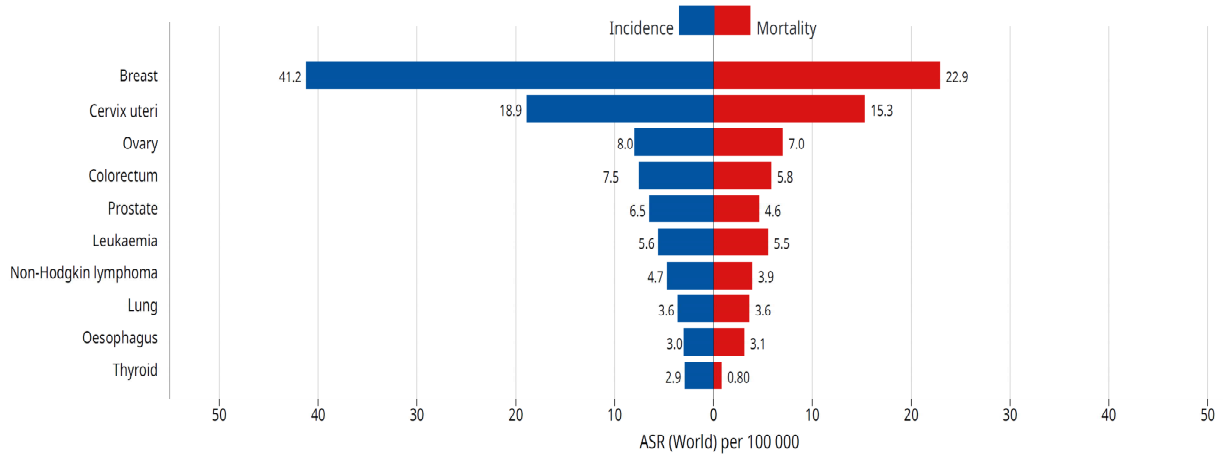


**Figure 2-2: Incidence and mortality rates of top ten cancers in Ethiopia in the year 2018 (http://gco.iarc.fr/today/data/factsheets/populations/231-ethiopia-fact-sheets.pdf).**

A study conducted on prevalence of HPV infection in Ethiopian women visiting health facility for any problem that may include gynecological complaints, showed that 15.9% of randomly selected women are HPV positive and 83.3% of the HPV positive are infected with cervical cancer causing hrHPV types (Ruland *et al.*, 2006). According to Fanta (2005), 92.6% of patients with cervical dysplasia and cancer, from Gondar area north western Ethiopia, are infected with HPV and HPV type 16 constitutes 76% of all HPV infections. A study conducted on patients with cervical pathologies at Jimma hospital reported 67.1% to be infected with HPV. From among the HPV infections the hrHPV type 16 constitutes the largest portion, the proportions being HPV types 16 (55.7%), 18 (8.2%), 56 (8.2%), 45 (4.1%), 39 (2.5%), 52 (1.6%), 31 (1.6%), 35 (1.6%), 58 (0.8%), 33 (0.8%), 59 (0.8%)(Bekele *et al.*, 2010). Another study on cervical

specimens from Ethiopia and Sudan (160 and 86 specimens respectively) showed higher prevalence (93%) of HPV infection among cervical cancer patients (Ebba Abate *et al.,* 2013). This study confirmed the highest prevalence of HPV16 (91% of all HPV positive samples) followed by HPV52 (25.5%), HPV58 (22%) and HPV18 (20.8%). Other high risk HPV types reported are HPV types 45(12.1%), 33(7.4%), 31(6.7%), 68(6%), 35(4%), 39(3.4%) and low risk types 6(10%) and 44(3.4%). A 100 percent infection with HPV in cervical lesions is also reported (Dawit Wolday *et al.,* 2018) with hrHPV constituting 92.5% of infections. HPV type 16 is the most prevalent with 44.1% followed by HPV types 35 and 45 comprising 10% each and types 39, 52 and 68 the least with 1.9%, 1.2% and 0.6% respectively of all HPV infections.

On top of the high incidence rate of cervical cancer, the cost it poses on the patients and their families is also unaffordable for most of the women. The average cost paid directly because of cervical cancer was estimated to be Birr 7,060.1 ($486.91) and productivity lost during illness and treatment amounts Birr 273,195 ($18,841. 03) for outpatients by the year 2011, and the cost for the hospitalized patients is even more (Alemayehu Hailu and Damen Haile Mariam, 2013).

Despite the enormous health and economic burden cervical cancer poses on women and their family, the women have very low awareness about the condition with only 31% of the study participants having good knowledge (Frehiwot Getahun1*et al.,* 2013; Zewdie Birhanu *et al.,* 2012). A study conducted with female university students revealed that only 40.5% of them had ever heard of cervical cancer l*et al*one the etiology, risk and prevention (Kalayu Birhane, 2018). The lack of awareness especially about the etiology of the cancer has led to misconception which is barrier to the treatment of the cancer or seeking the right remedy (Zewdie Birhanu *et al.,* 2012) and preventive measures which could have been taken.

## 2.3. Biology of cervical cancer

Cervical cancer arises from the epithelial cells covering the uterine cervix. Uterine cervix is covered by stratified squamous epithelium on the outer ectocervix and mucus secreting glandular columnar epithelium in the inner endocevix (Small *et al.,* 2017). The transition zone between the two is called squamocolumnar junction and it is the site with distinct type of epithelial cells

where majority of cervical cancer and its precursor lesions cervical intraepithelial neoplasias (CINs) and adenocarcinoma in situ (AIS) arise (Herfs *et al.*, 2012). Cervical cancer develops following the multistage carcinogenesis process passing through multiple precursor lesions (Plummer *et al.*, 2012).

Cervical cancer thus arises from different cellular origins, most of the time from the lining of ectocervix. Cancer arising from the ectocervix is termed as squamous cell carcinoma (SCC) and the type of cervical cancer that starts in glandular cells of the endocervix is called adenocarcinoma (Buckley *et al.*, 1982). There are also mixed type of cervical carcinomas called adenosquamous carcinomas. Squamous cell carcinomas usually arise at the junction of the squamous epithelial lining and the glandular lining, a region called transformation zone (TZ). Most of the cervical cancer is of the squamous cell type, followed by the adenocarcinoma of the glandular cells. Other types including the mixed cervical carcinoma and those arising from other tissues of cervix are rare (Brinton *et al.*, 1987; Small *et al.*, 2017). The proportion of histological origin however varies from population to population, proportion of adenocarcinoma for example being larger in the western population than in others (Martín, 2007).Transformation zone is particularly susceptible to cervical cancer initiation because of the presence of actively dividing basal cells associated with puberty and onset of sexual activity (Doorbar *et al.*, 2012).

Cervical cancer progresses through series of histopathological stages ranging from pre-cancerous lesions called cervical intraepithelial neoplasias (CINs) of the squamous cells or adenocarcinoma in situ (AIS) of the columnar glandular cells. These precursor lesions may be reverted spontaneously or may progress through various intermediate stages to invasive cancer of higher degree (metastatic) where the malignant cervical cells cross the basal membrane and spread to other parts of the body (Wiebea *et al.*, 2012). These precursor lesions especially CIN with three grades (CIN1, CIN2 and CIN3), are stepwise progression of cervical cancer (Richart, 1967). CIN1 refers to mild dysplastic lesions that regress in short time or does not progress to higher grade lesions while CIN2 is moderate dysplasia and CIN3 severe dysplasia and carcinoma in situ, with CIN2 more likely to regress than CIN3 and all of them associated with HPV infection (Schiffman and Wentzensen, 2013).

Though it was not possible to clearly determine its role as carcinogen, HPV was detected in various types of cervical lesions since the beginning of the third quarter of twentieth century (Torre *et al.*, 1978; Zur Hausen *et al.*, 1981). In 1980s HPV DNA was isolated from majority of cervical cancers and the viral *E6* and *E7* proteins were known to have oncogenic potential. HPV types whose DNA was frequently detected in cervical cancer are called high risk HPVs (hrHPVs) (Zur Hausen, 1991). Despite compelling evidence from both epidemiological and virological studies in 1980s, it was only by November 1991 that HPV16 was accepted to have etiological association with cervical cancer at the workshop by international agency for research on cancer (IARC) and WHO (Bosch *et al.*, 1992). HPV infection persists in only small portion of women and causes cervical cancer while majority of it is reverted by natural immune response. Thus HPV infection is deemed a necessary but not sufficient precondition for development of cervical cancer. Among other cofactors that interact with HPV infection in causing cervical cancer are various environmental exposures and genetic makeup of individuals (Wolf *et al.*, 2003).

## 2.3.1. Risk factors for cervical cancer

### 2.3.1.1. Infection by hrHPV

Persistent genital infection by oncogenic HPV is the primary risk factor for the development of cervical cancer. Association of HPV infection with malignant lesions and cancers (Rous and Kidd, 1938) and especially with cervical lesions and cancers (Torre *et al.*, 1978; Zur Hausen *et al.*, 1981) is known since long time. Since IARC's and WHO's joint international workshop of November 1991 (Bosch *et al.*, 1992) has declared association of HPV and cervical cancer, subsequent epidemiological (Walboomers *et al.*, 1999; Schlecht *et al.*, 2001), virology and molecular studies have showed the oncogenic role of HPV. Specific class of HPV types was repeatedly associated with cervical cancer while others are associated with non cancerous lesions (Durst *et al.*, 1983). The group of HPVs that associate with cervical cancer are known as oncogenic or high risk HPV (hrHPV) types. Currently 12 genital HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are classified as carcinogenic to human (hrHPV) (IARC, 2012). Although the role of hrHPV in cervical cancer is currently proved beyond any doubt,

necessity of other cofactors was also evident from the fact that only small portion of HPV infected women develop cervical cancer.

#### 2.3.1.2. Other infections and clinical conditions

Most of the genital HPV infections are cleared within short time without intervention (Elfgren *et al.,* 2000). This high rate of infection clearance is attributed to the properly functioning immune system (Maskey *et al.,* 2019). Infection persistence and hence progression to invasive cancer is much more common in women whose immune system is compromised either by other infections or medically induced immune suppression (Reusser *et al.,* 2015; Leon *et al.,* 2016; Cistjakovs *et al.*, 2018). Co-infection by sexually transmitted infections (STI) (Tao *et al.,* 2014; Li *et al.,* 2018; Dawit Wolday *et al.,* 2018; Kashyap *et al.,* 2019) and inflammatory diseases (Momenimovahed and Salehiniya, 2017) are also reported to increase the risk of cervical cancer development.

#### 2.3.1.3. Socio-demographic risk factors

Lifestyle and socio-demographic factors also play important role in putting women at greater risk of cervical cancer development. Exposures to domestic, environmental and occupational carcinogenic agents are known risk factors (Waggoner, 2003). Smoking cigarettes is among the risky lifestyle activities that increases the chance of cancer development (Elfgren *et al.,* 2000; Haverkos *et al.,* 2003; Li *et al.,* 2018). Women who began sexual intercourse at younger age are at higher risk than those who started later (Cooper *et al.,* 2007; Louie *et al.,* 2009; Plummer *et al.,* 2012; Kaur *et al.,* 2016; Cândido *et al.,* 2017). Having several sexual partners (Elfgren *et al.,* 2000; Li *et al.,* 2018), multiple full term pregnancies or giving birth to higher number of children (Muñoz *et al.,* 2002; Jensen *et al.,* 2013; Tao *et al.,* 2014; Dawit Wolday *et al.,* 2018) are also found to increase the risk. Lower socioeconomic status, illiteracy (Thulaseedharan *et al.,* 2012; Kaur *et al.,* 2016) and personal hygiene (Kashyap *et al.,* 2019) are also among risk factors that contribute to cervical cancer development (Li *et al.,* 2018). Residence and oral contraceptive use are also reported to associate with cervical cancer but in contradicting ways in different studies (Moreno *et al.,* 2002; Dawit Wolday *et al.,* 2018; Kashyap *et al.,* 2019; Jensen *et al.,* 2013). Moreno *et al.* (2002) and Kashyap *et al.*(2019) reported contraceptive use as risk factor while

Dawit Wolday *et al.* (2018) and Jensen *et al.*(2013) reported non association. Rural residence is reported to be risk factor in Dawit Wolday *et al.* (2018) but as protective factor in Kashyap *et al.*(2019).

#### 2.3.1.4. Role of genetics in cervical cancer

Epidemiological studies that show only small portion of women infected with hrHPV develop cervical cancer and findings from heritability studies led to investigations of genetic association studies. Several studies on hundreds of human gene variants reported different degree of association between the gene variants with HPV infection persistence and progression to cervical cancer. Many of polymorphisms in tumor suppressor genes, immune response genes, DNA damage repair and many in signaling pathways are among most commonly studied gene classes in various populations. Despite the number of researches undertaken, the data obtained in most of the genetic polymorphisms is insufficient to draw a sound and concrete relationship between cervical cancer and the genetic variants. This is basically because majority of cancer cases associate with several low penetrance cancer predisposing gene variants as gene variants that have higher penetrance of carcinogenicity are so rare that their contribution is so low (Turnbull *et al.,* 2018). Moreover different researches on different populations often come up with contradicting findings (Eltahir *et al.,* 2012; Koshiol *et al.,* 2009; Makni *et al.,* 2000; Mitra *et al.,* 2005; Luo *et al.,* 2012; Mei *et al.,* 2012).

Comparative studies of relative risks of sibs, half sibs and adoptive sibs (Rotkin, 1966; Magnusson *et al.,* 2000; Coimbra and Regateiro 2001; Hemminki and Chen 2006) and twins (Rotkin, 1966; Czene *et al.,* 2002) compared against risks of unrelated individuals, reveal familial clustering of cervical cancer. In these studies relatives of cervical cancer patients were found to be at increased relative risk than unrelated individuals. Some of these studies show the heritable factor contributes significantly (Czene *et al.,* 2002, 22% and Hemminki and Chen 2006, 64%) for susceptibility to cervical cancer. Even though the shared environment also contributes to the familial clustering, the relative risks are higher for the more close biological relatives than the non biological relatives.

Several epidemiological studies show familial aggregation of cervical cancer, but many of them attributed the observed familial aggregation (Lichtenstein *et al.*, 2000; Czene *et al.*, 2002) higher relative familial risks and heritability (Hemminki *et al.*, 1999) to environmental factors shared by members of the same family. For example, shared environment that makes family members susceptible to infection by hrHPV types or vertical transmission of hrHPV from mothers to daughters (Kaye *et al.*, 1996) were thought to be the reasons of familial risks. Incidence of cervical cancer in relatives of cervical cancer patients and healthy controls shows that heritable factors play great role in susceptibility to the cancer (Table 2-1) (Magnusson, *et al.*, 1999). A well designed study to partition between genetic and environmental shared factors (Magnusson *et al.*, 2000) revealed that heritable genetic factors play important role through either susceptibility to infection by and persistence of HPV or enhancing cancer progression. An epidemiological study with siblings (Hemminki and Chen, 2006) showed familial risk for full sibs of an affected proband is 1.84 while it is 1.40 and 1.27 for maternal and paternal half sibs respectively, which implies the importance of genetic factors.

**Table 2-1. Relative risks of relatives of patient compared with relatives of healthy controls**

| | Magnusson, *et al.*, 1999 | Hemminki and Chen 2006* | Coimbra and Regateiro 2001 |
|---|---|---|---|
| Biological mothers | 1.83 (1.77–1.88) | - | 1.83 |
| Biological full sisters | 1.93 (1.85–2.01) | 1.84 (1.81-1.88) | 1.93 |
| Adoptive mothers | 1.10 (0.76–1.54) | - | 1.10 |
| Non biological sisters | 1.15(0.82–1.57) | - | 1.15 |
| Half sisters | 1.45 (1.31–1.60) | 1.31 (1.24-1.37) | 1.45 |
| Paternal half sisters | 1.41(1.22–1.62) | 1.27 (1.18-1.36) | - |
| Maternal half sisters | 1.49(1.29–1.73) | 1.40 (1.30-1.51) | - |

*standard incidence ratio

A lot of molecular genetic studies ranging from candidate gene association studies and linkage analysis to genome wide association studies have also tried to map cervical cancer to certain gene variants but with little success. In an effort to document the large body of information from

genetic studies, Agarwal *et al.*, (2011) have built a cervical cancer gene database (CCDB). Only upon establishment in 2011, CCDB has compiled 537 genes which were experimentally validated to have some sort of involvement in cervical cancer development, from which mutations and polymorphisms constitute very small portion (Fig. 2-3).

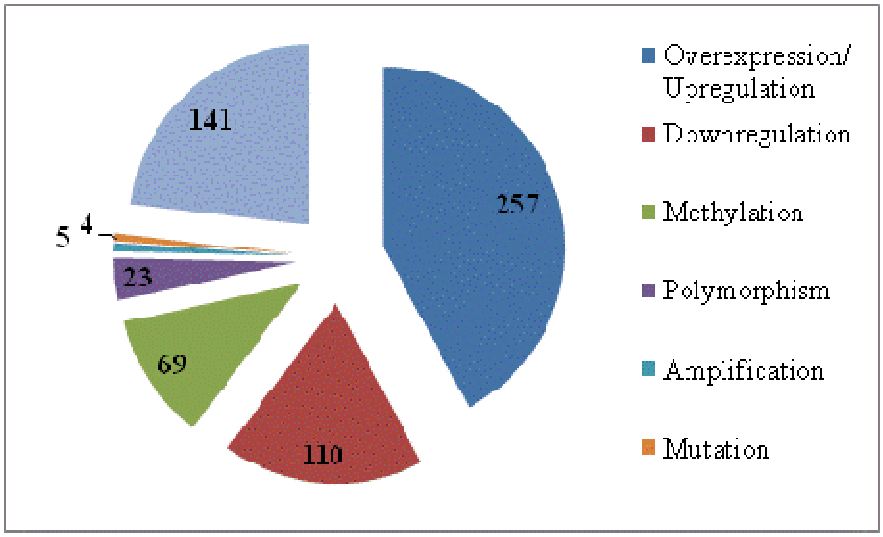


**Figure 2-3: Genes involved in cervical cancer (source CCDB, Agarwal *et al.*, 2011)**

### 2.3.2. Epigenetics in cervical cancer

Gene expression is mainly regulated by different epigenetic mechanisms that enhance or repress it (Taby and Issa, 2010). Since epigenetic regulation of gene expression plays crucial roles in the normal functioning of cells, anomalous epigenetic events referred to as epimutations, lead to abnormal conditions such as cancer, degenerative diseases and metabolic disorders (Fingerman *et al.* 2011). The role of epigenetics in cervical cancer is great for all the factors believed to cause or predispose to cervical cancer act mainly through epigenetic alterations by affecting expression of different classes of genes such as those involved in cancer suppression and immune response (Kabekkodu *et al.*, 2017).

Cervical cancer is found to associate with all sorts of aberrant epigenetic modifications. Several genes ranging in their functions from apoptosis, DNA repair, transcription factors, hormone receptors and key actors in cell cycle and cell differentiation pathways, show different degrees of

epigenetic alterations (Yang, 2013; Siegel *et al.,* 2015). Progressive global DNA hypomethylation with severity of pathology is documented in cervical cancer (Kim *et al.,* 1994). Various histone modifications such as increased acetylation and phoshorylation of histone protein H3, loss of acetylation and methylation respectively of $16^{th}$ and $20^{th}$ lysine residues of histone protein H4 (H4K16ac and H4K20me3) and up-regulation of histone deacetylase (HDAC) enzyme are evident in cervical cancer progression (Soto *et al.,* 2017; Sen *et al.,* 2018; Liu *et al.,* 2019). Loss of imprinting of growth regulator genes, insulin like growth factor 2 (IGF2) and H19 is also among epigenetic changes observed in cervical cancer (Dueñas-González *et al.,* 2005). Chromatin binding protein Bromodomain-containing protein 4 (Brd4) is one of genes whose function is modulated by HPV proteins through epigenetic processes. Brd4 is competitively inhibited by HPV *E2* protein while its viral early promoter repression function is interrupted by ablation of the necessary epigenetic marks (Soto *et al.,* 2017).

From epigenetic changes different studies have described different DNA methylation markers to be informative about the stages of cervical pathologies. Screening from 12 genes which are known to be differentially methylated in cervical or other human cancers, Wisman *et al.* (2006), have showed a panel of four genes methylation to detect cervical cancers with sensitivity of 89% which is comparable with that of cytology (89%) and hrHPV DNA tests (90%) and specificity of 100% which is much higher than cytology (83%) and hrHPV tests (68%). Kim *et al.* (2010), had screened methylation of 12 genes for their association with the stage of cervical lesions and demonstrated that combination of three genes (RAR-β, Twist and MGMT) methylation could differentiate between invasive cancer and precancerous lesions with specificity of 82.2% and sensitivity of 78.7%. Overmeer *et al.*(2011) reported down regulation of two tumor suppressor genes, cell adhesion molecule 1 (CADM1) and T-lymphocyte maturation associated protein (MAL) in cervical cancer. They have demonstrated that CADM1 and MAL promoter methylation when combined can detect cervical cancer with 78% specificity and 70% sensitivity. CADM1 and MAL promoter methylation is also associated with hrHPV infection status in addition to cervical pathology grades (Meršaková *et al.,* 2018), hrHPV positive patients showing higher level of methylation than hrHPV negative patients. Promoter methylation of CADM1 and MAL together with another gene implicated in cervical carcinogenesis, family with sequence similarity 19 member A4, C-C motif chemokine like (FAM19A4), evaluated in recent

study(Dankai *et al.,* 2019) has enhanced sensitivity of test for high grade cervical lesion especially when combined with hrHPV genotyping. Methylation of CpGs in promoter region of heparan sulfate D-glucosamyl 3-O-sulfotransfarase-2 (HS3ST2) and CDH1 gene encoding adhesion molecule were also higher in high grade cervical lesion and invasive cervical cancer (Shivapurkar *et al.*, 2007).

In an effort to enhance the sensitivity and specificity of the existing cervical screening tests, various studies had reported a lot of methylation markers to be used as triage test. Panel of four human genes (*JAM3, EPB41L3, TERT and C13ORF18*) promoter methylation in combination with HPV test is reported to improve accuracy of referrals to coloscopy (Eijsink *et al.*, 2011b). *EPB41L3* promoter methylation stood the best among 26 human genes screened for their performance in detecting CIN2 or worse cervical lesions (Vasiljevic *et al.*, 2014a).

DNA methylation changes that associate with cervical pathology are not only those of the human genes, but also those of the HPV. Lorincz *et al* (2013) demonstrate that methylation of CpG sites in HPV16 L1 and L2 genes increase progressively with severity of cervical lesions. Methylation of CpGs in L1 and L2 genes of HPVs 18, 31 and 33 is higher in cervical lesions worse than CIN2 (Vasiljevic *et al.*, 2014b). DNA methylation scores of various combination of gene both from human (Eijsink *et al.*, 2011b) and human and HPV combined (Brentnall *et al.*, 2014, Brentnall *et al.*, 2015, Lorincz *et al.*, 2016) were evaluated for their performance to be used as triage classifiers of cervical lesions.

Brentnall *et al* (2015) have formulated DNA methylation based triage test using *EPB41L3* promoter and HPV16 L1, HPV16 L2, HPV18 L2, HPV31 L1 and HPV33 L2 methylation scores and named it S5 classifier.

$$S5 = 30.9 \times EPB41L3 + 13.7 \times HPV16L1 + 4.3 \times HPV16L2 + 8.4 \times HPV18L2 + 22.4 \times HPV31L1 + 20.3 \times HPV33L2$$

Where; the gene names in the formula correspond to methylation percentage and proportions of CpG sites in the gene regions (described in the methods section).

S5 classifier was developed through series of modifications starting with methylation of HPV16 L1 and L2 regions (S1) and adding methylation in HPV16 URR (S2) (Lorincz *et al.*, 2013) and combining methylation data from *EPB41L3*, HPV16 L1 and L2, HPV18 L2 and HPV31 L1 (S4) (Brentnall *et al.*, 2014).

### 2.3.3. Cervical cancer precursor lesions intervention and management

Various intervention strategies and management procedures are devised and implemented in different settings. As in any health problem, prevention is the priority area in cervical cancer intervention also. Since cervical cancer is a kind of health problem that affect large number of people and its prevalence is almost global, WHO has produced standardized prevention (WHO, 2014a) and treatment (WHO, 2014b) guidelines to help countries to adopt screening and treatment methods that suit their situations. Cervical cancer has latent or asymptomatic premalignant stages which may regress spontaneously at younger ages (Basu *et al.,* 2018). Cervical precancerous lesions (CINs or AIS) occur and last decades before they progress to invasive cancer. Treatments of these non invasive precancerous cervical lesions may successfully prevent deaths and morbidities associated with the invasive cancer (Castle *et al.*, 2017).

#### 2.3.3.1. Cervical cancer screening

Cervical cancer screening is a program adopted by almost every nation aimed at testing for existence of cervical precancerous lesions, which have potential to progress to cancers, and then treat it (WHO, 2013). Detection of cervical precancerous lesions, at early curable stage which enables early treatment and increase positive outcomes and ultimately reduces incidence and mortality, is considered secondary prevention (WHO, 2014a). Effective prediction of the fate of the premalignant stages of cervical cancer is important to treat the lesions without both missing the progressive lesion as well as over-treating the lesions that could regress spontaneously. In addition to their performance, screening tests should be cost effective and accessible (Johnson, 2018) to those in need. Different countries have formulated different screening policies based on prevalence of the cases, cost of the tests, sensitivity of tests and age of women to be screened (de

Kok *et al.,* 2012). Currently available cervical screening tests are cytological, visual inspection and HPV testing, with a lot of others under development and awaiting aproval.

#### *2.3.3.2. Pap smear*

Cytological test as a means of screening for cervical cancer was first introduced in 1940s by George Papanicolaou and the test is termed Pap test after the inventor (Flanagan, 2018). Pap smear screening has substantially reduced cervical cancer incidence in western countries (Gustafsson *et al.,* 1997), even though the success is largely limited to squamous cell carcinomas (Dijkstra *et al.,* 2014). Pap smear has broad range of sensitivity which is affected by factors such as specimen quality, slide preparation and subjective detection and interpretation. Cytology based screening is improved by introduction of liquid based cytology (LBC) where the specimen collected is fixed in liquid preservative instead of direct smear on slide. The advantage of LBC is good quality smear as the cells are uniformly distributed and leftover sample can be used for other tests such as HPV detection (Chrysostomou *et al.,* 2018).

Cytological tests are valuable in diagnosing cervical cancer where clinical symptoms are evident in addition to screening asymptomatic women. It can detect cervical cancer with a sensitivity of greater than 90% in young women with symptoms, segment of population with higher frequency of symptoms but lower cases of cervical cancer (Lim *et al.,* 2016). Cytological screening however, has number of drawbacks, like limited sensitivity especially for precancerous lesions, need for repeated screening and shorter interval of re-testing, inability to detect adenocarcinoma and its precursor lesions AIS and subjectivity of interpretation (Chrysostomou *et al.,* 2018; Dijkstra *et al.,* 2014). Women with abnormal cytology are usually tested again, tested for HPV, referred to colposcopy examination and/or biopsy based histopathology (Basu *et al.,* 2018).

#### *2.3.3.3. Immunocytochemical (ICC) staining*

To improve sensitivity and specificity of cytology screening, immunocytochemical staining where expression of cyclin-dependent protein kinase inhibitor $p16^{INK4a}$ (also known as p16 for short) and nuclear antigen Ki-67 are stained using antibody specific to them is introduced (Sun *et al.,* 2019). Immunocytochemical staining also known as p16/Ki-67 dual staining relies on

detection of simultaneous expression of functionally antagonistic proteins p16 and Ki-67. Expression of p16 which normally serves as cell cycle control at G1 to S phase is lost in most cancers but it is enhanced in hrHPV induced cervical lesions because of actions of HPV oncogenes which deregulate the negative feedback of its expression (Celewicz *et al.*, 2018). Ki-67 is a nuclear proliferation marker expressed at all stages of cell cycle except $G_0$. Expression of these two in the same cervical cell denotes malignant transformation (Sun *et al.*, 2019).

Immunocychemical staining uses antibodies specific to p16 and Ki-67 and chemical stains that differentially stain cells with p16 and Ki-67 (Celewicz *et al.*, 2018). ICC staining not only detects high grade cervical lesions but is useful in determining the fate of hrHPV infected low grade cervical lesions (Hebbar and Murthy, 2017). Hence p16/Ki-67 dual staining is believed to enable cytological tests to identify lower grade lesions which are more likely to progress to invasive cervical cancer (Sun *et al.*, 2019).

#### *2.3.3.4. Visual inspection*

An alternative screening technique especially for resource limited settings is visual inspection with acetic acid (VIA) or visual inspection with Lugol's Iodine (VILI), where acetic acid or Lugol's iodine solution is applied to the cervix and observed for distinctly colored area by naked eyes. This technique is reported to work best for pre-menopausal women because in post-menoupausal women the transformation zone is hidden and hormonal changes may obscure the color change (Belinson *et al.*, 2001; Huchko *et al.*, 2015; Basu *et al.*, 2018). In women aged 35 to 45 years, VIA had sensitivity of 71% and specificity of 74% in detecting CIN2 or worse lesions (Belinson *et al.*, 2001). Visual inspection is method of choice especially in resource limited settings as it does not require laboratory setup and much equipment, no specimen transport and immediate test results (Burd, 2016; Chrysostomou *et al.*, 2018).

#### *2.3.3.5. HPV testing*

It was only at the onset of the 21$^{st}$ century that alternative screening method by detection of the hrHPV DNA came in to place. Pap smear and HPV testing have served together since, as co-testing to extend the testing intervals and enhance test acuracy. In 2012 findings of persistent

hrHPV infection by the DNA test assumed the status of screening positive. HPV testing and genotyping was approved to be used in women older than 25 years as screening test without concurrent cytology in 2015 (Flanagan, 2018).

Following recognition of causal relationship between persistent hrHPV infection and cervical cancer, series of HPV based tests are developed as screening test for cervical lesions. Various techniques are used to detect presence of HPV DNA, detect specific HPV type (genotyping) and quantify the HPV DNA and sometimes mRNAs to follow up changes (Eide and Debaque, 2012). Some of the techniques detect non-amplified DNA in the samples while others rely on amplification techniques of various types. The most commonly used amplification techniques are target amplification where the target HPV DNA is amplified by PCR followed by various types of analysis (Abreu *et al.,* 2012), signal amplification by intensifying the signal or probe amplification where only hybridized probes are amplified (Fu *et al.,* 2012). Loop mediated isothermal amplification (LAMP) was also successfully used to detect and type HPVs in cervical specimens (Rohatensky *et al.,* 2018).

Non-amplification techniques such as southern blotting, dot blot hybridization and in-situ hybridization were used in research laboratories that study HPV. In techniques that use southern blotting, DNA extracted from samples containing HPV is restriction digested, then run on gel electrophoresis and visualized by hybridization with HPV genomic probes (Capone *et al.,* 2000). In dot blot hybridization the target DNA is either restriction digested or PCR amplified and then hybridized with probes immobilized on membrane (Lindh *et al.,* 1992; Kang *et al.,* 2019). In in-situ hybridization intact cells suspected to contain HPV DNA are prepared and immobilized on microscope slides and hybridized with HPV specific probes which may be visualized by antibody and enzymes (Capone *et al.,* 2000). Limitations with these techniques are their lower sensitivity, requirement of large amount of DNA and sometimes radioactive labeling and their time and labor intensive procedure (Abreu *et al.,* 2012).

Signal amplification is where certain signal, following presence of specific species of molecules, is amplified by either compound probing or branching probes. One of the widely and long used HPV detection test hybrid capture assay uses RNA probes complementary to 18 HPV types

(Burd, 2016). DNA in the test sample is denatured and mixed with the RNA probe. The mix is then poured to antibody immobilized on the wall of microtiter plate that captures the DNA/RNA hybrid only. The captured hybrid is then detected by light emission after series of antibodies and enzyme treatment (Clavel *et al.,* 1998).

Probe amplification where probes complementary to specific HPV type DNA is amplified by PCR or ligase chain reaction (LCR) only in the presence of the target HPV DNA. Labeled probes with different meting temperature are designed for different target sequences (HPV genotypes in this case) so that their presence in the reaction mix is detected at their specific melting temperature. The probes are designed in such a way that they hybridize to target sequence either to serve as a primer or lie in primers bound region so that they can be hydrolyzed by 5' exonuclease activity of DNA polymerase. PCR amplification following hybridization will consume hybridized probes leaving unhybridized behind. Probe detection after hybridization reveals which probe has got its target sequence in the reaction mix (Fu *et al.,* 2012; Sakellariou *et al.,* 2019).

Target HPV DNA or RNA is PCR amplified and detected or further analyzed by techniques like hybridization or sequencing. Dozens of HPV detection, genotyping and viral load quantification and even HPV genome integration tests work based on PCR amplification of the viral DNA or RNA (Abreu *et al.,* 2012). PCR using consensus primers GP5/6 and MY09/11 has potential to detect all mucosal HPV types. Hybridizing PCR amplicons with type specific probes enables detection of specific genotypes (Villa and Denny, 2006; Eide and Debaque, 2012). PCR can be used for genotyping by the use of type specific primers or though hybridizing, restriction digesting (PCR-RFLP) or sequencing the PCR products (Abreu *et al.,* 2012). Multiplexed real time PCR is also used in detecting presence as well as quantification of DNA or RNA of specific HPV types (Josefsson *et al.*, 1999; Wang *et al*, 2015). PCR based tests are good to work with scarce material but suffer a lot from contamination and sequencing is cost inhibitory to be used as routine test (Shah *et al.,* 2016).

HPV testing is amenable for self sampling which increases the likelihood of women to take part in screening by as high as twofold due to its convenience and privacy by precluding barriers like

fear, shame, geographical barriers, time limitation, cultural and religious barriers (Yeh *et al.*, 2019). HPV tests have generally higher sensitivity than cytological test but large number of HPV test positives might not be of clinical significance (Chrysostomou *et al.,* 2018). This may lead to more unnecessary referrals while cytology has lower sensitivity and hence may lead to delay in receiving the appropriate treatment (Koliopoulos *et al.,* 2017). mRNA based tests with good level of sensitivity and specificity have better potential in both HPV detection and risk stratification by enumerating mRNA of HPV oncogenes which is direct player in the process of carcinogenesis (Macedo *et al.,* 2019).

There is a heated debate whether to use hrHPV testing alone or co-testing with cytology as screening test. The pro hrHPV testing argue that hrHPV testing is superior in preventing cervical cancer, cost effectiveness and acceptability (Chrysostomou *et al.,* 2018). HPV testing is likely to reduce cervical cancer incidence, and health system costs but increases number of colposcopies because of its lower specificity (Bains *et al.,* 2019). Using hrHPV testing reduces the cost as the testing interval is longer (at least five years) compared with the three years of cytology test interval (Diaz *et al.,* 2018). Comparative study of Pap test and HPV (HC2) test reported 39.2% superiority in sensitivity of HPV test while it is only 2.7% less specific (Mayrand *et al.,* 2007).

The opponents challenge this argument from the assumptions it is based on. hrHPV testing assumes that hrHPV DNA is found in 100% of cervical cancers, and those arguing against refute this argument citing studies demonstrating 9% cervical tissue in American cancer registry alone lacking detectable HPV DNA (Flanagan, 2018; Hopenhayn *et al*, 2014) and 37% of adenocarcinomas around the world are HPV negative (Pirog *et al.,* 2014).

Dudding (2016) argues screening by cytology and HPV testing have comparable sensitivity and the reason screening by HPV testing is preferred is because of only its extended interval of testing. Extended intervals jeopardizes its predictive potential putting the screened at risk. HPV based screening has 3-4% lower specificity because of its inability to distinguish between persistent and transient infection, but it is 20-30% more sensitive than cytology which also requires highly trained practitioner (Dijkstra *et al.,* 2014). Regardless of the debates, many

countries are shifting their screening programs from cytology and cytology –HPV co-testing to HPV testing alone (Flanagan, 2018; Bains *et al.,* 2019).

#### *2.3.3.6. HPV genotyping*

HPV genotyping- identifying the HPV type- has two purposes in cervical cancer prevention efforts. First knowing the most prevalent HPV types in cervical cancers help in developing type specific vaccination, and second, detecting infection by carcinogenic HPV genotype is crucial to clinical follow-ups and to treat precancerous lesions earlier (Gradíssimo and Burk, 2017). Currently available HPV vaccines all target the most common HPV types from prevalence data obtained through epidemiological studies.

Because most of commercial HPV genotyping tests are designed with the hrHPVs as their main targets, very little information about prevalence of other HPV types is available in most of the scientific literature. Meiring *et al.* (2012) have identified four HPV types (HPV-30, -74, -86 and -90) with significant prevalence among HIV positive patients using next generation sequencing. Though these types were not reported for their importance in cervical carcinogenesis, the authors recommended tests to be more inclusive so that other HPV types which may have medical significance could be detected.

### 2.3.4. HPV vaccine

The first HPV vaccination was licensed in 2006 (WHO, 2017) with a quadrivalent vaccine made of empty viral coat proteins with no infectious agent targeting the two most common oncogenic HPV types, HPV16 and HPV18 and the other two most common low risk types HPV6 and HPV11 (Cutts *et al.,* 2007). Other two vaccines a bivalent, against HPV 16 and 18, and a nonavalent targeting nine hrHPVs (HPVs 6, 11, 16, 18, 31, 33, 45, 52 and 58) made from *L1* coat protein, were licensed in 2007 and 2014 respectively (WHO, 2017). HPV vaccine is the second ever type of vaccine intended to prevent major cancer next to the hepatitis B vaccine (Kane, 2010).

It is also a success story as the quadrivalent and bivalent HPV vaccines have showed protection from precancers of more than 90% for up to five years after vaccination when evaluated (Cutts *et al.,* 2007). Moreover, antibody levels induced by all HPV vaccines are higher than that induced by natural HPV infection and remained stable for 7 to 12 years after immunization (Artemchuk *et al.,* 2019). This guards adolescent girls against infection during their both biologically and behaviorally most susceptible ages.

Since the highest incidence of HPV infection is during the age immediately following onset of sexual activity and the vaccines do not protect after exposure, vaccination at ages before sexual debut will have greatest benefit (Cutts *et al.,* 2007). WHO recommended vaccinating pre adolescent girls (9-14 years old) as a first priority and females older than 15 and males as second priority. Inclusion of HPV vaccination in national immunization programs is also recommended by WHO (WHO, 2017).

### 2.3.5. Treatment of precursor cervical lesions

Based on the results of screening tests discussed above women may be called for next routine screening test, repeat test if the previous is inconclusive or referred for colposcopy examination and treatments (Chrysostomou *et al.,* 2018). CIN2 or worse and AIS are recommended to be treated by cryotherapy, loop electrosurgical excision procedure (LEEP)/large loop excision of the transformation zone (LLETZ) or cold knife conization (CKC) (WHO, 2014b).

Cryotherapy, where very cold probe is used to freeze the precancerous tissue and thus induce necrosis to destroy the abnormal cells is a widely used treatment for CIN (Basu *et al.,* 2018). This technique uses liquefied gases usually nitrous oxide or carbon dioxide to cool the probe to around -90°C (Castle *et al.,* 2017). The cure rate of cryotherapy is 92% and 85% for CIN2 and CIN3 respectively. Cryotherapy is a good treatment option in resource limited settings as it doesn’t require anesthesia and can be performed by trained non-specialist practitioners. Availability of the refrigerant gases however is limiting factor for cryotherapy (Basu *et al.,* 2018).

Alternative to cryotherapy, where the refrigerant gases are not available, is thermal coagulation, treatment of the abnormal cervical tissue by applying hot probe for less than a minute (Castle *et al.,* 2017). This procedure is proved feasible and effective in resource limited setting where infrastructure for cryotherapy is lacking (Campbell *et al.,* 2016). Recently a new portable thermal coagulation device powered with battery was invented to increase accessibility of the service (Langell *et al.,* 2018). Thermal coagulation has generally higher cure rate (94%) than the cryotherapy (Basu *et al.,* 2018) and minor side effects (Viviano *et al.,* 2017).

Removal of the entire transformation zone by a wire loop electrosurgical instrument termed loop electrosurgical excision procedure (LEEP) or large loop excision of the transformation zone (LLETZ) is commonly used in clinical facilities. LEEP or LLETZ is performed under local anesthesia and has a cure rate of 90% (Basu *et al.,* 2018). Another excision technique used to treat AIS and micro invasive cancer is cold knife conization (CKC). CKC is performed only rarely as it is more complicated and requires anesthesia and hospitalization. LEEP/LLETZ and CKC can also be used as tissue sampling for diagnosis where biopsy testing is required (Castle *et al.,* 2017). In cases where CIN2 and CIN3 persist or recur or the above mentioned treatment options are not successful and for women with AIS who has completed child bearing hysterectomy is preferred treatment option (Massad *et al.,* 2013).

## 2.4. Human papillomavirus (HPV)

Human papillomaviruses (HPVs) are small non enveloped viruses of the Papillomaviridae family which is composed of different papillomaviruses including different animal papillomavirus (de Villiers *et al.,* 2004; Bernard *et al.,* 2010). Papillomaviruses are small (50-60nm in diameter) non enveloped DNA viruses with icosahedral shape. Taxonomy of papillomaviruses is based on nucleotide sequences of genomic or selected sub-genomic region (usually *L1* open reading frame) because phylogenies based on nucleotide sequences are highly correlated with pathogenicity and tissue tropism of the virus (Ranst *et al.,* 1992). The family Papillomaviridae is further classified in to genera, designated by Greek alphabets followed by the term ‘papillomavirus’(for example alpha papillomavirus, beta papillomavirus), species designated by the genus Greek alphabet followed by number (for example alpha 1, alpha 2) and type named by

an abbreviation HPV followed by number (for example HPV1, HPV2). Members of different genera share nucleotide sequence of less than 60%, species of the same genus have 60% to 70% sequence similarity and different types in the same species have 71% to 89% sequence similarity (de Villiers *et al.,* 2004). Papillomaviruses are classified and named by the international committee on taxonomy of viruses (ICTV) to the species level, but the types are assigned and named by the international HPV center (Mühr *et al.,* 2018). HPVs are assigned as type if nucleotide sequence in the selected region of *L1* gene differ at least 10% from other related types. Papillomaviruses which share 90% to 98% of their sequence in the selected *L1* region are considered subtype and a difference of less than 2% variant (Bernard, 2013a; Doorbar *et al.,* 2015).

Though the formal taxonomic levels recognized by biologists are in the species, genus, family etc levels, these taxonomic levels are not much used by HPV researchers especially by those focusing on medical importance (de Villiers *et al.,* 2004). The prominent level of HPV classification in the field is the type and subtypes researched by some epidemiological studies (Bernard *et al.,* 2010). Classification based on E6 genomic region which is directly involved in pathogenicity is also consistent with the type level classification based on the L1 region (Cornet *et al.,* 2012).

Currently there are more than 220 validated and confirmed types of HPVs which fall in to five genera (Alpha, Beta, Gamma, Mu and Nu) of family Papillomaviridae based on their genome sequences (Doorbar *et al.,* 2012). Recently genus gamma has acquired the largest number of HPV types and there are 98 HPV types in gamma, followed by alpha and beta with 65 and 54 HPV types respectively while mu has three HPV types and nu only one HPV type (Mühr *et al.,* 2018). There are also hundreds of novel papillomavirus types recently isolated and are awaiting validation (www.hpvcenter.se/human_reference_clones).

### 2.4.1. HPV genome

HPV genome is circular double stranded DNA of size ~7.9kb, encoding eight or nine gene products among which four, two capsid proteins and two regulatory proteins, are core proteins

expressed and highly conserved across all HPV type(Van Doorslaer *et al.*, 2017). Higher gene density in HPVs is attained through overlapping open reading frames (ORFs) and complex alternative splicing (Doorbar *et al.*, 2015). HPV genome is organized in to chromatin like structure wrapped with cellular histone proteins (Favre *et al.*, 1977) which helps the HPV to use cellular products and machinery for its genome replication and transcription.

The HPV core proteins the two capsid proteins called *L1* and *L2*, and two replication or transcription factors *E1* and *E2* are highly conserved (Doorbar *et al.*, 2015). The rest proteins encoded, *E4*, *E5*, *E6* and *E7*, vary from type to type in their size, sequences and sometimes presence (Doorbar *et al.*, 2012). These unconserved proteins may serve variety of functions such as adapting to specific host niches, establishing a viral replication competent environment, eluding the host immune system, and modifying the host cell to facilitate production and release of progeny virion (McBride, 2017).

The HPV genome is generally divided in three regions; i) the early region, that encodes *E1*, *E2*, *E4*, *E5*, *E6*, *E7* and in some HPV types different forms of *E8* viral early proteins; ii) the late region, that encodes *L1* and *L2* viral capsid proteins, and iii) the upstream regulatory region (URR) or long control region (LCR), a non coding regulatory region (Fig. 2-4). The LCR region is subdivided as 5'LCR, enhancer and promoter (Vinokurova and Doeberitz, 2011). It also contains important regulatory sequences such as replication origin and four *E2* binding sites (*E2*BS1, *E2*BS2, *E2*BS3 and *E2*BS4) for regulation of transcription downstream (Zheng and Baker, 2006).

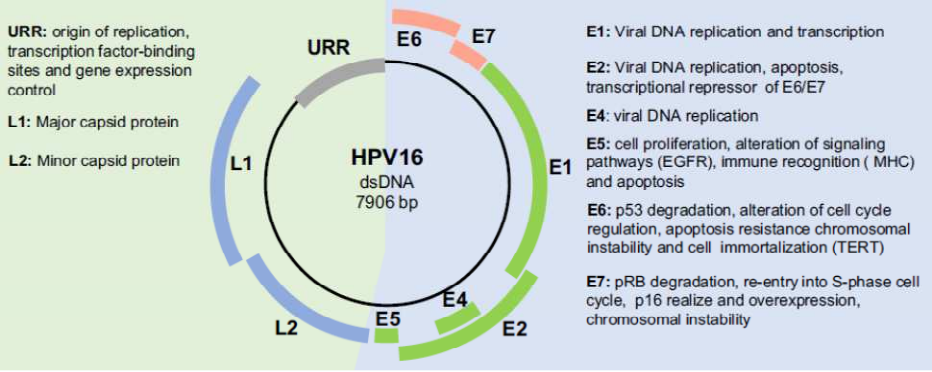


**Figure 2-4: HPV16 genome organization and structure (de Sanjose *et al.*, 2018)**

Early genes are transcribed from early promoter located in the LCR region while the late genes are transcribed from the late promoter located in the *E7* ORF (Bernard, 2013b). The early promoter is tightly controlled from the *E2* binding site located upstream that interacts with cellular transcription factors mostly recruited by the viral *E2* protein. Early primary transcript (pre-mRNA) is composed of three exons and two introns which yield *E1*, *E2*, *E6*, *E7*, *E1^E4*, *E4*, *E5* and in some HPV types *E8^E2* fusion proteins through alternative splicing (Moody, 2017). Transcription and replication regulatory protein E2 binds the early promoter and regulates the transcription of early genes including own mRNA. The late promoter is activated in differentiated keratinocytes only and is not controlled by E2. The late primary transcript is chimeric with early genes *E1*, *E2*, *E1^E4* and *E5* at its 5' terminus and late genes *L1* and *L2* at 3' terminus (Zheng and Baker, 2006, Moody, 2017). The late promoter is thought to be activated by differentiation specific cellular factors (Moody, 2017).

Though different papillomavirus types have different life cycle, all are adapted to epithelial niches (Doorbar *et al.,* 2012). The genus alphapapillomavirus is composed of HPVs that infect the mucosal and cutaneous epithelium while the others infect only the cutaneous epithelium (Crow, 2012). The group alphapapillomaviruses infect the anogenital mucosa mainly uterine cervix and are called genital HPVs.

Infection by HPV may cause cancer, papillomas or warts or may be asymptomatic or latent depending on the HPV type and tissue type or site of infection (McBride, 2017). A study conducted to assess prevalence of skin HPV infection in people with healthy skins found high prevalence (42-70%) in five populations from different parts of the world (Antonsson *et al.,* 2003). The infections often cause micro-lesions and sometimes no symptoms at all as a lot of them are isolated from healthy skins (de Villiers *et al.,* 2004). The mucosal alpha HPV types are further divided as high risk and low risk types while the cutaneous are all low risk types. The high risk HPVs (hrHPVs) also called oncogenic HPVs, causes invasive cancers of various types. Infection by hrHPVs is the major etiologic factor for several cancers of epithelial cells while the low risk HPVs cause common warts and flat warts (Doorbar *et al.,* 2012). Under different immune suppressing conditions however, the latent infections commonly result in different forms of lesions (Reusser *et al.,* 2015).

International agency for research on cancer (IARC) has classified carcinogenic agents in groups as group 1 carcinogenic, group 2A probably carcinogenic, group 2B possibly carcinogenic, group 3 not classifiable as to its carcinogenicity and group 4 probably not carcinogenic. Cervical alpha HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are classified as group1, type 68 as group 2A, types 26, 53, 66, 67, 70, 73 and 82 as group 2B and types 6 and 11 as group 3 (IARC, 2012). The 12 carcinogenic types also known as hrHPV types are well known to associate with cervical cancers (Smith *et al.*, 2007) and HPV types 16 and 18 alone cause about 70% of cervical cancers worldwide (WHO, 2013). The two major oncogenic proteins of hrHPV are designated as *E6* and *E7* and they are known to disrupt the function of cell cycle regulating proteins retinoblastoma (pRb) and p53 respectively (Martin, 2007 and Doorbar, 2006). In addition to cervical cancer hrHPV infection is also implicated in vulvar, vaginal, penile, anal, some oropharyngeal, head and neck as well as colorectal cancers (Stanley, 2010; Lace *et al.*, 2011).

### 2.4.2. HPV genes (proteins)

Genes of hrHPV are transcribed as polycistronic mRNAs from a single DNA strand. Transcription is initiated at two major viral promoter regions called early and late promoters (Longworth and Laimins, 2004). Each HPV gene is expressed in viral lifecycle dependent manner as HPV proteins have lifecycle stage specific role (Schwartz, 2013).

LCR/URR

The long control region contains no ORF but only regulatory elements. It contains the early promoter or transcription start site, replication origin and four *E2* binding sequences called *E2*BSs and other control elements such as binding sites for cellular factors in ~850bp region (Bernard, 2013b). Flanked by the L1 and E6 genes the LCR is divided in to three segments; the 5', central and 3' segments. The 5' segment contains one E2BS, the central segment is known for its transcription enhancer function and the 3' segment contains two E2BSs, E1 binding site and origin of replication (Ribeiro, *et al.*, 2018).

*E1*

Papillomavirus E1 protein ranging in its size from 593 to 681 amino acids and weight of 67.5 to 76.2 kDa (Wilson *et al.,* 2002) it is one of HPV genes to be expressed first in infected cells (Stubenrauch and Laimins, 1999) and in higher amount in later stage of viral life cycle (Graham, 2017). *E1* encodes a highly conserved virus-specific protein with DNA helicase activity and it mainly serves in viral genome replication (Doorbar *et al.,* 2015). HPV16 and 18 E1 regulate expression of cellular genes involved in cell proliferation, migration and metastasis in addition to immune response gene which are modulated by E1 of HPV16, 18 and 11 (Castro-Muñoz *et al.,* 2019). *E1* is entirely not essential for maintenance replication but is important for establishment and productive replication of the HPV16 genome in human keratinocytes (Egawa *et al.,* 2012).

*E2*

*E2* is the major HPV regulatory protein that controls transcription, replication and partitioning of viral genomes to daughter cells during cell division (Doorbar *et al.,* 2015). Its expression is higher in the middle to upper epithelial layer which coincides with HPV genome amplification (Graham, 2016). *E2* protein interacts with both viral and cellular genomes and is involved in variety of functions from viral genome replication and transcription regulation to hacking the cellular products and processes in favor of the viral replication and persistence (Doorbar *et al.,* 2015). Evidences are there that E2 interacts with almost all HPV proteins at different stages of viral infection cycle (Graham, 2017).

*E4*

Contained in the *E2* ORF, *E4* is expressed at the later stage of viral life cycle regardless of its location in the early gene region (Doorbar, 2013). E4 of HPV types 1, 16, 18 and 31 exists in the form of *E1^E4* fusion protein formed from the first five amino acids of *E1* and *E4* protein (Yajid *et al.,* 2017). E4 is involved in release of the mature assembled viral particles from the cell surface. E1^E4 protein interacts with various cellular structures and is known to arrest cell cycle which means suppress proliferation (Raj *et al.,* 2004).

*E5*

*E5* exists in very diverse forms in the HPV, even a single HPV type expressing different forms. Most hrHPV types encode an *E5* protein with around 80 amino acids, HPV16 E5 is membrane localized protein with 83 amino acids (Venuti *et al.,* 2011). E5 is more consistently expressed at the early stage of HPV infection cycle and in premalignant lesions (Venuti *et al.,* 2011). Evidences from in-vitro experiments show that hrHPV *E5*, though weakly, is involved in cellular transformation by enhancing anchorage independent growth, growth in low serum and DNA synthesis via synergistic interaction with epidermal growth factor (EGF) (reviewed in DiMaio and Petti, 2013). Evidences from both in vitro and in vivo experiments show that protein E5 interact with various cellular processes and components such as growth factors, cell cycle, apoptosis, stress responses and immune response (Venuti *et al.,* 2011).

*E6*

Expressed at low level, HPV E6 is protein with approximately 150 amino acids (Tomaic, 2016). E6 is expressed in the lower and middle upper epithelium but decreases in the upper layer in the productive HPV infection cycle (Graham, 2017). Interacting with large number of cellular proteins, *E6* protein alters cellular processes in array of ways including immortalizing or transforming, telomere activation, p53 degradation, transcription modulation, DNA replication, apoptosis and immune response suppression (reviewed in Pol and Klingelhutz, 2013).

*E7*

Conserved among different HPV types, it is a protein with 98 amino acids post translationaly modified by proteosome and phosphorylation (Tomaic, 2016). Like the E6 protein E7 is also expressed at the lower and middle upper layers of epithelia in productive life cycle (Graham, 2017). *E7* of the widely studied HPV16 has no enzymatic activity or DNA binding domain (Munger at al., 2004). HPV16 E7 is localized in nucleus and cytoplasmic region of the infected cells (Roman and Munger, 2013). Viral protein *E7* of hrHPV alters transcription of host cell genes, via chromatin remodeling, histone modification and DNA methylation, binding transcription factors and other promoter interacting factors and micro RNAs, in such a way that

the host cell can support the productive replication of HPV genome (reviewed in Songock *et al.*, 2017). Mainly it binds and degrades retinoblastoma tumor suppressor (pRb) which regulates the cell cycle, deriving cell cycle in differentiated keratinocytes and creating cellular environment suitable for DNA replication (Tomaic, 2016).

*E8^E2*

A fusion protein, *E8^E2* made from *E8* and the C terminal half of *E2* (Moody, 2017), is essential for the maintenance of the persistent, non-productive phase of infection. It is encoded by a spliced transcript and modulates viral replication and transcription (McBride, 2017).

*L1*

*L1* protein is the major viral coat protein which makes the viral capsid assembled in to 72 capsomers of five *L1* proteins (Doorbar *et al.,* 2015). Since viral assembly takes place in the upper strata of epithelium during the later stage of infection cycle, L1 protein is also expressed in this part of the epithelia (Stubenrauch and Laimins, 1999).

*L2*

Also known as minor capsid protein, the *L2* protein is a component of the viral capsid with not yet established proportion. It is composed of nearly 500 amino acids and weighs around 64-78kDa. L2 is expressed at later stage of productive HPV infection cycle in the upper layer of epithelia (Stubenrauch and Laimins, 1999). It has also numerous functions throughout the viral lifecycle from cell entry, vesicular and nuclear transportation, gene expression regulation, viral assembly and others not clearly defined (Wang and Roden, 2013).

### 2.4.3. Transmission and infection cycle

#### 2.4.3.1. HPV transmission

HPV infection is common and widespread in global population. The rate varies from time to time and place to place depending on various factors such as prevension programs such as vaccination

and specific HPV type circulating in populations. Some studies have reported more than 65% of women and comparable proportion of men have been infected some time in their lives (Giles, 2003). Factors such as age of onset of sexual contact, number of sexual partners or number of sexual partners of sexual partner raises the odds of getting infected (Stanley, 2010).

Direct body to body physical contact or indirect through environmental contact between HPV infected tissue and normal skin often with micro wound is sufficient for natural transmission of HPV (Christensen, 2016). HPV transmission is common between sexual partners, the route of transmission being not only penetrative sex. In other words, the transmission is not restricted to genital to genital but genital to other body parts which frequently come in contact with the genitals and vise versa (Hernandez *et al.* 2008). Genital HPV infection is mainly acquired through sexual intercourse penetrative or non penetrative and consistent proper use of condoms may reduce transmission by about 60% (Stanley, 2010).

A family study to determine the possibility and routes of HPV transmission in the family concludes that mode of transmission is complex with different potential factors such as strength of immune response taking part in it (Rintala *et al.,* 2005). Transmission from mothers to infants prenatally is also of significant importance as considerable proportion of genital HPV positive women transmitted to their newborns during pregnancy or delivery (Rombaldi *et al.,* 2009). A study analyzed HPV transmission by type of delivery suggested the possibility of inutero transmission via trans-placental route (Hong *et al.,* 2013).

#### 2.4.3.2. Infection cycle

Many HPV infections culminate in only productive lesions where stable and persistent infection is accompanied by steady release of virions. HPV genome in this kind of life cycle is maintained as a circular non integrated episomal form (Martin, 2007). Productive life cycle of HPVs takes advantage of the hosts' means of cell replenishment and renewal of stratified epithelium by tightly coupling with differentiation steps of stratified epithelial cells (Stubenrauch and Laimins, 1999; Pyeon *et al.*, 2009; McBride, 2017; Schiffman *et al.*, 2016). Productive life cycle causes

only transient lesions that usually regress spontaneously without causing clinical symptoms (Stanley, 2010).

Stratified epithelium of the cervix is composed of basal, parabasal and suprabasal layers. The basal layer contains epithelial stem cells and transit amplifying cells that divide and serve as reservoir of cells for the other upper layers. Cells born in the basal layer migrate upwards through parabasal layer to suprabasal layer and get differentiated and lose proliferating ability. Upon arriving at the suprabasal layer epithelial cells become keratinized and divide no more (Fig. 2-5).

HPVs infection occurs if the viruses get access to the actively dividing basal cells through microwounds of the stratified epithelium that expose cells in the basal lamina that consist of stem cells and other actively dividing (transit amplifying) cells (Longworth and Laimins, 2004). Once the HPV viral particles reach the basal cells of stratified epithelium, they interact with cell surface proteins and other receptors such as integrin and heparin (Stubenrauch and Laimins, 1999), heparan sulfate (Giroglou *et al.*, 2001) laminins and growth factor receptors (Doorbar *et al.*, 2012) to enter the cells. In-vitro assay with HPV16 virus like particle (VLP) showed higher VLP binding to cells with higher expression of cell surface receptor alpha 6 integrin, suggesting that α-6 integrin to be receptor for HPV infection (Yoon *et al.*, 2001). Different HPV types are shown to interact with different types of cell surface heparan sulfate proteoglycans (HSPGs) that may lead to their preference to different tissue types (Johnson *et al.*, 2009). HPV virions associate with laminin 5 (LN5) that is secreted by wound healing keratinocytes and this association is thought to guide the viral particles to the right type of cells (Culp *et al.*, 2006).

In the cell the viral particles will be transported through various types of endosomal pathways to the cell nucleus (Letian and Tianyu; 2010; McBride, 2017). The viral genome enters the nucleus during the nuclear envelop breakdown during mitotic division. The mitotic division believed to be induced by the wound healing or other developmental processes serves as a gateway for the virus (Doorbar *et al.*, 2012).

Once in the nucleus the viral genome undergoes transient replication extra chromosomally (as episome) to approximately 50 -100 copies per cell and this process is termed establishment replication (Moody, 2017). Following this stage is maintenance replication where the viral DNA copy number per cell is maintained low, replicating only along the cellular genome and it is partitioned among the newly formed daughter cells (Stubenrauch and Laimins, 1999). Viral proteins *E1* and *E2* are expressed and undertake various activities. *E1* binds to the viral replication origin to unwind the double strand while *E2* binds to the viral DNA and recruits replication and transcription machinery including *E1*. *E2* also regulates the transcription process by activating the early promoter when it is at low concentration and suppressing it when it is at higher concentration (Longworth and Laimins, 2004). This self control ability of *E2* keeps the viral copy number at lower level in the basal cells at this stage.

As cells migrate up from the basal layer through the epithelium to the surface to replace older cells, they pass through different levels of differentiation and will cease to enter cell cycle rather they go into senescence. However HPV infected cells fail to differentiate terminally so that they enter cell cycle and sustain DNA replication. In cervical interaepithelial neoplasia (CINs) the viral genome is maintained episomally and the viral gene expression tightly regulated. HPV genome is integrated in the cellular genome in invasive cervical cancer leading to E6 and E7 overexpression. In lower grade squamous interaepithelial lesions which correspond to CIN1 and CIN2, the virus undergoes productive cycle which is aborted in the higher grade CIN3 lesion (Fig. 2-5) (Crosbie *et al.*, 2013).

Thus cells enter an S phase of cell cycle and produce DNA replication factors which are used to replicate the viral genome (Longworth and Laimins, 2004). With the cellular supplies synthesized during the S phase, viral genome continues to replicate in the G2 stage as well (Reinson *et al.*, 2015). Along with this aberrantly sustained cell cycle goes productive replication, where the viral genome reaches thousands of copies per cell. Soon viral late genes start to be expressed and progeny virion assembly takes place in the upper most layer of the epithelium where terminally differentiated cells are located. This helps the virus to evade the immune system as this part of the epithelium is not under strong immune surveillance (McBride, 2017).

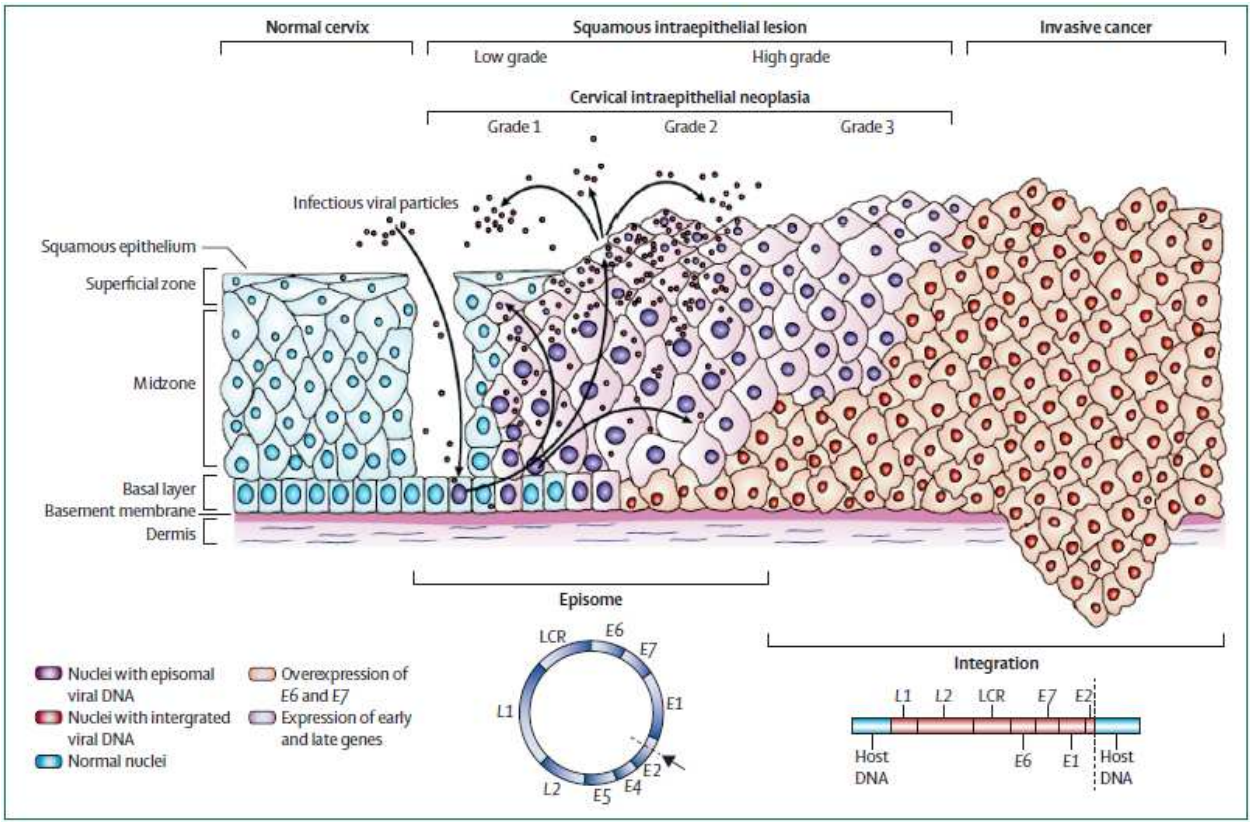


**Figure 2-5: Schematic presentation of HPV life cycle and transformation of cervical cells (Crosbie *et al.*, 2013)**

Following infection, initial transient phase of genome replication occurs, but viral genome maintained episomally at lower (50 – 200) copy number per cell. hrHPV *E6* and *E7* oncoprotein driven basal and parabasal cell proliferation is also evident during this stage initiating neoplastic lesions (Doorbar *et al.,* 2012). The HPV genome is partitioned among the daughter cells by attaching to the host cell mitotic chromosomes which is thought to be accomplished by viral protein *E2* (McBride, 2017). When HPV infected and proliferating cells reach the suprabasal layer late viral genes important for the assembly of mature virions are expressed and new virions are shed from the surface of epithelium (Moody, 2017).

Viral oncogene induced proliferation results in lesion expansion in the suprabasal layer of the epithelium and the would be differentiated cells remain actively dividing. Expression of *E6* and *E7* of the hrHPV in the upper layer of the epithelia also force the infected cells to re enter S-phase, which along with the activity of other viral proteins helps viral genome amplification

(Doorbar *et al.,* 2012). The E4 HPV protein arrests cell cycle at the G2 phase so that the viral genome synthesis proceeds without cells entering the division phase (Moody, 2017).The viral life cycle is completed by the encapsulation of the viral genome and release of the viral particles. This stage requires expression of the viral major (*L1*) and minor (*L2*) coat proteins (Doorbar *et al.,* 2012).

### 2.4.4. Carcinogenesis

Carcinogenicity of papillomaviruses was known as early as 1930s when rabbit experimentally inoculated with papillomavirus developed cancer (Rous and Kidd, 1938). Research has since been undertaken to determine the role HPV may have in carcinogenesis. Strong association between HPV infection and various levels of cervical lesions was becoming clear arousing speculations if the association is causal (Torre *et al.,* 1978; Zur Hausen *et al.,* 1981). HPV16 was identified for the first time as new type that prevails in malignant lesions while condylomata acuminate or anogenital warts harbors previously known HPVs type 6 and 11 (Durst *et al.,* 1983)

Following the confirmation of its carcinogenicity, research efforts were shifted towards determining the mechanisms. Biochemical and in vitro molecular studies revealed that the HPV proteins *E6* and *E7* interact with proteins that control cell cycle and proliferation inactivating or degrading them (Sen *et al.,* 2018). It is important for the virus to immortalize the infected cells as it is through cellular proliferation that the virus replicates its genome. However, the HPV *E6* and *E7* oncoproteins cause malignant transformation less often as their expression is tightly regulated (Kajitani *et al.,* 2012). Under normally regulated viral genome replication and expression condition, HPV undergoes productive infection where the viral genome is maintained and regulated in its intact circular stable episome and do not cause malignant transformation (Martin, 2007).

Infection with hrHPV is so common that around 80% of sexually active women become infected. However most (80-90%) infections are cleared by cell mediated immune response, in periods ranging from months to years, but HPV16 and HPV18 often persisting longer (Doorbar *et al.,* 2015). The only reason behind this small portion of infection to progress to invasive cancer is

changes that may occur to expression rates of HPV genes. Polymorphisms in the LCR, which does not code for any ORF but regulates transcription in the other genomic regions, are implicated in cervical cancer progression (Pientong *et al.,* 2013) suggesting carcinogenicity is mainly effected by regulation of HPV early genes.

The major mechanism by which HPV gene expression is altered is through integration of HPV genome in to the cellular genome. HPV genome integration in the host genome frequently disrupts the *E1* or *E2* genes and deregulates expression of the viral *E6*/*E7* oncoproteins causing their persistent and high level expression which may in turn lead to genomic instability and cell proliferation (Pett *et al.,* 2004; Motoyama *et al.,* 2004). *E2* loss of function is implicated in cellular transformation either by integration associated disruption of the *E2* ORF or repression of *E2* function by inhibition of *E2* protein association with its binding sites (by methylation) (Doeberitz and Prigge, 2019). In cervical cancer *E1*/*E2* gene disruption is common event in HPVs 18 and 45 when compared with HPV16 and is inversely related with the LCR CpG methylation (Amaro-Filho *et al.,* 2018). This implies existence of different mechanisms of carcinogenesis associated HPV gene expression deregulation and the possibility that they are type dependent.

HPV genome integration into the cellular genome is important event in invasive cancer development and bad prognosis (Das *et al.,* 2015). Study on adenocarcinoma which is predominantly caused by HPV18, found a 100% integration of the viral genome. In more than 80% of cervical cancer of all type, HPV genome is found integrated in the cellular genome and this may affect both the viral gene regulation and host genome stability (McBride and Warburton, 2017).

The hrHPV *E6* and *E7* proteins are viral oncoproteins, *E6* mainly binding the tumor suppressor protein p53 and leading to its degradation while *E7* binds the retinoblastoma (Rb) family of tumor suppressors and other proteins involved in cell cycle control. hrHPV *E7* also binds and degrades p105 and p107 proteins that control cell cycle entry (Longworth and Laimins, 2004). HPV16 E6 is also involved in expression of telomerase enzyme, which is implicated in cancer cell immortalization, through interaction with transcriptional repressor NFX1-91 (Gewin *et al.,*

2004). Over-expression of these HPV proteins is the major driving molecular event in the process of HPV mediated cervical carcinogenesis (Graham, 2017).

### 2.4.5. Regulation and expression of HPV genes

HPV depends on the host cell machinery both for replication and transcription (McBride, 2017). HPV genome is tethered to the cellular chromatin by the means of *E2* protein's chromatin binding ability and this helps the virus to escape host immune response, to ensure efficient propagation among daughter cells upon cell division, to stay transcriptionally active by association with active host chromatin and induce DNA damage repair response to use the cellular DNA damage repair machinery for the viral replication in differentiated cells (McBride *et al.,* 2012). This dependence on the host cellular machineries and resources necessitates the virus to alter the host cellular processes (Moody, 2017).

Viral protein *E2* is the major factor that controls both replication and transcription of HPV genome. At lower level *E2* binds to one of its binding sites *E2*BS1 and activates transcription from the early promoter. This process increases the level of all early genes including *E6*, *E7* and *E2* itself which when at high level will bind to the other binding sites (*E2*BS2, *E2*BS3, *E2*BS4) and represses transcription from the early promoter down regulating the early gene products (Doeberitz and Prigge, 2019). This way E2 keeps the gene expression profile of HPV in check so that the induced cellular proliferation is not out of control and the productive cycle is maintained (Graham, 2017).

### 2.4.6. Epigenetic control of HPV genome

Viral genome is assembled with host histone proteins in to chromatin similar to the host's (Favre *et al.,* 1977; McBride, 2017) and hence maintained and regulated in similar fashion and by similar cellular factors. HPVs express diverse proteins from a short genome through alternative splicing and polyadenylation making RNA processing an important gene regulation (Graham and Faizo, 2017, Schwartz, 2013). The HPV transcription regulator protein E2 is in charge of regulation of the whole viral life cycle through regulation of HPV gene expression (Kajitani *et*

*al.*, 2012). The functions of E2 protein are manipulated by viral genome methylation (Johannsen and Lambert, 2013) and chromatin (histone) modification (Bernard, 2013b). The major HPV transcription factor *E2* binding is sensitive to the binding site methylation (Johannsen and Lambert, 2013).

### 2.4.7. HPV DNA methylation in cervical cancer

Genome binding ability of the major HPV regulatory protein *E2* is modulated by DNA methylation in a complex way as the different *E2* binding sites (*E2*BSs) serve different functions with regard to transcription and replication activation (Vinokurova and Doeberitz, 2011). Methylation of CpGs at the *E2*BS1 represses the HPV early promoter by hindering *E2* binding to this site while methylation of CpGs at the other three *E2*BSs (*E2*BS2, *E2*BS3 and *E2*BS4) enhance the viral early promoter activation by inhibiting *E2* binding which is repressive if bound at these sites (Kim *et al.*, 2003). A lot of studies discovered differential methylation level of HPV genome in association with severity of cervical pathology (Louvanto *et al.*, 2015; Yang-chun *et al.*, 2017), which in turn is attributed to viral genome deregulation (Middleton *et al.*, 2003).

Different levels of CpG island methylation is observed in 3'LCR of HPV16, 18 and 45 in cervical cancer samples. Higher level of methylation is recorded in HPV16 (Amaro-Filho *et al.*, 2018). Whether de novo HPV DNA methylation is laid as the viral immune escaping mechanism (Vinokurova and Doeberitz, 2011) or as the cellular immune response to suppress expression of intruding viral genome (Kalantari *et al.*, 2004), remains to be elucidated. Vinokurova and Doeberitz, (2011) showed CpG islands in the LCR region show complex methylation pattern in a viral life cycle and pathologic grade dependent manner. All the CpGs in the LCR region are hypermethylated in cervical cells adjacent to HPV 16 transformed cells with no morphological change and sign of HPV infection, suggesting that the viral particles in these cells are latent. However, in basal and parabasal cells of lesions CpGs in cellular transcription factors binding sites are methylated whereas all *E2*BSs are unmethylated, but in the fully differentiated suprabasal cells CpG islands in the promoter region are completely methylated (Vinokurova and Doeberitz, 2011).

Generally de novo methylation of viral genome increases with increased cervical malignancy with the highest level of methylation observed in CpG sites in the viral *L1* ORF (Kalantari *et al.,* 2004) and those in *E2*BS1 (Vinokurova and Doeberitz, 2011). Increased methylation of CpG sites in the *L1* and *L2* genes of HPV genome is associated with increased level of neoplastic lesion of the cervix (Clarke *et al.,* 2018). DNA methylation in the HPV16 *L1*/*L2* region (nucleotide positions 5600 and 5609) strongly associates with cervical cancer progression (Bryant *et al.,* 2014). Hypermethylation of CpGs at *E2*BS1 is consistent with its role of activating the early promoter and hence increased transcription of the viral oncogenes.

## 2.5. *EPB41L3*

Erythrocyte membrane protein band 4.1 like 3 (*EPB41L3*), also known as differentially expressed in adenocarcinoma of the lung (DAL-1), is tumor suppressor membrane protein. The protein DAL-1 also known as protein 4.1B is localized at cytoplasmic side of cell-cell junction (Tran *et al.,* 1999). *EPB41L3* was first characterized as its expression was lost in more than 60% of non small cell lung cancer in 1999 by Tran *et al. EPB41L3* is mapped to chromosome 18p11.3 using fluorescent in-situ hybridization (FISH) technique. Role of *EPB41L3* as growth regulator in pathogenesis of meningiomas was soon reported in 2000 by Gutmann *et al.,* (2000) adding experimental evidence to its tumor suppressor activity.

Lines of evidence are there for tumor suppressor function of *EPB41L3* or DAL-1. Its tumor suppressor activity is evident through its differential expression and frequent loss of heterozygosity of its chromosomal locus in non-small cell lung cancer (Tran *et al.,* 1999). Production of miR-223 which targets *EPB41L3* is higher in certain type of lung cancers and this is proved to promote tumor cell invasion (Liang *et al.,* 2015). *EPB41L3* is expressed in normal epithelial cells but absent or minimal in cancer cells of ovarian (Dafou *et al.,* 2010) and breast (Takahashi *et al,* 2012) epithelial cells, suggesting its tumor suppressor function. *EPB41L3* expression was also found to be reduced in esophageal squamous cell carcinoma cell lines as well as specimens from patients (Zeng *et al.,* 2018). Data from its loss in different cancer types of the nervous system (meningiomas and schwannomas) and its over-expression in cell lines

from these same cancer types (Gutmann *et al.*, 2001) showed that *EPB41L3* has a tumor suppressor activity in specific cell types but not in every cell type.

From in vitro and cell line experiments, *EPB41L3* is reported to interact with protein arginine N-methyltransferase 3 (PRMT3) (Singh *et al.*, 2004) and PRMT5 (Jiang *et al.*, 2005), an enzyme that methylates arginine in mammalian cells. The interaction of *EPB41L3* with PRMT enzymes modulates their activity in a substrate specific manner in such a way that cell growth is suppressed (Jiang *et al.*, 2005). Interacting with a membrane glycoprotein TSLC1, *EPB41L3* is also involved in actin filament cytoskeleton regulation and determines cell adhesion and motility in epithelial cells (Yageta *et al.*, 2002). Both cell motility and adhesion are important aspects of cellular transformation. It's up regulation in mammary epithelial cells during pregnancy (time of mammary cells proliferation) and the increase of mammary epithelial cell proliferation up on its loss, shows *EPB41L3*'s role in regulating epithelial cell proliferation (Kuns *et al.*, 2005).

A general mechanism by which *EPB41L3* suppresses tumor formation and growth is evident from an experiment that induced expression of *EPB41L3* in breast cancer cell lines (Jiang and Newsham, 2006). It was found to activate apoptotic pathway through its protein methylation modulation described in (Jiang *et al.*, 2005). Over-expressing *EPB41L3* gene in esophageal squamous cell carcinoma cell lines inhibited cell proliferation and induced apoptosis and G2/M cell cycle arrest (Zeng *et al.*, 2018).

Epithelial to mesenchymal transition (EMT) is an early cellular process that promotes metastasis, through loss of the epithelial properties such as cell-cell adhesion and polarity and acquisition of mesenchymal properties. Inhibition of *EPB41L3*/4.1B expression promotes EMT and hence migration and invasion ability of cancer cells while its re-expression made the dedifferentiated cancer cells to regain epithelial properties (Chen *et al.*, 2015). *EPB41L3* hinders EMT by suppressing expression of heat shock protein 5 (HSPA5) that promotes the transition through array of signaling pathways (Qiu *et al*, 2017) and regulating expression of other EMT marker products such as cadherins, beta catenin and vimentins (Chen *et al.*, 2015). Another possible mechanism for *EPB41L3*'s tumor suppressor activity is through stabilizing cell adhesion and

regulating motility suppressing metastatic invasion of cancer cells(Yageta *et al.,* 2002) or through signaling cell contact growth inhibition (Gutmann *et al.,* 2000).

### 2.5.1. *EPB41L3* promoter methylation

*EPB41L3* has a GC content of 62% upstream of the transcription start site which indicates that its expression is controlled through methylation of CpG islands in the promoter region (Tran *et al.,* 1999). *EPB41L3* is hypermethylated in brain tumors of astrocytic and oligodendroglial lineage (Perez-Janices *et al.,* 2015) and epithelial ovarian cancers (Dafou *et al.,* 2010). *EPB41L3*/4.1B expression is suppressed by promoter hypermethylation in invasive breast cancers (Takahashi *et al.,* 2012) and non small cell (NSCLC) and small cell (SCLC) lung cancers (Kikuchi *et al.,* 2005).

Methylation analysis of the promoter region of *EPB41L3* gene in esophageal squamous cell carcinoma revealed its potential as therapeutic target and prognostic indicator (Zeng *et al.,* 2018). Treatment with an epidrug, methyl transferase inhibitor 5-aza-2'-deoxycytidine restored *EPB41L3*/4.1B expression in cell lines initially lacking the *EPB41L3* gene expression (Kikuchi *et al.,* 2005). Expression of methylation silenced *EPB41L3* gene could be enhanced to many folds by artificial transcription factors that can bind to the methylated promoter region, though it is transient. Epidrugs augmented with reactivation of *EPB41L3* expression could reduce cell proliferation in vitro (Huisman *et al.,* 2015).

### 2.5.2. *EPB41L3* in cervical cancer

*EPB41L3* promoter region is among frequently hypermethylated human genes by HPV *E6* and *E7* induction and it is suggested to have diagnostic potential to serve as non invasive molecular marker for early detection of cervical cancer (Sen *et al.,* 2018). Kelly *et al.,* (2018) showed increase in DNA methylation at the promoter region of *EPB41L3* gene with increasing grade of cervical intraepithelial neoplasia. A lot of studies showed CpG methylation in the promoter region of *EPB41L3* has potential to differentiate different stages of cervical pathologies (Blanco-Luquin *et al.,* 2015; Louvanto *et al.,* 2015). DNA methylation based cervical cancer triage

classifiers that included *EPB41L3* promoter region and four HPV DNA sites methylation could predict from precancerous lesion to cervical cancer with better specificity and sensitivity than both cytology and hrHPV tests (Lorincz *et al*, 2016; van Leeuwen *et al*, 2019).

# Chapter 3

# 3. Materials and Methods

## 3.1. Study population

This study was conducted with Ethiopian women attending Tikur Anbesa general specialized hospital and family guidance association of Ethiopia (FGAE) clinic. Tikur Anbesa general specialized hospital is a tertiary level teaching hospital where patients are referred from all over Ethiopia for chronic and critical cases. FGAE clinic is a clinic run by the nonprofit making association and mainly focused on family planning and reproductive health services. Participants in this study include women with various degrees of cervical pathology (ranging CIN1 to stage-IVB invasive cancer) (cases) and healthy women with normal uterine cervix (controls). The women included in this study were in the age range from 20 to 75 years. Women with any other cervical or gynecological problems and those with undefined status of their cervical lesions were excluded from the study.

## 3.2. Study design

This study was an observational case control study. Cases were defined as women with invasive cervical cancer and precursor lesions (cervical intraepithelial neoplasias CIN1, CIN2 or CIN3) where CIN2, CIN3 and invasive cervical cancer were histopathologicaly confirmed but CIN1s were cytology positive. Controls were women with normal cervix who had negative Pap test results. The data was also analyzed based on the stage of cervical cancer including the healthy (controls), CIN1, CIN2, CIN3 and clinical stages IA, IB, IIA, IIB, IIIA, IIIB, IVA and IVB cancer. There were no CIN2 and cancer stage IA as there were no eligible women with these conditions in the participants. Alternative categorizations used were ‘level’ and ‘risk’ where level referred to pathologic severity at three levels as normal, CIN1 and CIN3 or worse (CIN3+) and risk was meant by level of cervical cancer risk where healthy and CIN1 were classified as low risk and CIN3 and worse as high risk.

## 3.3. Sample and data acquisition

Samples were collected from a total of 135 women (96 cases and 39 controls). The sampling technique followed was convenience sampling where all eligible women who had visited the study sites during the study perion and had consented to participate in the study were included. Determinants in the sampling of this study were the visit of the patients during specific time and willingness to participate. Both of these factors has no direct impact on all the variables studies and hence cannot cause systematic sampling bias. However, the fact that both cancer referral facility and cervical cancer screening services are available in the city may bais the distribution of samples with regard to residence.

Samples were collected during period from August 2015 to December 2018. All samples from invasive cervical cancer cases and cervical intraepithelial neoplasia grade 3 (CIN3) were obtained from Tikur Anbesa general specialized hospital while all the healthy controls and cervical intraepithelial neoplasia grade 1 (CIN1) were obtained from FGAE clinic. All the study participants were informed about the study protocol and had given written consent to participate in the study by providing their demographic data and allowing the use of samples collected for diagnosis and screening tests to this study. Socio-demographic variables were collected by a questionnaire (Annex 1-A) filled in by trained nurses and the researcher asking the participants in Amharic, Afan Oromo and by translation of other languages. All the samples were coded to protect the privacy of the study participants. Demographic and clinical data obtained by the questionnaire was also coded accordingly so that any finding that arises from the research cannot be traced back to the individual participants.

Most of the demographic data were self reported and some were not reliable and some inconsistently reported by different participants. Only data that were consistently reported across all the participants and were more reliable were included in the analysis. History of any chronic illness and associated treatments and family history of cancer were not consistent and reliable because it was based on personal memory because of lack of organized registration system in place. Number of life time sexual partners and exposure to cigarette smoke were also reported inconsistently as some of the participants were not willing to disclose such personal information.

Occupation, domestic fuel smoke exposure and staple food were excluded as they were difficult to enumerate.

The demographic data included in this study were current age, residence (as urban and rural), parity (as number of full term pregnancies or deliveries), age at first sexual intercourse (AAFSI) and hormonal contraceptive use (for those who used for at least a year). Clinical staging of the precancerous lesions and cervical cancer cases were collected from the clinical records (patient record cards). Infection status of hrHPV (as positive and negative), hrHPV genotypes and methylation profile of the human (host) *EPB41L3* gene and HPV L1 and L2 genes were generated from laboratory analysis.

### 3.3.1. Cervical punch biopsy samples

Samples from invasive cervical cancer and CIN3 were collected by cervical punch biopsy performed for histopathological diagnosis of patients who were referred to confirm the cancer status. Portion of cervical punch biopsy was taken for this study while the other portion was sent for histopathology examination.

Tissue punch biopsy samples were put in cryovials and kept in liquid nitrogen (flash frozen) within maximum of 5 minutes after biopsy was taken and transferred to and kept in -80$^{o}$C freezer until DNA was extracted. DNA was extracted after the histopathology result of each sample was obtained from the pathology laboratory. All punch biopsy samples collected were kept until later screened by histopathology reports and clinical diagnosis. Samples with missing clinical information, ambiguous or negative histopathology results or insufficient material were excluded from this study. Only samples from clinically diagnosed and biopsy (histopathology) proven cases were included (Fig. 3-1).

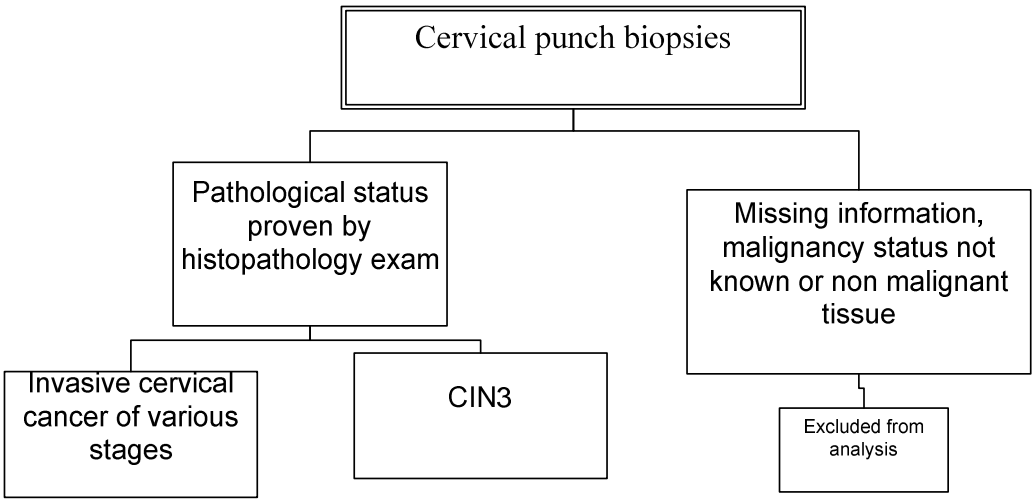


**Figure 3-1: Flow chart of cervical punch biopsy samples classification**

### 3.3.2. Cervical swab samples

Cervical swabs were collected at FGAE clinic from clinically healthy women. Cervical swabs were collected from women visiting the clinic for routine cervical cancer screening. The swabs were taken in duplicates from ectocervix and endocervical regions for Pap smear. Cervical swabs leftover from smear slide preparation (Pap test) were saved for this research. Swabs from both Pap negative (normal cytology) and from women with CIN1were used in this study (Fig. 3-2).

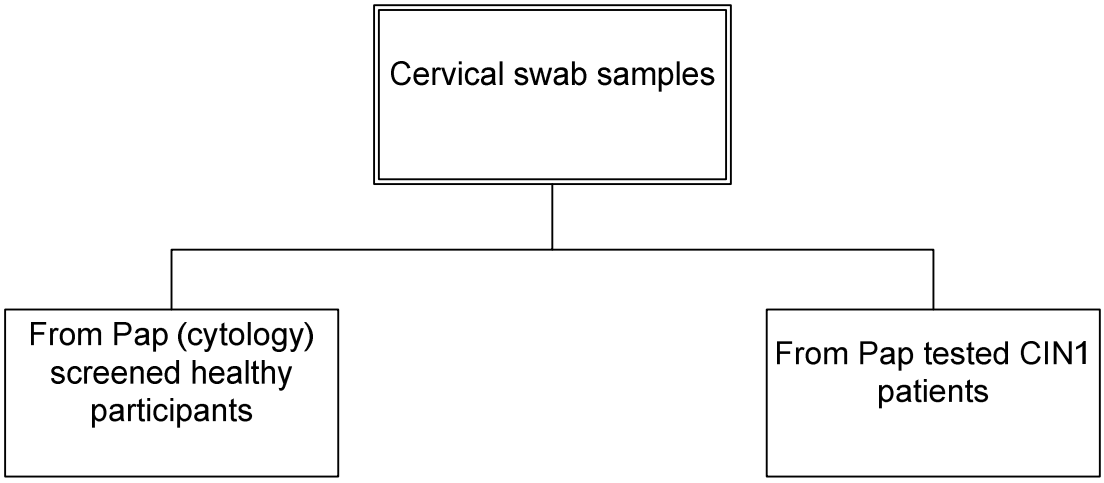


**Figure 3-2: Flow chart of cervical swab samples classification**

## 3.4. DNA extraction

DNA was extracted manually following an in-house optimized DNA extraction protocol (annex 3) modified from Aidar and Line (2007) and Küchler *et al* (2011). The cotton swabs were immersed in 1.5 mL lysis buffer containing 10 mM tris-HCL, 5 mM ethylene diamine tetra acetate (EDTA), 0.5% sodium dodecyl sulphate (SDS) at pH 8 and proteinase K (100µg/mL) and incubated at 55°C for six hours. The swabs were removed and cell lysate was then treated with 750 µL cold 8 M ammonium acetate solution to precipitate cell debris and denatured undigested proteins. The precipitate was settled by centrifuging at 6000 RPM for 20 minutes. DNA was precipitated by transferring 750 µL aliquot of the supernatant to microcentrifuge tubes containing equal amount (750 µL) of cold isopropanol and centrifugation at 13000 RPM for 10 minutes. DNA pellet was air dried and washed with 1 mL 70% ethanol and re-suspended in 100 µL 1XTE buffer (10 mM Tris and 1 mM EDTA, pH 8) and stored at -20°C until analyzed.

DNA from the punch biopsy samples are extracted by mincing the tissue with clean scalpel as finely as possible on clean (new) plates. Then the tissue is treated with lysis buffer containing 100µg/mL proteinase K in similar way as cotton swabs (mentioned above).

Quality and quantity of the DNA was evaluated by agarose gel electrophoresis and UV spectrophotometry (NanoDrop, Thermo scientific 2000) respectively. Intense bands of high molecular weight were considered intact genomic DNA. Negative controls were used at every step from DNA extraction to the electrophoresis based quality checking and spectrophotometric quantification.

DNA quantity was checked using absorbance of UV light by NanoDrop spectrophotometer (NanoDrop Thermo scientific 2000) which calculates DNA concentration and absorbance ratios at 260 nm to 280 nm and 230 nm to 280nm to indicate contamination.

To check for existence of any PCR inhibitors in the DNA, test PCR was run with various consensus and human specific primers. None of the samples failed to amplify by PCR. The extracted DNA was then stored at -20$^{o}$C until used.

## 3.5. Primer selection and design

Sequences for degenerate primers MY09 (5’ CGT CCM ARR GGA WAC TGA TC 3’) and MY11 (5’ GCM CAG GGW CAT AAY AAT GG 3’) that target a 450bp consensus sequence in the L1 ORF region of all HPVs were taken from literature (Venceslau *et al.,* 2014). Primers for the human beta globin gene which was used as an internal control for DNA quality and PCR protocols were designed using Primer 3 software (Untergasser *et al.,* 2007) and human genome reference sequence (accession number NC_000011) from NCBI database (https://www.ncbi.nlm.nih.gov/). Primers for DNA methylation analysis were designed using Pyromark Assay Design software (Qiagen, Germany) and human and HPV reference sequences.

## 3.6. HPV detection and genotyping

### 3.6.1. Non specific test for HPV DNA

DNA extracted from all samples were tested for the presence of HPV DNA using a degenerate primers MY09 (5’ CGT CCM ARR GGA WAC TGA TC 3’) and MY11 (5’ GCM CAG GGW CAT AAY AAT GG 3’) as forward and reverse primers. Amplification of segment of human beta globin gene with primers Hbtgl1 (5' ACACAACTGTGTTCACTAGC 3') and Hbtgl2 (5’ CAACTTCATCCACGTTCACC 3’) as forward and reverse was also multiplexed to serve as internal control of the DNA quality and PCR success. PCR components and composition was 5 µM of each primer, 200 µM of each dNTPs, 2 mM $MgCl_2$ solution, 3 µL BD PCR buffer (HiMedia laboratories Pvt. Ltd., Mumbai India) 1U Taq polymerase, and 18.75 µL nuclease free water in 25 µL reaction mix. The PCR profile was initial denaturation at 95°C for five minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 45 seconds and extension (polymerization) at 72°C for 45 seconds and final extension at 72°C for 7 minutes. The PCR amplicon was then electrophoresed on 1.6% agarose gel with 100 bp DNA ladder (Bioneer Inc., Korea) with Ethidium Bromide staining and gel photo taken with Biorad gel doc

system. Upon gel-electrophoresis presence of ~450 bp band was interpreted as HPV positive and ~110 bp band as PCR success and presence of human (internal PCR control). All DNA samples were expected to have the ~110 bp band unless the PCR process failed, but only samples positive for HPV DNA have the ~450 bp band.

### 3.6.2. HPV genotyping

The Papilloplex High Risk HPV test (GeneFirst, UK), a multiplex probe amplification based system that simultaneously genotypes the 12 hrHPV types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58and 59) and the possibly carcinogenic HPV66 and probably carcinogenic HPV68 was used to identify HPV infection and genotype according to manufacturers instruction. The assay was based on multi-probe amplification technology which allowed differentiation of up to six targets per each of the following florescence channels: FAM, JOE/HEX, ROX and Cy5. Genotyping was run on QuantStudio 5 Real-Time PCR System (Thermoficher Scientific, UK) and targeted the L1 region of all the genotypes described. Papilloplex High Risk HPV internal negative and positive controls were used as baselines. Cycle threshold (ct) value of $\leq$ 36 for positive result is predetermined by the manufacturers. Samples with ct value of greater than 36 were considered negative. Each sample was run in duplicates and the papilloplex system software automatically interpretes the curves in to positive and negative results.

## 3.7. DNA methylation analysis

100 ng of DNA was used in the bisulfite conversion reaction where un-methylated cytosines were converted to uracils with the EZ DNA methylation kit (Zymo Research, Irvine, USA). This step prepared the genomic DNA for gene-specific DNA methylation analysis. qPCR analysis was performed on Quantstudio 5 (ThermoFisher, UK). A region of the β-Actin gene that contains no CpG sites (hence no methylated cytosine) was tested with qPCR to check bisulphite conversion efficiency (Malentacchi *et al*., 2009). The qPCR was performed in duplicates with primer sets (Table 3-1) targeting bisulphite converted and unconverted targets. The threshold for conversion efficiency was set at 95%.

**Table 3-1. Sequences of primers used to measure bisulphite conversion efficiency**

| Primer | Primer name | Primer sequence 5' to 3' |
|---|---|---|
| **Converted β-Actin Control** | ACT (+) F | TGGTGATGGAGGAGGTTTAGTAAGT |
| | ACT (+) R | AACCAATAAAACCTACTCCTCCCTTAA |
| **Unconverted β-Actin Control** | ACT (-) F | TGGTGATGGAGGAGGCTCAGCAAGT |
| | ACT (-) R | AGCCAATGGGACCTGCTCCTCCCTTGA |

The bisulfite converted template DNA was diluted with nuclease free water in 1:5 ratio. The 12.5 µL reaction mix consisted of 6.25 µL Kapa SYBR Fast Master mix (Sigma-Aldrich, Germany), 0.625 µL (12.5 µM) of forward and reverse primer mixes, 4.375 µL RNAse Free Water (Qiagen, Germany) and 1.25 µL (~25 ng) bisulfite converted DNA. The qPCR amplification conditions were, initial denaturation at 95°C for 20 minutes followed by 40 cycles of, denaturation at 95°C for 1s and annealing/extension at 60°C for 20s.

Further, the converted DNA was amplified by methylation independent PCR primers to fish out the genomic regions containing CpG sites of interest. PCR primer sequences with their annealing temperature, number of cycles and primer concentrations are given in Table 3-2. 20 ng converted DNA was used per PCR reaction. For each 25 µL PCR reaction master-mix, the following were added: 2.75 µL CoralLoad 10x (Qiagen, Germany), 4.5 µL RNAse Free Water (Qiagen, Germany), 13.75 µL Pyromark PCR mix (Qiagen, Germany), 1.5 µM $MgCl_2$ (Qiagen, Germany) and 0.2-0.3 µM primer mix (Sigma-Aldrich, Germany).

**Table 3-2. Primer sequences and PCR conditions used to amplify segments of the genome with CpG sites of interest**

| Gene | Primer name | Primer sequence 5' to 3' | Annealing T (°C) | No Cycles | Primer conc. (mM) |
|---|---|---|---|---|---|
| ***EPB41L3*** | *EPB41L3* F | GGGGGATTTGTGTAAATTGG | 54 | 45 | 0.2 |
| | *EPB41L3* R (B) | (btn) ACCTAAAAACCTCCCTAAAATC | | | |
| **HPV16L1** | HPV16 L1.3 short F (B) | [btn]-AGTGAAGTTTTATTGGATATTTGTAT | 51 | 40 | 0.3 |
| | HPV16 L1.3 short R | CAACAACACCAACCCTATTAAAT | | | |
| **HPV16L2** | HPV16 L2 F | GTATGTTTTATAAAGTTGGGTAG | 51 | 50 | 0.3 |
| | HPV16 L2 R (B) | [btn]-TTAATAAACTATTATCACTTAACAATAC | | | |
| **HPV18L2** | HPV18L2 6CpG F | GTATAGGTTGTTTTATATAGTGTATTGT | 54 | 50 | 0.2 |
| | HPV18L2 6CpG R | [btn]-TCCACCTTAAAAACAACATCAAATAA | | | |
| **HPV31L1** | HPV31 L1 6284 F (B) | [btn]-ATTTGTGTATTTGAAGTAATTATGGAG | 49 | 45 | 0.2 |
| | HPV31 L1 6284 R | TCCAAATTATCTTAAAATAATTACTAAACC | | | |
| **HPV33L2** | HPV33 L2 F | AGGTAGGTATATTGTGGTTTTATTAGGT | 53 | 45 | 0.2 |
| | HPV33 L2 R (B) | [btn]-CACATCTAACCCATTTATTCCTATTTC | | | |

Known controls to each HPV type and human genome with, 0%, 50% and 100% methylation were included. Temperature profile of the thermal cycler used was; initial denaturation at 95°C

for 15 minutes followed by; number of cycles mentioned in Table 3-2 at 94°C for 30s, at annealing temperature (Table 3-2) for 30s and at 72°C for 30s.

The amplicons were pyrosequenced on a PyroMark Q96ID (Qiagen, Germany) for DNA methylation analysis on CpG islands of *EPB41L3* promoter (CpG 438, 427, 425) and viral late genes (L1 and L2) coding for proteins involved in viral capsule formation: HPV16L1 (CpG 6367, 6389), HPV16L2 (CpG 4275, 4268, 4259, 4247, 4238), HPV18L2 (CpG 4256, 4261, 4265, 4269, 4275, 4282), HPV31L1 (CpG 6352, 6367) and HPV33L2 (CpG 5557, 5560, 5566, 5572) as detailed by Lorincz *et al* (2016). Percentage methylation scores were taken as the mean of methylation percentage of each CpG sites in a specified region involved in each case. Sequencing primers are described in Table 3-3.

**Table 3-3. Sequences of sequencing primers and CpG positions targeted**

| Gene | Primer name | Primer sequence 5' to 3' | Target CpG positions |
|---|---|---|---|
| ***EPB41L3*** | *EPB41L3* s | GGGATTTGTGTAAATTGG | 438, 427, 425 |
| **HPV16L1** | HPV16L1.3 6367 s | CTAACAAACATTTATTCCCTTC | 6389, nonCpG, 6367 |
| **HPV16L2** | HPV16L2.5CpG 5s | TTTTATAAAGTTGGGTAGT | **4275**, 4268, **4259**, 4247, **4238** |
| **HPV18L2** | HPV18 L2s | TGTATTTTTGTAATAAAAGTATGGTA | 4256, 4261, 4265, 4269, 4275, 4281 |
| **HPV31L1** | HPV31 6284 sB | CATTTTTTTAATAAATCAAACAC | 6352, 6364 |
| **HPV33L2** | HPV3333 L2 5572s | GGATATTTGTAAAAAAATATGG | **5557, 5560, 5566**, 5572 |

## 3.8. S5 methylation assay

S5 cervical cancer risk classifier was calculated based on DNA methylation panel of CpG sites in the human *EPB41L3* gene promoter region, HPV16 L1 and L2 regions, HPV18 L2, HPV31 L1 and HPV33 L2 regions (Brentnall *et al.*, 2015). S5 scores were calculated by the formula;

$$S5 = 30.9 \times EPB41L3 + 13.7 \times HPV16L1 + 4.3 \times HPV16L2 + 8.4 \times HPV18L2 + 22.4 \times HPV31L1 + 20.3 \times HPV33L2$$

where, *EPB41L3* is mean of methyaltions at nucleotide positions 438, 427 and 425; HPV16-L1 is mean methylation at nucleotide positions 6367, 6389; HPV16-L2 is proportion of CpGs methylated at nucleotide positions 4238, 4259, 4275; HPV18-L2 is mean methylation at nucleotide positions 4256, 4261, 4265, 4269, 4275, 4281; HPV31-L1 is methylation at positions 6352 and 6364 and HPV33L2 is mean methylation of CpG sites 5557, 5560 and 5566 (Brentnall *et al.*, 2015).

## 3.9. Diagnostic performance evaluation

Formerly developed DNA methylation based cervical cancer risk classifier score S5 (Brentnall *et al.*, 2015) and *EPB41L3* promoter methylation were evaluated for their capability to differentiate healthy cervix from various degrees of cervical lesions. Diagnostic performance of the two types of methylation assays were also compared using parameters of diagnostic tests. Sensitivity and specificity of the assays (S5 and *EPB41L3* methylation) were calculated for each value as a cut-point and were plotted against the cut points to manually select the best cutoff points. Cutoff points and corresponding positive and negative predictive values were also calculated. Area under the curve (AUC) of receiver operating characteristic (ROC) curve which is indicator of the overall performance of a diagnostic test was also calculated (Mandrekar, 2010).

## 3.10. Determining methylation cutoff values

Optimal cutoff values were determined as it is one of important aspects in diagnostic test development as it helps in minimizing misclassifications. Different approaches were used in deciding the optimal cutoff values, all aimed at maximizing sensitivity and specificity of the test. The point where both specificity and sensitivity are at their maximum was obtained from the plot of these indices against cutoff values by locating the intersection of the two graphs. Other ways of getting optimal cutoff values used were by maximizing sensitivity and specificity maintaining the differences between the two lower. Their performance was evaluated by receiver operating characteristic (ROC) curve analysis. Maximizing the sum or product of sensitivity and specificity and maximizing Youden's index were used in addition to minimizing the difference between

sensitivity and specificity. Cutoff values obtained in such a way were evaluated and compared by their corresponding sensitivity, specificity and AUC.

## 3.11. Statistical analysis

Different demographic, clinical and laboratory data were compared among cases and controls and among various stages of cervical pathology. Means of the quantitative variables were compared using Mann Whitney test and Kruskal Wallis test to test for statistical significance of variation of the variables among different groups. Correlation of the variables among themselves and with sevierity of the cancer was also calculated using Spearman correlation coefficient. Proportions of the categorical variables (HCU, residence and hrHPV infection status) were compared between cases and controls usin chi square test. Distributions of the categorical variables among the groups (cases and controls or cancer stages) were used to calculate odds ratios.

Differences between groups were compared using Mann Whitney and Dunn's multiple comparison tests and the Cuzick test for trend to assess any methylation trend with disease progression. Further differences of the methylation levels of invasive cervical cancer samples were assessed via pair-wise Wilcoxon test. Odds ratios for the risk factors were also calculated using binary logistic regression analysis.

The receiver operating characteristic (ROC) curves were constructed to assess the diagnostic potential (Mandrekar, 2010) of methylation scores in discriminating invasive cervical cancer from normal specimens in the exfoliated samples. The area under the curve (AUC) was used to evaluate the extent of prediction of methylation of the different conditions. Specificity and sensitivity were also calculated based on the ROC curve. Unconditional logistic regression was also used to measure the strength of the associations between methylation in the invasive cervical cancer group, stage of cancer, type of cancer, age, demographics and HPV status. For the purpose of quantitative statistical analysis severity of cancer or cancer stages were transformed into numerical values as described in Annex 4. Analysis was performed with statistical packages

within R v 3.5.3 (R Core Team, 2019). P-values less than 0.05 were considered statistically significant.

## 3.12.Ethical considerations

This study protocol was approved by the research ethics committee of college of natural and computational sciences Addis Ababa University, institutional review board of college of health sciences Addis Ababa University (protocol number 016/14/Bioch) and the national research ethics review committee (Annex 2). All the study participants had signed a written consent (Annex 1B). The study participants were assured that their participation in the study will in no way affect the services they require and the study protocol is in agreement with the standard diagnosis and treatment available at the facilities. The protocol also ensured the safety and privacy of the participants.

# Chapter 4

# 4. Results

## 4.1. Cervical cancer risk factors

A total of 135 Ethiopian women aged 20 to 75 years were enrolled in this study, of which 96 were cases with abnormal cervical cytology confirmed by histology and 39 were controls, women who had normal uterine cervix upon cytology screening. Alternatively categorized by stage of lesions, 39 were normal, 16 CIN1 and 80 were CIN3 or worse. The CIN1 and normal grouped together under category low risk were 55 while the CIN3 or worse categorized as high risk were 81(Table 4-1).

**Table 4-1. Summary of cervical pathology**

| Stage | Number | Level | Risk | type |
|---|---|---|---|---|
| Healthy | 39 | normal (39) | low (55) | Controls (39) |
| CIN1 | 16 | CIN1 (16) | | |
| CIN3 | 2 | CIN3+ (80) | High (80) | Cases (96) |
| IB | 2 | | | |
| IIA | 7 | | | |
| IIB | 19 | | | |
| IIIA | 7 | | | |
| IIIB | 21 | | | |
| IVA | 19 | | | |
| IVB | 3 | | | |

Rural and urban residents were nearly of equal proportion among the cases but number of urban residents was disproportionately higher in the control group. Greater percentage of women in the control group used hormonal contraceptives than women in the cases group. Prevalence of hrHPV is also higher in the cases than in the controls group (Table 4-2 and Fig. 4-2).

Mean and median age and parity of the cases was greater than that of the controls while age at first sexual intercourse was lower in the cases than in controls (Table 4-2). Mean number of children born to women in the cases group is 5.49(±3.01) (95% CI = 4.84 - 6.04), while 2.03(±2.04) (95% CI = 1.38 - 2.67) children born to women in control group (Table 4-2).

Women in the cases group have had first sexual experience at an average age of 16.58(±4.04) (95% CI = 15.76 - 17.37) and those in the control group had had at mean age of 20.18(±4.24) (95% CI = 18.85 - 21.51).

Most women in the control group had less than five children and only few had more than five while the proportion of women who had more than five children was higher in the cases group (Fig. 4-1A). More women in the cases group had started sexual intercourse at age of 15 years and younger compared with those in the control group (Fig. 4-1B).

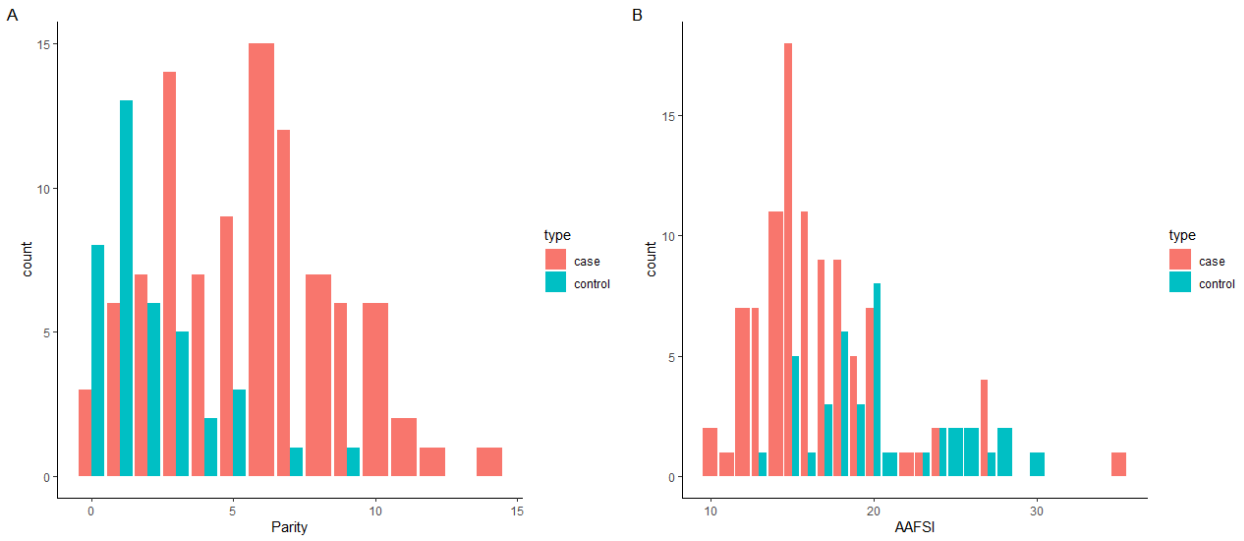


**Figure 4-1: Barrgraph of (A) parity and (B) age at first sexual intercourse by pathological group (cases and controls)**

Table 4-2. Summary of demographic variables

| | | **cases (n=96)** | **controls (n=39)** | **p value** |
|---|---|---|---|---|
| Residence | | | | |
| | Rural | 49(51%) | 6(15.4%) | |
| | Urban | 47(49%) | 33(84.6%) | <0.001 |
| Hormonal contraceptive use(HCU) | | | | |
| | No | 59(61.5%) | 21(53.8%) | |
| | Yes | 37(38.5%) | 18(46.2%) | 0.4146 |
| hrHPV infection status | | | | |
| | Negative | 19(19.8%) | 33(84.6%) | |
| | Positive | 77(80.2%) | 6(15.4%) | <0.001 |
| Age | | | | |
| | Median (IQR) | 50(13) | 38(17) | |
| | Mean (95% CI) | 48.5(46.6-50.4) | 38.41(35.0 - 41.8) | <0.001 |
| Parity | | | | |
| | Median (IQR) | 6 (4) | 1(2) | |
| | Mean (95% CI) | 5.49(4.89 - 6.09) | 2.03(1.38 - 2.67) | <0.001 |
| Age at first sexual intercourse (AAFSI) | | | | |
| | Median (IQR) | 16(4) | 20(6) | |
| | Mean (95% CI) | 16.58(15.77 -17.39) | 20.18(18.8 - 21.5) | <0.001 |

The cases group was composed of 16(16.7%)CIN1, 2(2.1%)CIN3 and 78(81.2%) invasive cervacal cancer of stages IB, IIA, IIB, IIIA, IIIB,IVA and IVB (Fig. 4-2A). Only few of women with normal cervix and lower grade lesion were residents of rural area while majority of women with advanced cancer were from rural (Fig. 4-2B).

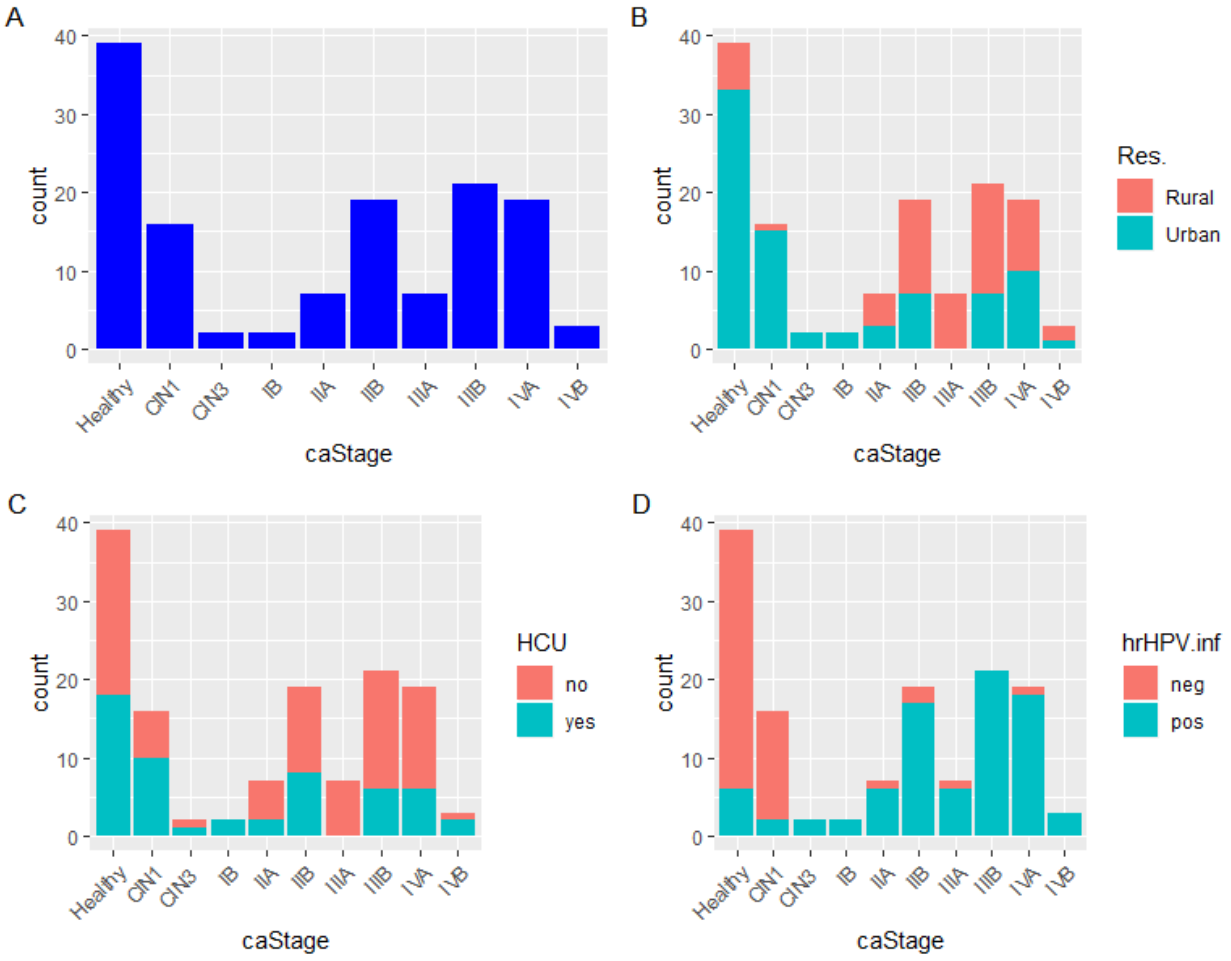


**Figure 4-2: Distribution of the study participants by; (A) cervical pathology status and cancer stage, (B) residence, (C) hormonal contraceptive use and (D) hrHPV infection status.**

### 4.1.1. Association of socio-demographic risk factors with cervical cancer

Comparing means of the quantitative factors by the categorical factors and between cases and controls, different patterns of variations were observed using Wilcoxon test. Parity and AAFSI differed with respect to hormonal contraceptive use, hrHPV infection status, residence and cervical pathology status (Fig. 4-3). The differences in age by hrHPV infection status and residence were statistically not significant ($p = 0.07$ and 0.24 respectively), but it is significant by cervical pathology ($p<0.0001$) and hormonal contraceptive use ($p= 0.0001$). Mean age of the cases group was higher than the controls group and women who had reported no use of hormonal contraceptive were older than those who used. Parity significantly differed by cervical pathology, hrHPV infection status and residence but not significantly differed by HCU. Women who were in the cases group, hrHPV positive and rural residents tend to have more children than those in the control group, hrHPV negative and urban residents. Age at first sexual intercourse also differed significantly by cervical pathology, hrHPV infection and residence but not significant by HCU. Women in the cases group, who were hrHPV positive and rural residents had had sexual intercourse at younger age than those in control group, were hrHPV negative and urban residents.

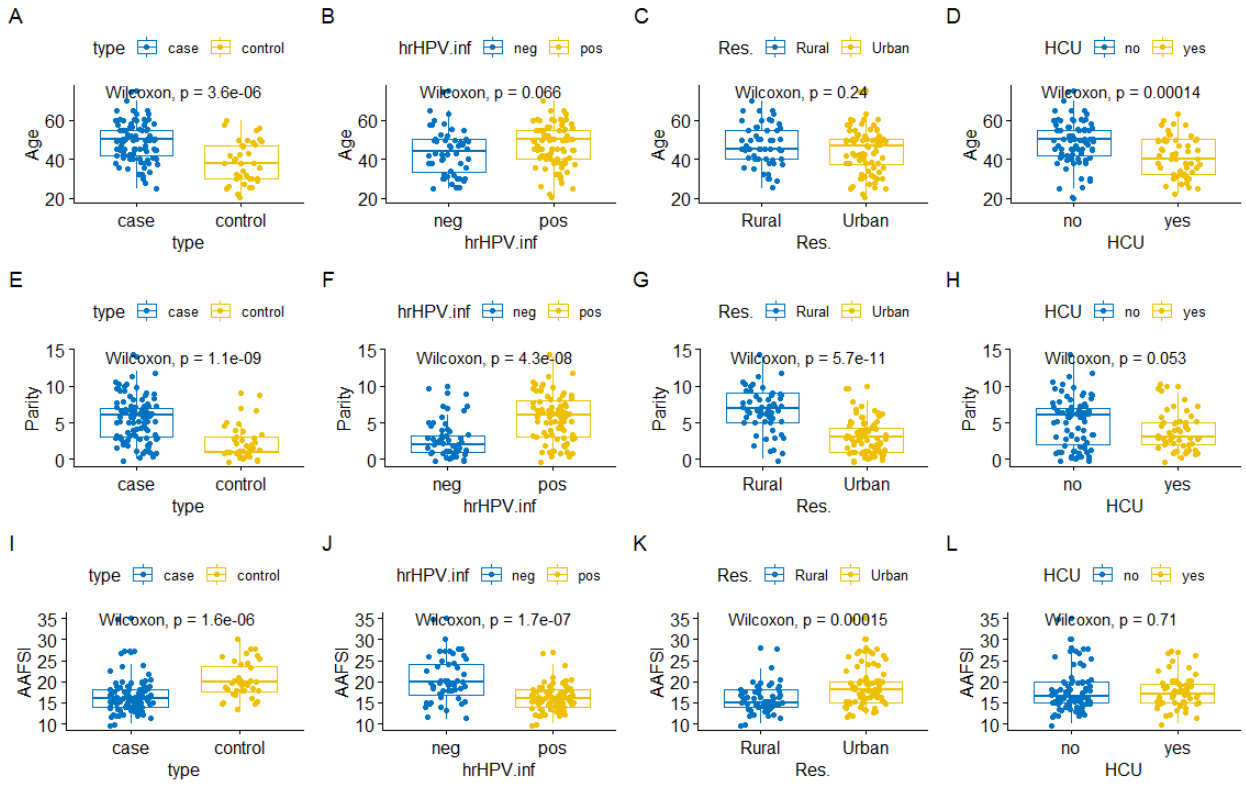


**Figure 4-3: Box plot of (A-D) age, (E-H) parity and (I-L) age at first sexual intercourse (AAFSI) by pathology status(as cases and controls), hormonal contraceptive use, hrHPV infection status and residence. mean age of women in the cases group, hrHPV positive, rural residents and who did not use hormonal contraceptives was higher than the controls, hrHPV negatives, urban residents and who used oral contraceptives. Mean parity tends to be higher among women who were in the cases group, hrHPV positive, rural residents and who had not used hormonal contraceptives than those in control group, hrHPV negative, urban residents who used hormonal. Age at first sexual intercourse was lower among women in the cases group, hrHPV positive and rural residents than women who were in the control group, hrHPV negative and urban residents.**

Mean ages of women in the cases group and women who did not use hormonal contraceptives were higher than those in control group and who had used hormonal contraceptive (Fig. 4-3A and D). Variation of age by hrHPV infection status and residence were not significant, p = 0.066 and 0.24 respectively (Fig. 4-3B and C).

Two samples Mann-Whitney test showed parity of women in the cases group significantly higher ($p < 0.0001$) than those in the control group. Similarly parity is high among high risk women compared to low risk group. Mean number of children born to women in the case group was greater than ($p<0.001$) those in the control group (Fig. 4-3E). Difference in parity by hrHPV infection status and residence was significant ($p<0.001$), while use of hormonal contraceptives didn't confer significant difference ($p = 0.053$) in parity (Fig. 4-3F-H).

Age at first sexual intercourse (AAFSI) was significantly lower in cases and low risk group than in controls and high risk group by two samples Mann-Whitney test ($p = 0.000$). AAFSI also differed with hrHPV infection status and residence. Women who are hrHPV positive and rural residents had started sexual intercourse at younger age compared with hrHPV negative and urban residents. AAFSI didn't differ significantly ($p = 0.71$) by use of hormonal contraceptives (Fig. 4-3L).

Chi squared test performed to assess association of the demographic factors with cervical cancers in the sample population showed no significant variation in hormonal contraceptive use between cases and controls ($\chi^2 = 0.39$, $p = 0.50$) but residence and hrHPV infection significantly vary among cases and controls with $\chi^2$ values of 13.17 ($p = 0.0003$) and 46.51 ($p < 0.0001$) respectively.

### 4.1.2. Correlation of the socio-demographic factors

In addition to variation by the two classes (cases and controls), parity and age at first sexual intercourse (AAFSI) had showed correlation with cancer stages and with cancer score (an ordinal scale of the cancer clinical stages). Spearman rank correlation of the risk factors showed all the quantitative parameters; parity, age at first intercourse, age and cancer score, had significant correlation with each other (Fig. 4-4 and Table 4-3). Parity had a positive correlation of 0.6 ($p<0.001$) and 0.5 ($p = 0.001$) with cancer stage and age respectively while AAFSI had a negative correlation of -0.5 ($p = 0.001$) with both cancer stage and parity.

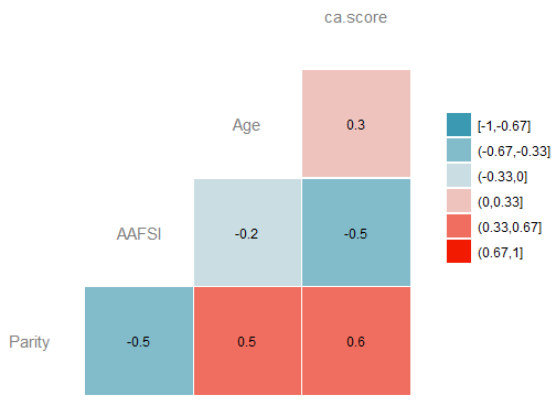


**Figure 4-4: Correlation heat map of parity, age at first sexual intercourse, age and cancer score**

**Table 4-3. Spearman correlation coefficients with respective p values**

| | Parity | AAFSI | Age |
|---|---|---|---|
| AAFSI* | -0.53 (p<0.001) | | |
| Age | 0.49 (p<0.001) | -0.23 (p = 0.007) | |
| ca.score. | 0.56 (p<0.001) | -0.51 (p<0.001) | 0.27 ( p = 0.002) |

* AAFSI = age at first sexual intercourse

Among the quantitative variables, parity and AAFSI showed stronger correlation (r = 0.6 and r = 0.5 respectively) with cancer score in addition to correlation with each other and age (r = 0.5, p<0.001). Age had weaker correlation with cancer score (r = 0.3, p = 0.002) and AAFSI (r = 0.2, p = 0.007) while it had stronger correlation (r = 0.5, p<0.001) with parity (Fig. 4-4 and Table 4-3).

### 4.1.3. Variability of parity and AAFSI among different stages of cervical cancer

Kruskal-Wallis test for comparison of parity and age at first intercourse (AAFSI) among the levels of cancer stage showed significant variation (p value <0.0001) (Fig. 4-5). However, pair

wise comparison of means (Wilcoxon test) showed that the variability was significant only between women with normal cervix and advanced cervical cancer stages and between CIN1 and advanced cancer stages. Parity significantly differed between women with normal cervix and stage IIB- IVB cervical cancer patients and between CIN1 and stage IIB- IVA (Annex 5). Variation in AAFSI was significant between normal and stages IIA –IVA cervical cancer and between CIN1 and stages IIA –IVA cervical cancer (Annex 6). Differences in parity between healthy and CIN1, CIN3, stage IB and stage IIA cancer, between CIN1 and CIN3, stage IB and IIA cancer and between CIN3 and all stages of invasive cancer were not statistically significant. The differences in AAFSI between healthy and CIN1, CIN3 and stage IB cancer, between CIN1 and CIN3 or stage IB cancer and between CIN3 and all the stages of invasive cancer were also not significant. The overall variations in mean parity and AAFSI among the stages of cervical cancer due to the greater variation exhibited between the lower risk group and the hgher risk group. These variables are not significantly different among the stages of the high risk group. Fig. 4-5 shows that only the healthy and CIN1 groups have distinctly lower AAFSI and higher parity than the other stages, while mean AAFSI and parity of the other stages differe only slightly.

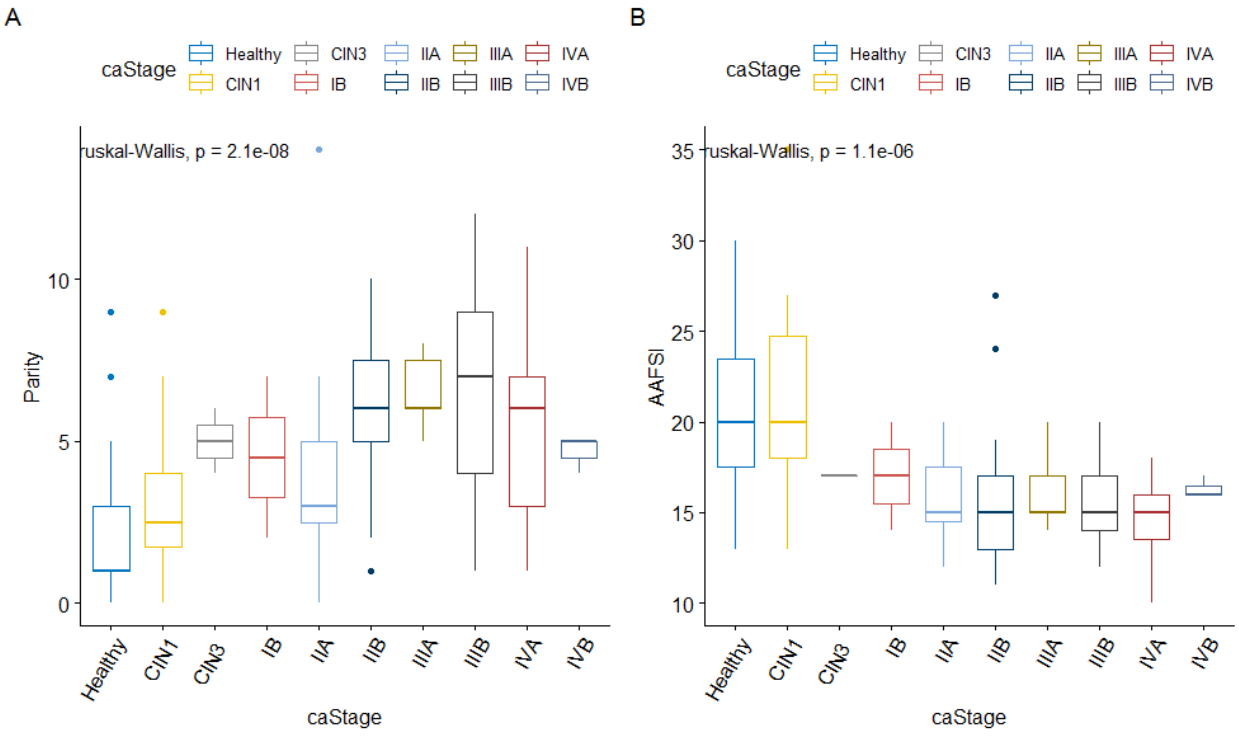


**Figure 4-5: Box plot of (A) parity and (B) age at first sexual intercourse (AAFSI) by cancer stage. Mean values are indicated by heavy line in or on the edge of the boxes. Lines extending from the boxes represent the range of values while the dots indicate outliers.**

### 4.1.4. Role of the risk factors in predicting risk levels

Regression analysis was performed to show if cervical cancer risk levels and infection by hrHPV could be predicted from the risk factors. Variables that were either not significantly correlated with the cervical pathological condition (HCU) or were affected by sampling (residence) were excluded. The binary logistic regression model included the variables which were significantly correlated with cancer score (parity, AAFSI and age). Binary logistic regression model showed parity and AAFSI can predict both risk level and hrHPV infection status significantly. Having one more child or full term pregnancy increases the odds of a woman to be in a high risk group by 36% and to be hrHPV positive by 38% (Table 4-4). Starting sexual intercourse one year earlier also increases the odds of women to be in high risk group by 31% and odds of being hrHPV positive by 18%. Both Wald's test and likelyhoood ratio p-values were less than 0.005 in all cases indicating that Parity and AAFSI are significant predictors of risk level and infection by

hrHPV. Age was non significant predictor of both risk level and hrHPV infection status with odds ratio of approximately one and non significant p values.

**Table 4-4. Binary logistic regression model of AAFSI, parity and age against risk level and hrHPV infection with odds ratios**

| Predicting Risk level high versus low | | | | |
|---|---|---|---|---|
| | crude OR (95%CI) | adj. OR (95%CI) | P (Wald's test) | P (LR test) |
| Parity cont. var. | 0.58 (0.48-0.7) | 0.64 (0.51-0.81) | < 0.001 | < 0.001 |
| AAFSI cont. var. | 1.47 (1.27-1.7) | 1.31 (1.12-1.53) | < 0.001 | < 0.001 |
| Age cont. var. | 0.95 (0.92-0.99) | 1.02 (0.97-1.07) | 0.401 | 0.401 |
| Predicting hrHPV infection positive versus negative | | | | |
| | Crude OR (95%CI) | adj. OR (95%CI) | P (Wald's test) | P (LR test) |
| Parity cont. var. | 1.48 (1.27-1.73) | 1.38 (1.13-1.69) | 0.001 | < 0.001 |
| AAFSI cont var. | 0.75 (0.67-0.84) | 0.82 (0.72-0.93) | 0.002 | < 0.001 |
| Age cont. var. | 1.03 (0.99-1.06) | 0.97 (0.93-1.01) | 0.175 | 0.172 |

## 4.2. Prevalence of high risk HPV

The non specific HPV test with MY09/11 PCR showed 80 (83.3%) of the cases and 7 (17.9%) of the controls were HPV positive while 17 (16.7%) of the cases and 32(82.1%) of the controls were HPV negative. In this test detection of a 450bp band in the PCR amplicon was dubbed HPV positive with a 109bp segment of human beta globin gene as internal positive control for the success of the PCR reaction and amplifiability of the DNA samples (Fig. 4-6). The MY09/11 HPV PCR test detects only presence of a 450bp segment of L1 gene of any mucosal HPV regardless of its genotype and carcinogenicity.

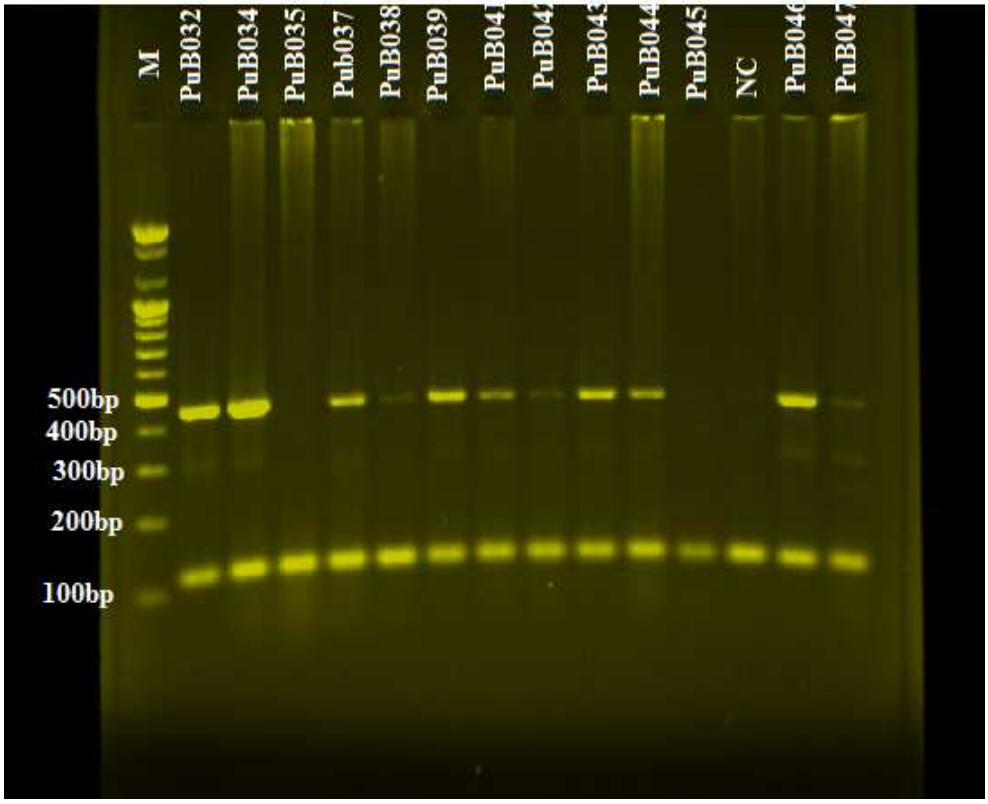


**Figure 4-6: Gel photo of MY09/11 PCR product; 109 bp section of human beta globin gene as positive control and 450bp HPV DNA amplicon run with 100bp DNA ladder (M) and HPV negative control (NC).**

HPV genotyping test performed on all samples using the Papilloplex hrHPV genotyping assay detected and genotyped eight hrHPV types in the sampled population. In the cases group 77 (80.2%) and only 6 (15.4%) in the controls group were hrHPV positive (Table 4-5).

**Table 4-5. Prevalence of high risk human papillomavirus**

| | | Cases (N=96) | | Controls (N=39) | |
|---|---|---|---|---|---|
| **HPV genotype** | **Single/multiple infection** | **Prevalence N(%)** | **co-infection with HPV type** | **Prevalence N(%)** | **co-infection with HPV type** |
| **HPV16** | | **67(69.8)** | | **2(5.1)** | |
| | single | 54(56.3) | | 2(5.1) | |
| | multiple | 13(13.5) | 2*16,18; 16,31; 16,33,45; 6*16,45; 16,52; 2*16,59 | 0 | |
| **HPV45** | | **14(14.6)** | | **1(2.6)** | |
| | single | 5(5.2) | | 0 | |
| | multiple | 9(9.4) | 16,33,45; 6*16,45; 31,45; 45,18 | 1(2.6) | 45,59 |
| **HPV18** | | **4(4.2)** | | **1(2.6)** | |
| | single | 1(1) | | 1(2.6) | |
| | multiple | 3(3.1) | 2*16,18; 45,18 | 0 | |
| **HPV31** | | **3(3.1)** | | **0** | |
| | single | 1(1) | | 0 | |
| | multiple | 2(2.1) | 16,31; 31,45 | 0 | |
| **HPV33** | | **2(2.1)** | | **0** | |
| | single | 1(1) | | 0 | |
| | multiple | 1(1) | 16,33,45 | 0 | |
| **HPV59** | | **2(2.1)** | | **2(5.1)** | |
| | single | 0 | | 1(2.6) | |
| | multiple | 2(2.1) | 16,59; 45,59 | 1(2.6) | |
| **HPV52** | | **1(1)** | | **0** | |
| | single | 0 | | 0 | |
| | multiple | 1(1) | 16,52 | 0 | |
| **HPV56** | | **0** | | **1(2.6)** | |
| | single | 0 | | 1(2.6) | |
| | multiple | 0 | | 0 | |
| **Total hrHPV positive** | | **77(80.2)** | | **6(15.4)** | |
| | single | 62(80.5) | | 5(83.3) | |
| | multiple | 15(19.5) | 2*16,18; 16,31; 16,33,45; 6*16,45; 16,52; 2*16,59; 45,18; 31,45; 45,59 | 1(16.6) | |
| **hrHPV Negative** | | **19(19.8)** | | **33(84.6)** | |

The figure followed by * indicated the number of that specific multiple infection

From the hrHPV types HPV16 was the most abundant (69.8%) followed by HPV45, HPV18 and HPV 31 each with prevalence of 14.4%, 4.2% and 3.1% respectively in the cases. HPV59, HPV33 and HPV52 were also prevalent with 2.1%, 1% and 1% respectively. In the controls group HPV 16 and HPV59 had the highest prevalence of 5.1% each. HPV18, HPV45 and HPV56 had prevalence of 2.6% each and HPV31 and HPV33 were not detected in the controls.

Infection by single HPV type contributes to 80.5% (62 of 77 hrHPV positives) of the total hrHPV infections in cases while 18.18% (n=14) were double infections and 1.3% (n=1) was triple infection. Only one of the six hrHPV positive controls (16.7%) was co-infected by two HPV types and the rest five (83.3%) were single infections. HPV16 was detected in the highest number (13 multiple infections) of multiple infections including the triple infection, followed by HPV 45 which was detected in 9 multiple infections. Seven of the 15 multiple infections including the triple infection are HPV16/45 co-infections (Table 4-5).

## 4.3. HPV DNA methylation in healthy and abnormal cervical cells

Total of 83 hrHPV positive (77 with cervical pathology and 6 with normal) cervical specimens were analyzed for methylation of CpG sites in HPV L1 and L2 regions. HPV16 was detected in 67 of the cases (54 single and 13 in multiple infections) and 2 of the controls (both single infections). HPV18, HPV31 and HPV 33 were detected in four, three and two cases respectively and only one HPV18 in controls but no HPVs 31 and 33. Hence methylation level of the CpG sites in HPV16 L1 sites could successfully be measured in 50 cases and two controls and HPV16 L2 sites in 48 cases and two controls, HPV18 L2 in three cases and one control and HPV31 L1 in two and HPV33 L2 in one cases.

Mean and median percent methylation of CpG sites in HPV16-L1, HPV16-L2 and HPV18-L2 regions in the cases was higher than in the controls (Table 4-6). HPV16 L1 region contained CpG sites with the highest level of methylation in cases (76.4%) compared to the other HPV genomic sites. CpG sites in the HPV16 L1, HPV16 L2 and HPV18 L2 regions were generally more methtylated in cervical cancer cases (with mean methylation of 75.95%, 43.62% and

17.34% respectively) than healthy cervical tissue (mean methylation 15.89%, 3.44% and 1.46% respectively).

**Table 4-6. Mean and median methylation levels in the HPV L1 and L2 genomic regions**

| CpG location | Cases | | | Controls | | |
|---|---|---|---|---|---|---|
| | | percent methylation | | | percent methylation | |
| | n | median | mean | n | median | mean |
| HPV16 L1 | 50 | 84.08 | 75.95 | 2 | 15.89 | 15.89 |
| HPV16 L2 | 48 | 36.72 | 43.62 | 2 | 3.44 | 3.44 |
| HPV18 L2 | 3 | 17.46 | 17.34 | 1 | 1.46 | 1.46 |
| HPV31 L1 | 2 | 73.69 | 73.69 | - | - | - |
| HPV33 L2 | 1 | 63.99 | 63.99 | - | - | - |

The distribution of the HPV DNA methylation values in cases and controls was distinct with only some overlaps (Fig. 4-7). HPV16 L1 and L2 methylation values in the normal cervical tissue were confined to the lower edge of the graphs. Methylation values in the cancerous tissue were distributed over wider range (Fig. 4-7) with median HPV16 L1 methylation being 84.08% and that of HPV16 L2 36.72% (Table 4-6).

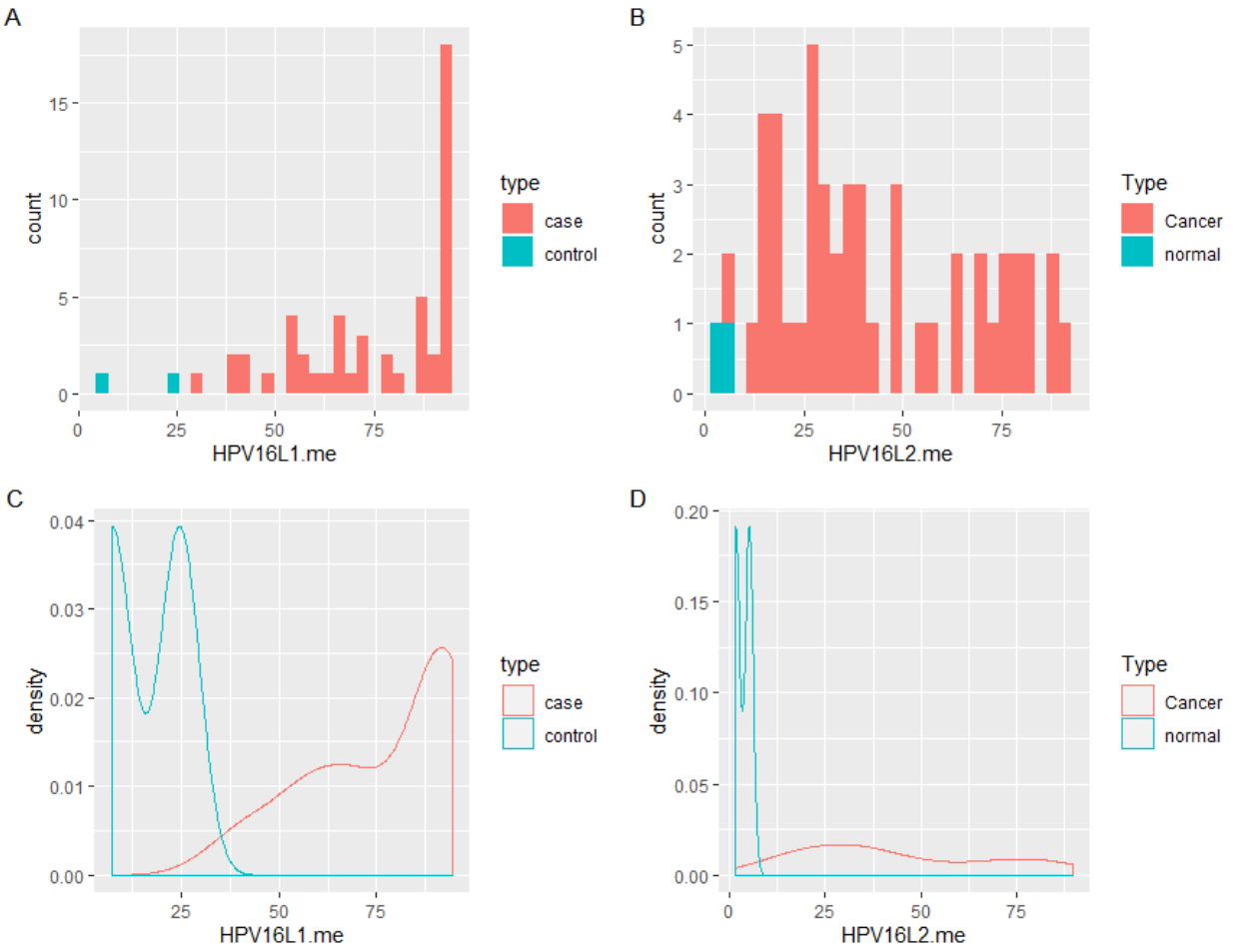


**Figure 4-7: Distribution of HPV16L1 and L2 methylation values with respect to healthy cervical tissue and tissue with lesion.**

Methylation levels of the CpG sites in HPV16 L1 and L2 regions vary significantly between cases and controls (p= 0.0016 and 0.0018 respectively) (Fig. 4-8) by Wilcoxon test.

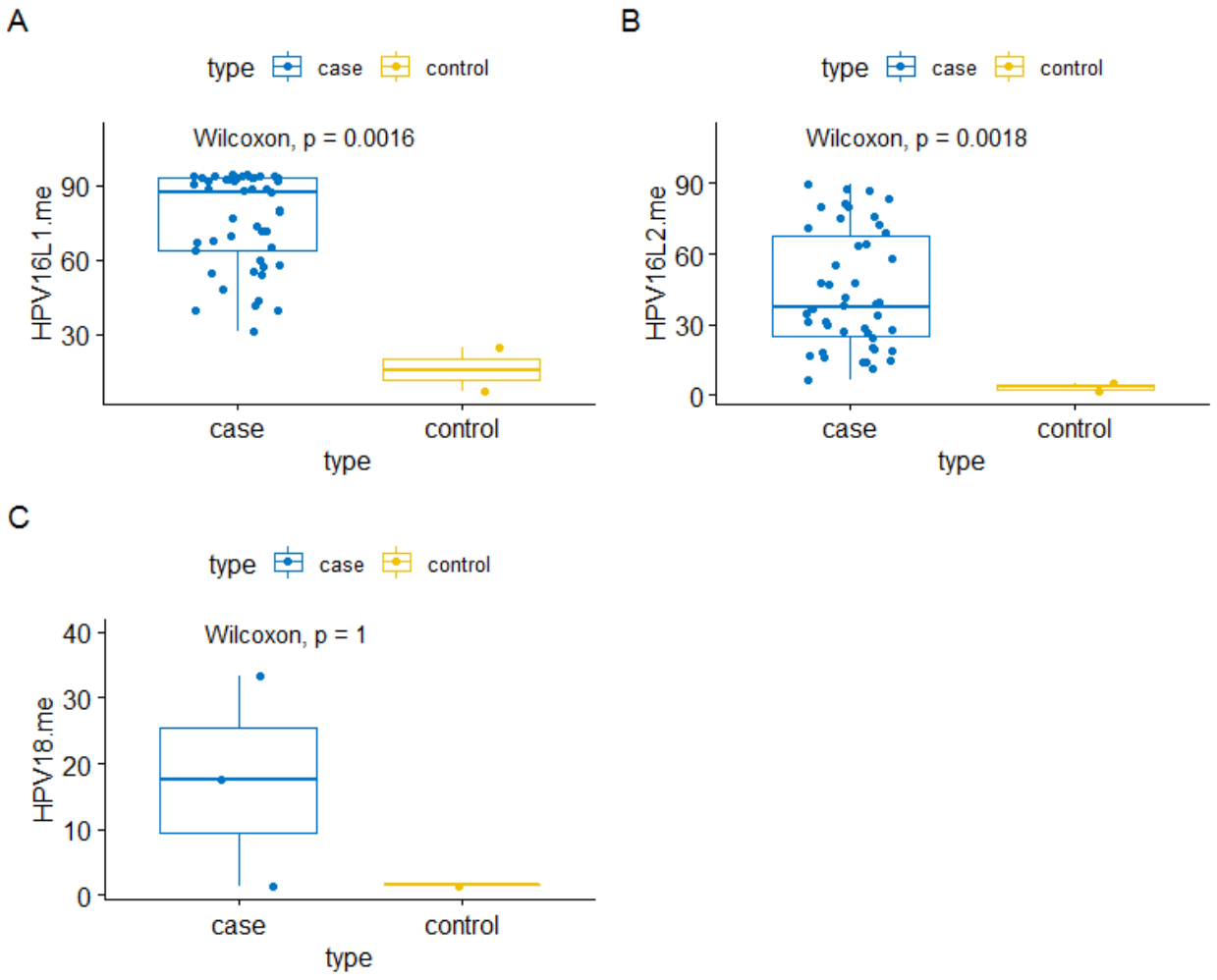


**Figure 4-8: Comparison of HPV DNA methylation among healthy cervical tissue and cervical cancers. HPV16 L1 and L2 methylation had distinct distribution of values for cases and controls**

### 4.3.1. HPV DNA methylation by cancer stage

Though the methylation levels show clear variation between the cases and controls groups, the variation between the different cancer stages within the cases was not significant. Average methylation of CpG sites in both HPV-L1 and L2 regions showed a steady increase from healthy to stage IB and from stage IB to stage IIA and then to IIB, but with no specific trend of change across the other cancer stages (Fig. 4-9A and B). Pair-wise comparison of mean methylation of CpG sites in HPV16 L1 and L2 regions among cancer stages (Annex 7 and 8) showed

statisticaly significant differences only between normal cervix and invasive cancers. Methylation values are distinct between the low risk and high risk groups too (Fig. 4-9 C and D).

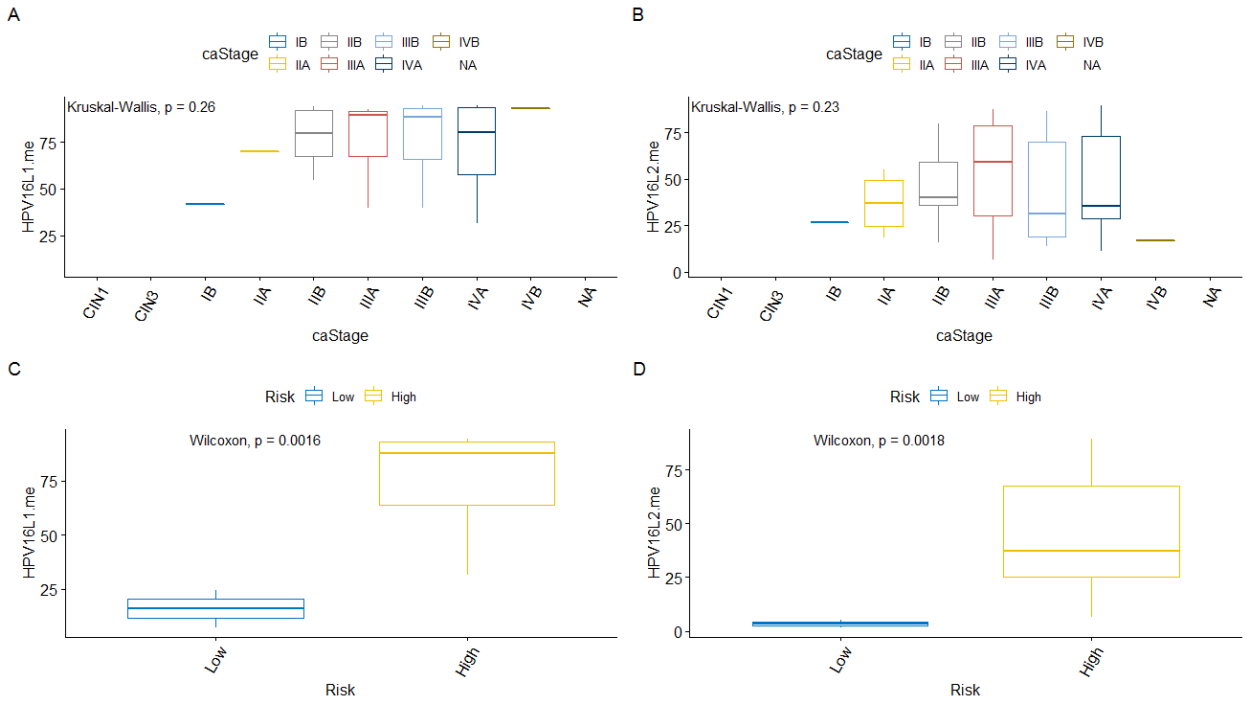


**Figure 4-9: Methylation of HPV DNA by cervical cancer stages. Both HPV16 L1 and L2 methylation levels variation was not statistically significant among different stages of cervical cancer (A&B), but significantly different between healthy cervical tissue and CIN3 and worse (C&D).**

### 4.3.2. Role of HPV DNA methylation in predicting cancer stages

HPV16 L1 showed significant (p = 0.0008) regression with cancer score with regression coefficient of 0.0222 (95% CI = 0.0097 to 0.0346). Coefficient of regression of HPV16 L2 methylation with cancer score was not statistically significant (p = 0.0903) with the value of 0.0105 (95% CI = -0.0017 to 0.0227) (Fig. 4-10). Thus methylation in the L1 region is stronger predictor of the cancer stages.

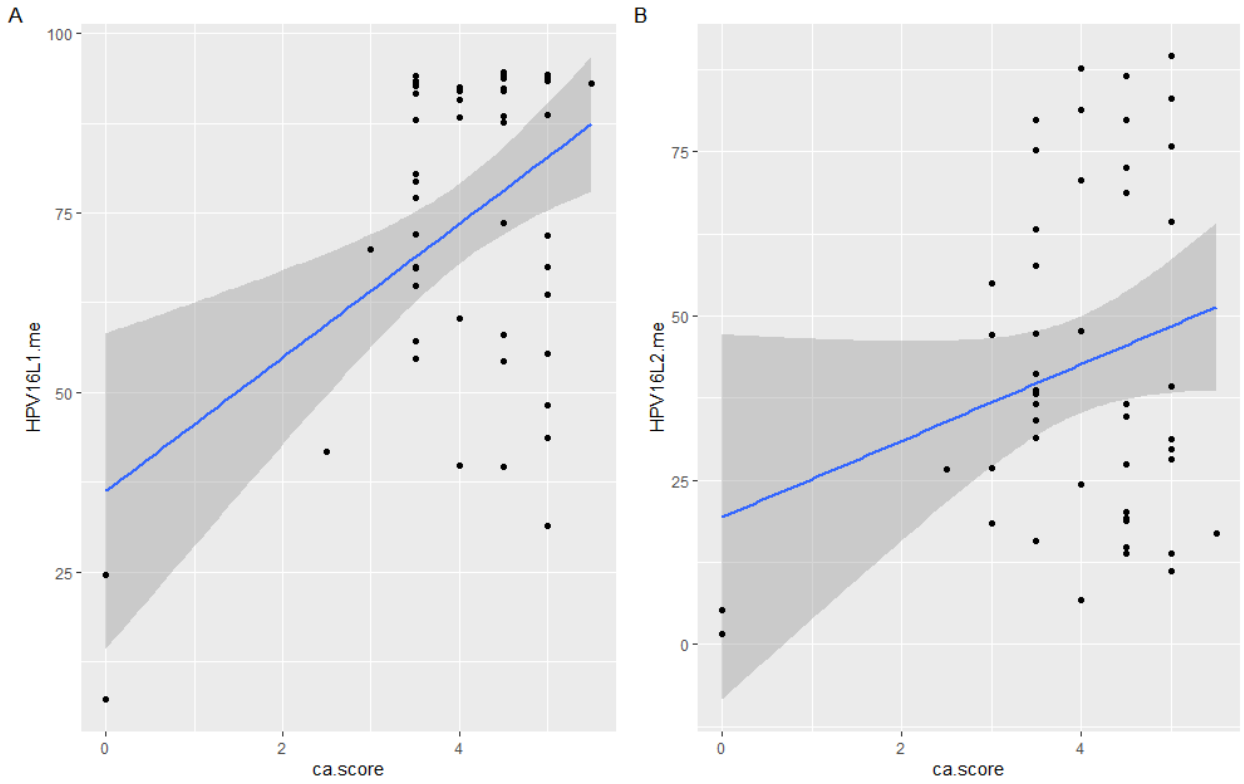


**Figure 4-10: Regression line with 95% confidence interval region. Regression of HPV16 L1 and L2 methylation values with cancer scores shows (A) HPV16L1 methylation has steeper regression slope than (B) HPV16 L2 methylation indicating that the L1 methylation is stronger predictor of cancer stage (represented here by cancer score)**

## 4.4. Distribution of *EPB41L3* promoter methylation among healthy and abnormal cervical tissue

Methylation of *EPB41L3* promoter region was successfully measured in all the 135 participants. Methylation level was compared by cases and controls where controls was composed of 39 women with normal cervix and cases group composed of 96 women diagnosed with CIN1, CIN3, and cervical cancer of stages IB, IIA, IIB, IIIA, IIIB, IVA and IVB. Comparison was also made between the participants categorized by risk as women with normal cytology and CIN1 designated as low risk and women with CIN3 or worse (CIN3+) designated as high risk. To check for relationship between *EPB41L3* promoter methylation and severity of cervical lesions,

the methylation level was compared between levels of lesion and normal cervical tissue as normal, CIN1 and CIN3+.

Higher level of average *EPB41L3* promoter region methylation was observed in cervical tissue with lesions or cancer (cases) (35.53%, 95% CI =30.15-40.90%) compared with healthy cervical tissue (controls) (2.95%, 95% CI= 1.47- 4.44%). Women in the high risk group also had higher *EPB41L3* methylation than those in the low risk (Table 4-7). Except a single individual with methylation level of 30.6, *EPB41L3* promoter methylation of all women with healthy cervix was within arrange of zero to 5.11 (Fig. 4-11). Despite the fact that *EPB41L3* promoter methylation confined to the lower level, that of the cases was distributed throughout from zero to 82.97.

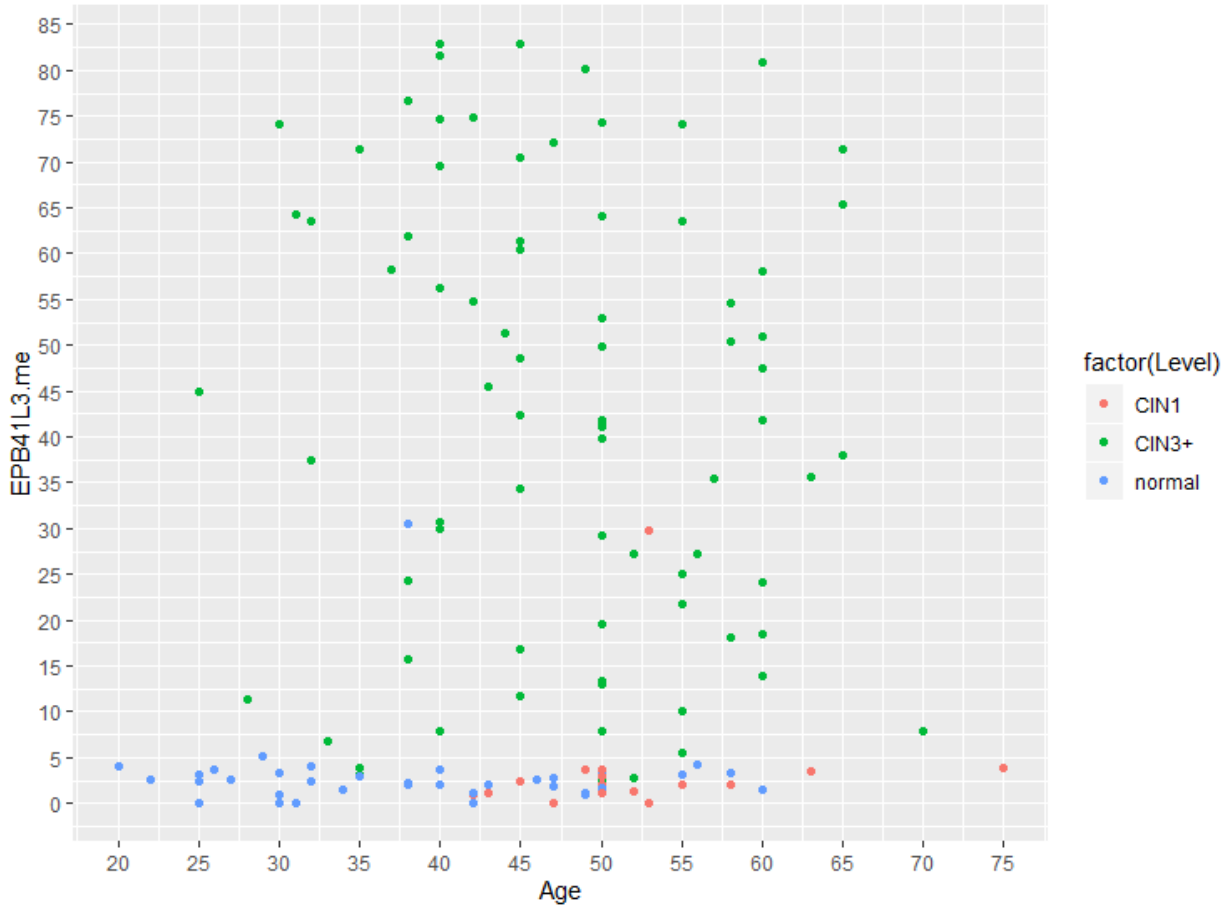


**Figure 4-11: *EPB41L3* methylation by age. Methylation level plotted against age of individuals categorized by tissue type as case (normal) and control (abnormal with lesion)**

Scatter plot of *EPB41L3* methylation by age (Fig.4-11) shows methylation was not affected by age but cervical pathology since older women with healthy cervix has lower methylation and younger women with cervical cancer had higher level of methylation.

**Table 4-7. Summary of *EPB41L3* promoter methylation and age by cases and controls**

| | | **cases (n=96)** | **controls(n=39)** |
|---|---|---|---|
| Age | Minimum | 25 | 20 |
| | Median | 50 | 38 |
| | Mean (**95% CI**) | 48.67(46.7-50.6) | 38.41(35.0 - 41.8) |
| | Maximum | 75 | 60 |
| *EPB41L3* methylation | Minimum | 0 | 0 |
| | Median | 35.37 | 2.48 |
| | Mean (**95% CI**) | 35.53 (30.15-40.90) | 2.95 (1.47- 4.44) |
| | Maximum | 82.97 | 30.58 |

*EPB41L3* promoter better classifies healthy cervical tissue and CIN1 together and CIN3 and invasive cervical cancers together than the cases and controls grouping used in this study (Fig. 4-12). In the case versus control comparison, where CIN1 was also classified as cases, considerable number of cases had lower *EPB41L3* promoter methylation. But in the four level classification (normal, CIN1, CIN3 and Cancer), normal and CIN1 had lower methylation level while CIN3 and cancer had larger methylation levels (Fig. 4-12B and D). Hence *EPB41L3* promoter methylation categorizes the study participants as low ris and high risk than as normal, CIN1, CIN3 and invasive cancer.

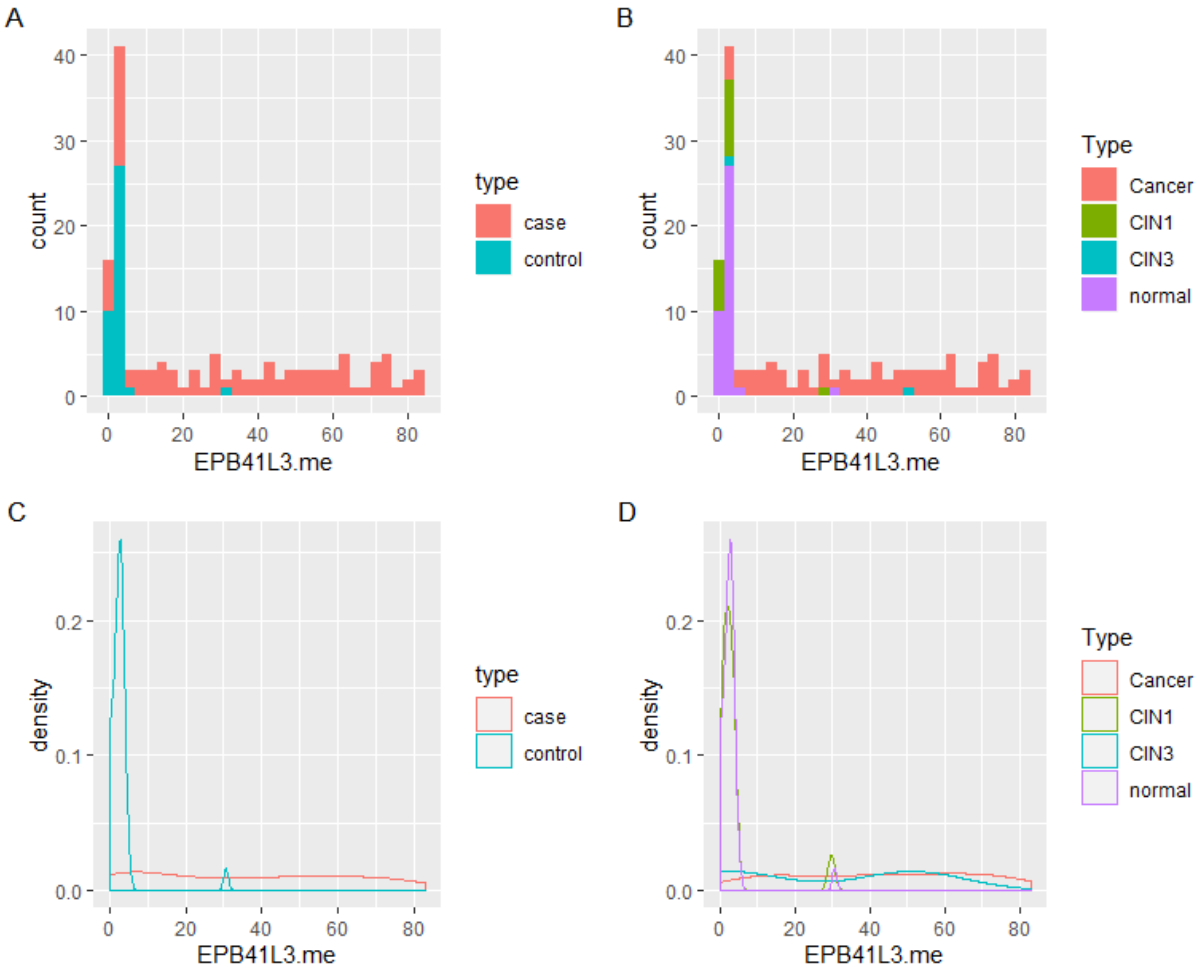


**Figure 4-12: Distribution of *EPB41L3* promoter methylation values by cervical tissue type (normal and abnormal). (A) histogram of *EPB41L3* promoter methylation by cases and control, (B) histogram of *EPB41L3* promoter methylation by lesion type as normal, CIN1, CIN3 and cancer, (C) densityplot of *EPB41L3* promoter methylation by cases and control, (D) densityplot of *EPB41L3* promoter methylation by lesion type as normal, CIN1, CIN3 and cancer. Normal cervical tissue and CIN1 generally showed lower level of methylation while CIN3 and invasive cervical cancer tend to have higher level over extended range.**

Variation of *EPB41L3* promoter methylation with respect to cases and controls as well as different stages of pathology (normal, CIN1, CIN3 and cancer), was significant by Kruskal Wallis test (Fig. 4-13). Distribution of methylation values among normal cervical tissue and CIN1was very similar. Pair wise comparison of means however showed variation was significant only between normal and cancerous tissues and between CIN1 and cancerous tissues (Fig.4-13B).

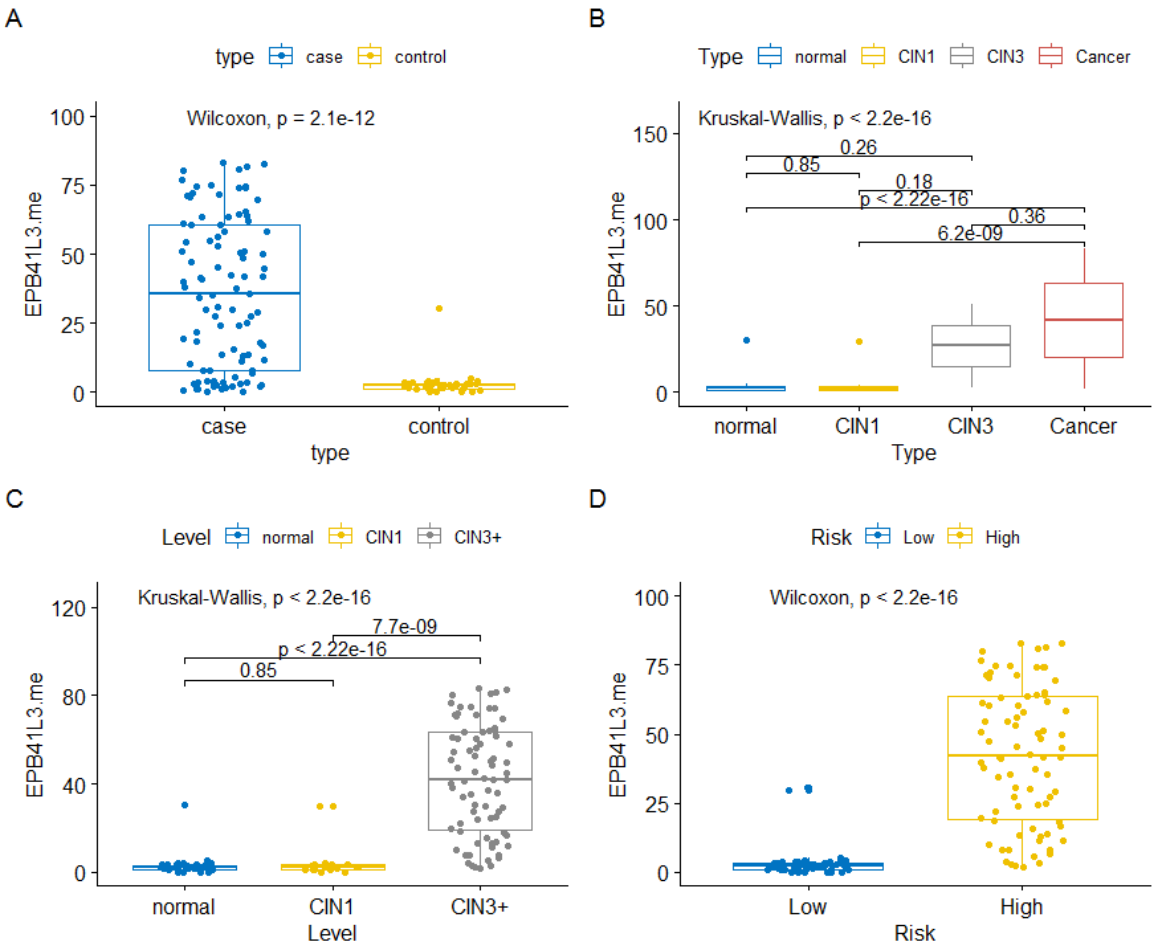


**Figure 4-13: Comparison of *EPB41L3* promoter methylation among different pathological states. Methylation values pair-wise comparison (A) between healthy cervical tissue and tissue with any sort of dysplasia, (B) between healthy, CINs and invasive cancer, (C) lesions by risk level as healthy, CIN1 and CIN3 or worse and (D) by risk level considering healthy and CIN1 as low risk and CIN3 and higher as high risk.**

Normal cervical tissue and CIN1 had similar mean level of *EPB41L3* promoter methylation (Fig. 4-13C) while the variation between CIN1 and CIN3 being statistically not significant (p = 0.18) (Fig. 4-13B). CIN3 also had methylation level that differed in statistically non significant way (p = 0.36) from invasive cancer (Fig. 4-13B). Risk categorization which grouped normal and CIN1 as low and CIN3+ as high risk showed significant (p<0.001) variation in methylation level (Fig. 4-13D). This shows *EPB41L3* promoter methylation is plays great role in progression from healthy and low grade lesion to high grade lesion and invasive cancer.

### 4.4.1. Average *EPB41L3* methylation by cancer stage

There is a general trend that *EPB41L3* promoter methylation increases with severity of cervical pathology. Healthy cervix and CIN1 having the lowest level of methylation with gradual increase with increasing severity of the lesion, but drops at the worst pathological stages (Fig. 4-14).

Pair-wise Wilcoxon test (Annex 9) revealed that variation in methylation is significant between normal cervical tissues and all stages (IB – IVB) of invasive cervical cancer. It also differentiates between CIN1 and all stages of invasive cervical cancers but stage IB. Differences in methylation between healthy and CIN1, CIN1 and CIN3, CIN3 and invasive cervical cancers were not significant.

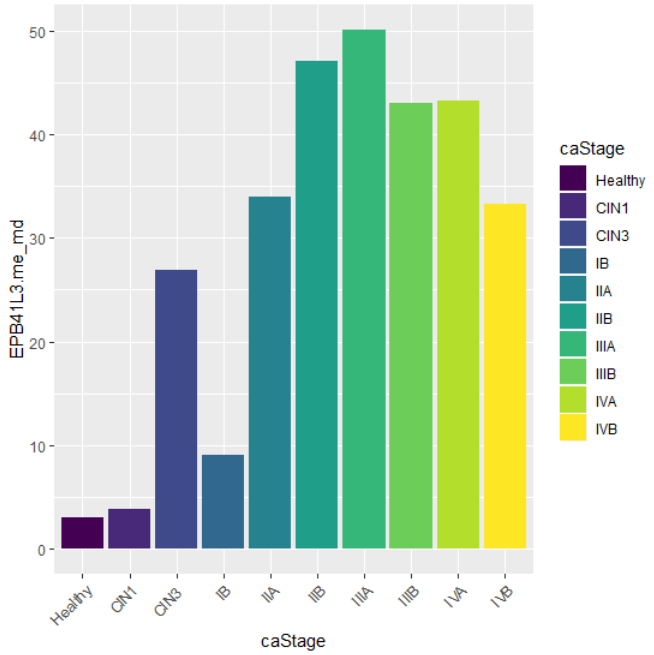


**Figure 4-14: *EPB41L3* methylation by cervical cancer stages. Mean level of methylation by level of cervical cancer with healthy control and precancerous lesions**

*EPB41L3* promoter methylation and cancer stages were strongly correlated (r =0.68, 95% CI = 0.58 – 0.76) and their correlation was statistically significant ($p < 0.0001$). Age and *EPB41L3* promoter methylation showed slight correlation (r = 0.11, 95% CI = -0.06 -0.27) which is

statistically not significant (p = 0.21). Age also correlates with cancer stage (r = 0.28, 95% CI = 0.11 – 0.42) with p value of 0.0011 (Fig. 4-15, Table 4-8).

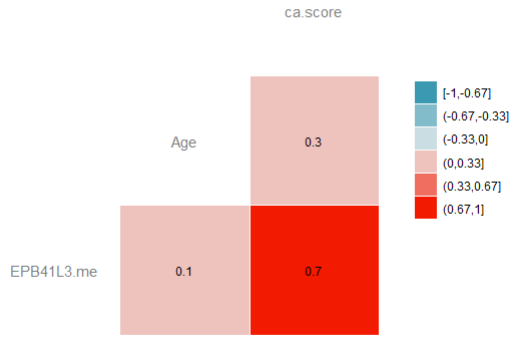


**Figure 4-15: Correlation of *EPB41L3* promoter methylation, age and cancer stage.**

**Table 4-8. *EPB41L3* promoter methylation, age and cancer score correlation with statistical significance**

| | *EPB41L3*.me | Age |
|---|---|---|
| Age | 0.11 (95% CI = -0.06 – 0.27) (p = 0.21) | 0.28 95% CI = 0.11 – 0.42) (p = 0.001) |
| ca.score | 0.68 (95% CI = 0.58 – 0.76) (p = 0.000) | |

### 4.4.2. *EPB41L3* promoter methylation models to predict cervical pathologies

Simple linear regression models showed that *EPB41L3* promoter methylation value to be good predictor of severity of cervical lesion with regression coefficient 0.053 (95% CI = 0.043-0.062) which was significant with p value of less than 0.0001. This analysis also showed that *EPB41L3* methylation predicted cancer stage than it did age of the women or the vice versa. The methylation values had non-significant (p = 0.16) regression coefficient (0.30, 95%CI= -0.12 -

0.73) to the age of the women. Age as predictor of cancer stage had regression coefficient of 0.05 (0.02 – 0.08) and p value 0.002. The slope of the regression lines (Fig. 4-16) also showed that *EPB41L3* promoter methylation to be stronger predictor than age of the women

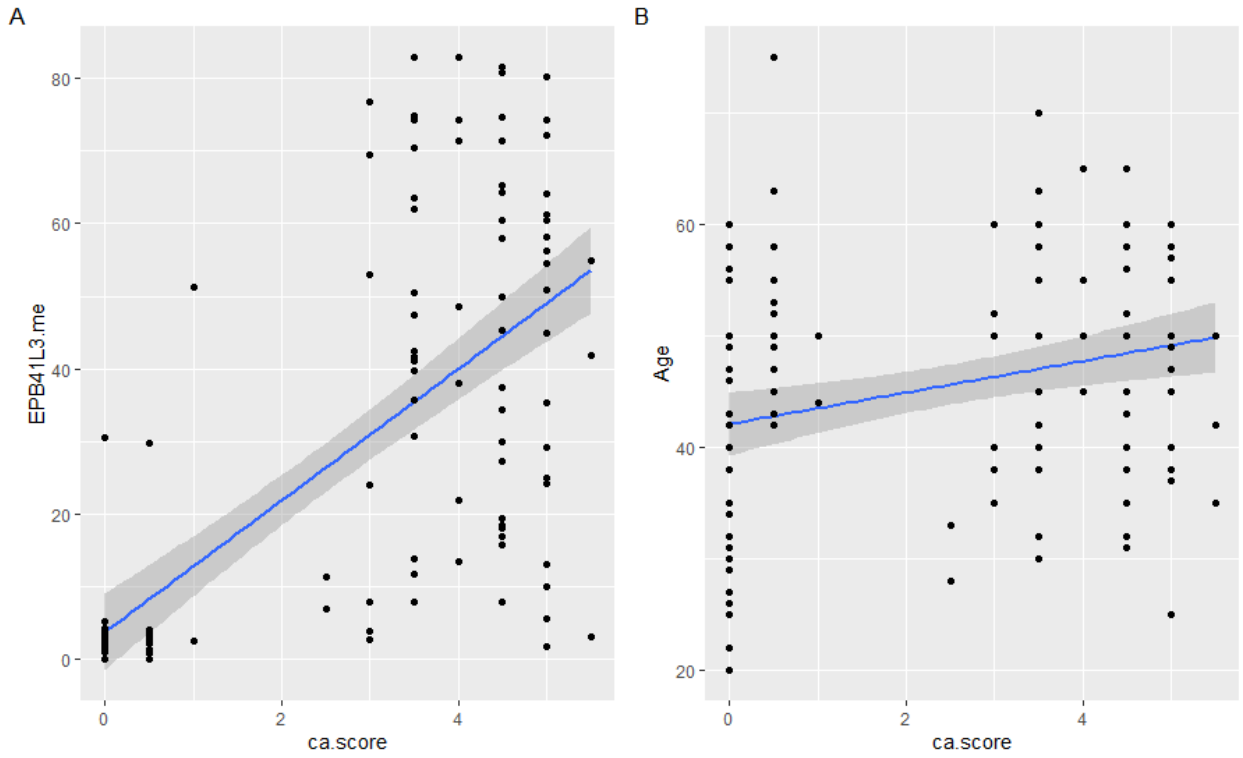


**Figure 4-16: Regression lines to compare *EPB41L3* methylation and age prediction of cancer stage. Regression line of *EPB41L3* methylation with cancer score (A) and age with cancer score (B) both with 95% confidence interval.**

## 4.5. Diagnostic performance of methylation markers

### 4.5.1. Correlations and variations among methylation assays and pathological status

Both *EPB41L3* methylation score and S5 cervical cancer classifier assay scores had strong correlation among themselves as well as with cancer score (Table 4-9). Correlation coefficients for S5 and *EPB41L3* assays with age were not statistically significant (p = 0.29 and 0.16

respectively). Comparatively S5 had stronger correlation with cancer score (r = 0.79, p <0.0001) than the *EPB41L3* methylation with cancer score (r = 0.69, p<0.0001).

**Table 4-9. Correlation coefficients and p values of S5, *EPB41L3* promoter methylation, age and cancer score.**

| | ***EPB41L3*.me** | **S5** | **Age** |
|---|---|---|---|
| S5 | 0.89(p = 0.000) | | |
| Age | 0.12(p = 0.1618) | 0.19(p = 0.294) | |
| ca.score | 0.69(p = 0.000) | 0.79(p = 0.000) | 0.27(p = 0.0017) |

Age showed statistically significant but weak correlation with cancer stage (r =0.27, p =0.0017) being weak predictor of risk of cervical cancer compared with the methylation markers (Fig. 4-17). From the positive correlation of methylation assays with cervical cancer stages, we can deduce that there is a general trend of increasing methylation levels with severity of cervical cancer.

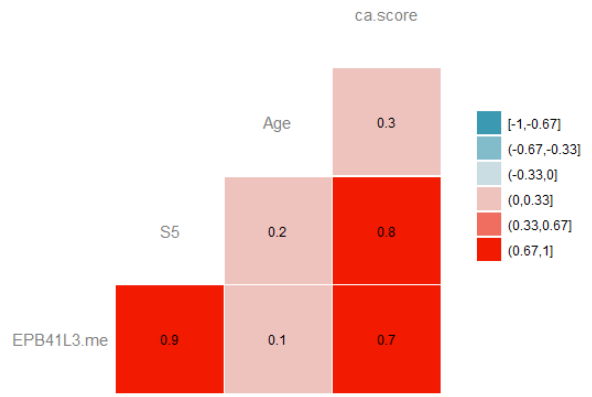


**Figure 4-17: Correlation heat map of *EPB41L3* methylation, S5 classifier, age and cancer stage**

Mean values of both S5 and *EPB41L3* promoter methylation vary significantly among low and high risk (p<0.001). Both assays discriminate well between healthy and CIN3 or worse (p <0.001) as well as CIN1 from CIN3 or worse (p<0.001). However, methylation assays do not vary significantly between normal cervical tissue and CIN1 (p = 0.66 and 0.85) (Fig. 4-18).

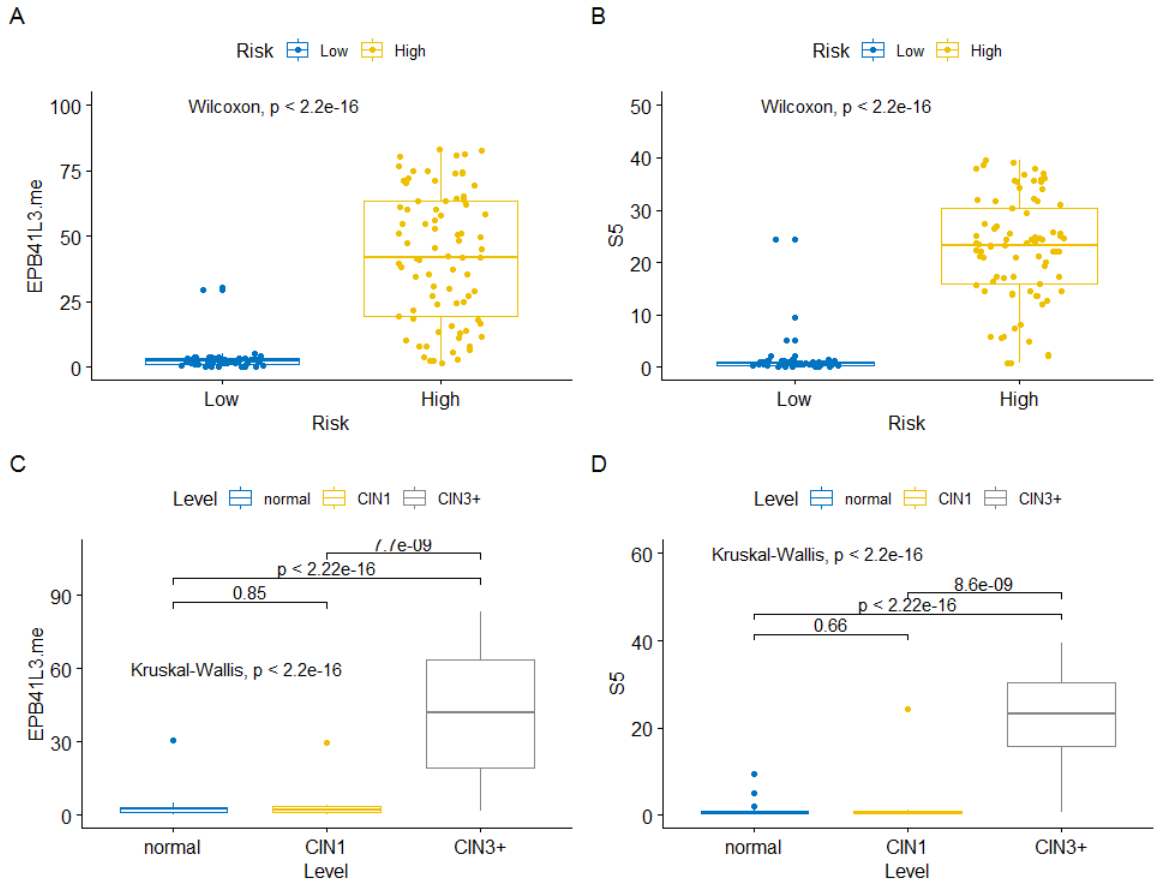


**Figure 4-18: Variation in *EPB41L3* methylation and S5 by case- control and by level of lesion. Variation between (A and B) women lower risk to develop caner of uterine cervix (low risk) and those at higher risk of the cancer (high risk) and (B and C) comparison between normal, CIN1 and CIN3 and worse.**

### 4.5.2. Performance of methylation assays

Sensitivity and specificity calculated with each methylation and S5 scores as cut points to asses discrimination potential of the scores showed sharp drop in sensitivity and rise in specificity in the lower short range of the values. Moreover, specificity and sensitivity curves showed sharp turn where these indices enter only slight change (Fig. 4-19). Intersection of the sensitivity and specificity graphs, usually used as cutoff value was at around value of 5 for both *EPB41L3* methylation and S5.

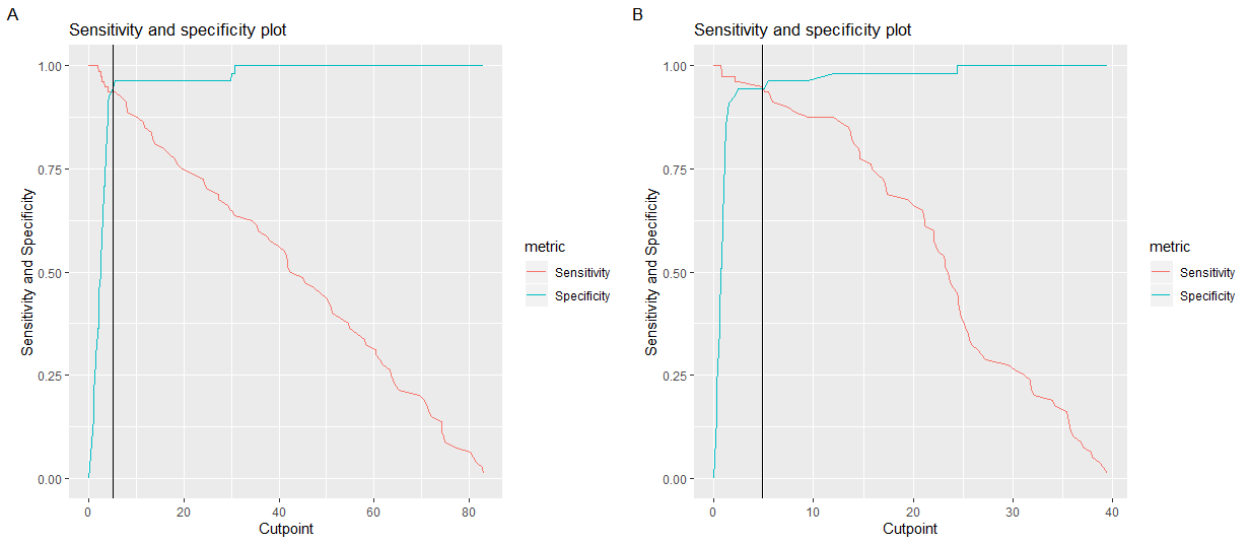


**Figure 4-19: Sensitivity and specificity of S5 and *EPB41L3* methylation. Sensitivity and specificity was computed for each value of (A) *EPB41L3* methylation and (B) S5 score as a cutoff point. The two indices plotted together to display their relative values against all possible cutoff values.**

### 4.5.3. Determining optimum cut points and evaluation of the assays

Optimal cut point value for *EPB41L3* methylation derived from ROC curve analysis using a method that minimizes the difference in sensitivity and specificity (intersection of sensitivity and specificity curves on Fig. 4-19A) was 5.11(Table 4-10). Using a method that maximizes Youden's index or the sum of sensitivity and specificity values, 5.56% methylation of *EPB41L3* promoter serves as optimal cut point. Using minimizing difference method, S5 assay can best discriminate between low and high risk women at cut point 4.85, but using Youde's index 2.44 appeared the best cut point. For *EPB41L3* methylation, cut points obtained both ways were almost similar except slightly higher specificity at cut point 5.56. Cut point 2.44 yielded slightly better sensitivity and negative predictive value (NPV) than cut point 4.85 for S5.

Though the differences from this data are only slight, S5 assay showed better sensitivity, AUC and NPV than *EPB41L3* methylation. *EPB41L3* methylation in turn had slightly higher specificity and PPV than the S5 assay.

**Table 4-10. Summary of reciever operating characteristic (ROC) curve analysis for the *EPB41L3* methylation and S5 assays to differentiate between cases and controls**

| Optimal cut point | Method | Index | specificity | sensitivity | AUC | PPV | NPV | outcome | predictor |
|---|---|---|---|---|---|---|---|---|---|
| 5.56 | youden | 0.9 | 0.96 | 0.94 | 0.97 | 0.97 | 0.91 | Risk | *EPB41L3*.me |
| 2.44 | | 0.91 | 0.95 | 0.96 | 0.98 | 0.96 | 0.95 | Risk | S5 |
| 5.56 | Sen + spe | 1.9 | 0.96 | 0.94 | 0.97 | 0.97 | 0.91 | Risk | *EPB41L3*.me |
| 2.44 | | 1.91 | 0.95 | 0.96 | 0.98 | 0.96 | 0.95 | Risk | S5 |
| 5.11 | \|sen-spe\| or | 0.01 | 0.95 | 0.94 | 0.97 | 0.96 | 0.91 | Risk | *EPB41L3*.me |
| 4.85 | sen = spe | 0 | 0.95 | 0.95 | 0.98 | 0.96 | 0.93 | Risk | S5 |

AUC= area under ROC curve, PPV= positive predictive value, NPV= negative predictive value

# Chapter 5

# 5. Discussion

## 5.1. Higher parity and early age at first sexual intercourse are risk factors for cervical cancer among Ethiopian women

Prevalence of hrHPV is higher in cases than in controls, which is in line with hrHPV infection being the major risk factor to develop cervical cancer. Significant differences in mean parity and AAFSI was observed between normal controls and invasive cervical cancer as well as between low risk and high risk groups. This dichotomy in parity and AAFSI with the malignant and non malignant states of cervical tissue is important in determining women at risk of cervical cancer. These differences were not significant between healthy controls and precancerous lesions and among different stages of invasive cervical cancer. Higher parity and having sexual intercourse at earlier age were shown to have increased the chance for infection by HPV and development of cervical cancer. Binary logistic regression analysis also showed that increased parity, earlier age of first sexual intercourse and infection by hrHPV are among the risk factors that are strong enough to predict the risk of cervical cancer development. This implies that women with higher parity and/or lower AAFSI are at higher risk than those with low parity and later AAFSI.

Greater parity in cases than in controls indicates having many children is risk factor as reported in several previous studies (Muñoz *et al.*, 2002; Jensen *et al.*, 2013; Dawit Wolday *et al.*, 2018). Sexual intercourse at younger age is shown to increase the risk of cervical cancer as the mean AAFSI is much lower (16.57) in cases than in controls (20.18). This is also in agreement with other studies (Plummer *et al.*, 2012; Ribeiro *et al.*, 2015; Kaur *et al.*, 2016; Cândido *et al.*, 2017) that report starting intercourse at earlier age increases the risk of cervical cancer. Parity and AAFSI can thus serve as prioritizing characteristics in cervical cancer prevention interventions along with other risk factors.

Cervical epithelia undergo natural changes including the position of transformation zone (Castle *et al.*, 2006) and metaplastic changes during puberty (Giroux and Rustgi, 2017) that affects both physical and physiological conditions of the tissue which may in turn modulate its susceptibility to infection by hrHPV and carcinogenesis. Several anatomical and physiological changes mediated by hormonal changes as well as mechanical strains are also evident in the uterine cervix in association with pregnancy and delivery (Timmons *et al.*, 2010). The metaplasic changes and other events associated with pregnancy and delivery increase susceptibility of the cervical tissue to infection by HPV (Hwang *et al.*, 2012). Hence the reason that higher parity and earlier AAFSI may increase the risk of cervical HPV infection and hence cervical cancer is the natural course of physiological and physical changes during puberty and pregnancy and trauma during delivery.

In the selected women, the cases are older with mean age 48.5 (95% CI, 46.6-50.4) years than controls 38.41(95% CI, 35.0 - 41.8) years, which means women attending cervical cancer screening are younger than those referred for histopathology examination. This variation in age may arise from two major reasons. The first is long latency of hrHPV infection and multistage progression of the pathology (Schlecht *et al.*, 2001) so that cancer develops and medical attention sought at later age. Second, the younger generation is more aware of cervical screening because of better exposure to information and is better educated to attend cervical screening services.

The higher number of urban residents in the controls group is because of lack of screening programs in the rural areas. Hence sampling bias makes it inconclusive about any association of cervical cancer with rural residence. However, proportional presence of rural residents in the cases group indicated that cervical cancer is not restricted to specific residence type. Since all the study participants were selected randomly, all the variables except residence are representative of the cases and controls group. Since the variation in HCU is not statistically significant, it cannot be accounted for neither as risk for nor as protective against cervical cancer. Studies from Brazil (Ribeiro *et al.*, 2015), Peru (Almonte *et al.*, 2011) and Kenya (Ermel *et al.*, 2019) also found no statistically significant association between hormonal contraceptive use and risk of HPV infection and cervical cancer. There are also other studies which reported hormonal contraceptive use as risk factor. For example a research team called ‘International Collaboration

of Epidemiological Studies of Cervical Cancer' (2007) found use of hormonal contraceptive for ten years of more to be a risk factor. Another study on Indonesian women also reported HCU as risk factor to cervical cancer (Paramita *et al.*, 2010). Therefore the impact of hormonal contraceptive use should be further studied as it is difficult to clearly conclude its role in cervical cancer development.

The socio-demographic factors are interdependent and one affects the other. HCU for example varies with age and parity and AAFSI with residence. Since both age and parity vary with cervical pathology, the two have confounding effect. However statistically significant correlation coefficients indicate which variable have strong effect. Parity correlates with AAFSI, age and cancer score with coefficients of -0.5, 0.5 and 0.5 respectively. Starting sexual intercourse at earlier age leads to have more children, older age is also a factor to have more children and having many children increases the risk of cervical cancer development. AAFSI is correlated with age and cancer score with coefficients of correlation -0.2 and -0.5. Older women tend to have started intercourse earlier than younger ones (which might be due to traditional early marriage) and starting sexual intercourse earlier increases risk of cervical cancer. The correlation coefficient of age and cancer score is 0.3 meaning cancer risk increases with age. Stronger correlations with cancer score are observed in parity and AAFSI. Therefore parity and AAFSI have greater role in cervical cancer development.

## 5.2. Prevalence and genotype distribution of hrHPV types among Ethiopian women with various stages of cervical lesions

The non-specific HPV test by PCR detected more HPV infections than the genotyping test because the later is specific to only 14 hrHPV types while the PCR amplifies any DNA of any mucosal HPV. HPV prevalence of 82.5% and 17.9 % was observed in women with cervical lesion and normal cervical cytology respectively. This is somewhat different from previous studies in Ethiopian women, which identified HPV in 149 of 160 (93%) of cervical lesions (Ebba Abate *et al.*, 2013) and in all of 92 (100%) women with abnormal cytology and in 69 of 141 (48.9%) women with normal cytology (Dawit Wolday *et al.*, 2018). The differences may arise

from the samples and methods used for HPV detection and genotyping. HPV negative cervical lesions was higher (17.5%) in the current study compared with recent study conducted in women from Texas USA (Ge *et al.*, 2019) which reported 8.3% HPV negative biopsy confirmed high grade lesions or worse. In another study on Finish women, 10% of women with high grade lesion and older than 45 years was hrHPV negative (Aro *et al*., 2019). This high rate of HPV negative cervical lesions is attributable to sampling errors and integration of the HPV genome in to the host cell genome that interrupts the L1 region that is targeted by the tests (Walboomers *et al.*, 1999) or presence of HPV negative cervical lesions (Sun *et al.*, 2018).

HPV16 is the most prevalent type with 69.8% in the cases and 5.1% in the controls, which is in line with all previous reports on Ethiopian women with cervical lesion which are 91.2%(Ebba Abate *et al.*, 2013) and 62% (Dawit Wolday *et al.*, 2018). However, with prevalence of 14.6%, HPV45 is the second most prevalent HPV type in the cases which is not common in other studies. A meta analysis on global population (Li *et al.*, 2011), a hospital based studies on Danish women (Hammer *et al.*, 2015), Korean women (So *et al.*, 2019), Italian women (Rassu *et al.*, 2005), Australian women (Stevens *et al.*, 2009) and USA (Hopenhayn *et al.*, 2014) all reported HPV 16 to be the most prevalent followed by HPV18. HPV45 occupied the rank of top four in none of the above studies. Studies on UK (Mesher *et al.*, 2014) and Ghanian (Awua *et al.*, 2016) women reported HPV45 as the third most prevalent HPV types. A systematic review of HPV prevalence in three eastern Africa countries, Ethiopia, Kenya and Sudan (Matifan and Gemechis, 2019), showed that HPV45 is the third most common type following HPV16 and HPV18 in all the three countries. In the current study and one previous study from Ethiopia (Dawit Wolday *et al*., 2018), HPV45 is the second most prevalent type among Ethiopian women with cervical lesions and cancer. The fact that the current study and Dawit Wolday *et al* (2018) are both from Ethiopian populations indicates higher prevalence of HPV45 to be peculiar epidemiological characteristic to this specific population.

Considerable amount (19.5%) of all hrHPV infections in cases were multiple infections. The two most prevalent types HPV16 and HPV45 were found in most of the multiple infections. From the total of 15 multiple infection 7(46.67%) were HPV16-HPV45 co-infection. 19.4% of HPV16 and

64.3% of HPV45 infections were in multiple infections. This emphasizes the importance of these two genotypes in Ethiopian patients.

Cervical hrHPV infection was higher in women who had started sexual intercourse at younger age. Majority of cervical cancer patients are infected by hrHPV types. HPV16 is the most prevalent type in Ethiopian cervical cancer patients. It is the most common type in both single infection and multiple infections. Infection by high risk HPV and other demographic factors make women prone to cervical cancer. HPV vaccination with HPV16 as target is a good choice but since the next most common hrHPV type is type 45, in this study as well as in another in Ethiopia (Dawit Wolday *et al.,* 2018), vaccines that target HPV45 as well are important for Ethiopia more so than those targeting HPV18. This indicates a need for the nonavalent vaccine which could target not only single infections but also considerable amount of multiple infections among Ethiopian women.

## 5.3. DNA methylation pattern in the L1 and L2 genes of HPV in hrHPV infected women with cervical lesions

Distribution of the HPV L1 and L2 methylation values and its pattern assumed generally distinct trend in the healthy and malignant cervical cells. CpG sites in HPV16-L1, HPV16-L2 and HPV18-L2 showed higher level methylation in cancerous cervical tissue than in healthy ones. It was not possible to compare methylation level of CpG sites in the other (HPV31-L1 and HPV33-L2) regions due to lack of healthy cells infected with the corresponding HPV types, however the methylation level was generally higher in cancerous specimens. The increased level of methylation in the abnormal cervical cells indicates association of the HPV epigenome modification with malignant transformation. In healthy cells, methylation of the evaluated sites was almost negligible while in malignant cells it ranges all the way from non- methylated to more than 80% methylated. This implies that DNA methylation at these sites is a conditional epigenetic change in the cervical cancer progression (Torres Rojas *et al.*, 2018).

Wilcoxon rank sum comparison of HPV16-L1 and L2 region methylation between cancerous and healthy cervical tissue showed statistically significant difference between low risk and high risk

women. Methylation values for the HPV18 L2 sites didn't show significant variation, which is likely due to small sample size in both healthy and cases groups.

Despite the significant difference in the healthy and malignant tissues, comparison among the various stages of cervical cancer yielded non significant variation in DNA methylation level of HPV16 L1 and L2 regions. Mean methylation level in both L1 and L2 regions of HPV 16 showed a steady increase in the earliest stages (stage IB through stage IIB) but levels out in the higher stages. This indicates that HPV DNA methylation change in the L1 and L2 region is among early epigenetic events in cervical cancer progression. Previous works with breast cancer (Rauscher *et al.,* 2015) and other cancer types (Guo *et al.,* 2019) revealed that DNA methylation changes are evident in the initial stages of the cancers.

Linear regression model revealed that methylation in the L1 region to be a better predictor of various stages of cervical cancer. Methylation in the HPV16 L2 region is a weak predictor with statistically non significant regression coefficient ($p = 0.0903$). Bowden *et al* (2019) in their meta analysis showed that methylation of CpG sites in the L1 region was much higher in high grade cervical lesions than in the low grade lesions compared to those in the L2 region.

DNA methylation of CpG sites in the HPV 16 L1 and L2 ORF regions was among epigenetic changes that have statistically significant association with cervical cancer development. Methylation of these sites may be either driver or passenger epigenetic changes in the process of cervical cell transformation, but regardless of mechanisms, the methylation levels are a very strong biomarkers of cervical cancer risk. Variation in levels of methylation by pathological status of cervical tissues can be used in various ways in the fight against the cancer. First the variation can serve as diagnostic marker both to predict as well as to diagnose the disease status. Second it adds to the understanding of the process of cervical carcinogenesis. Third it may serve in designing a therapeutic intervention either serving as treatment target or through helping in uncovering the pathways involved in the process. The underlying biological mechanisms of these associations must hence be the subject of future studies in this area.

## 5.4. Hypermethylation of CpG sites in human *EPB41L3* gene promoter region in cervical cancer

*EPB41L3* promoter methylation is higher in cervical cells from cancerous tissue than in those from normal tissues. With the exception of one woman who had high level of methylation (who may have had an occult cancer) methylation in normal women was quite low, implying that low methylation level of CpG sites in the *EPB41L3* promoter region are associated with active expression of the DAL-1 tumor suppressor protein 4.1B in healthy epithelium but not in carcinoma cells (Dafou *et al.,* 2010). Methylation level was lower not only in healthy tissue but also in CIN1 lesions compared with invasive cancer. Level of methylation in CIN3 was difficult to judge due to presence of only two cases in this category. Hypermethylation of the *EPB41L3* gene promoter in cervical cancer cells observed in this study is also similar with what was previously reported in cervical cancer (Eijsink *et al.,* 2011a, Kelly *et al.,* 2018) as well as other cancer types (Kikuchi *et al.,* 2005, Takahashi *et al.,* 2012). This makes *EPB41L3* promoter methylation a potent diagnostic marker that signals cervical cell transformation as at least one of the many molecular aberrations underlying the malignancy. Methylation level of *EPB41L3* promoter in healthy and the CIN1 lesion (low risk) compared with CIN3, a lesion which is higher risk to progress to invasive cancer, and invasive cancer (high risk) is lower. The methylation percentage for the two groups (low risk and high risk) was distinct. Hence *EPB41L3* promoter methylation is important in predicting level of risk of cervical cancer development.

DNA methylation especially of gene promoter regions, as an epigenetic marker can be affected by various biological variables such as age (Xie *et al.,* 2019). Age as one of the major epigenetic modulator was assessed for its association with the *EPB41L3* promoter methylation and showed no significant association which could however be biased by a residual effect of our biased age distribution among cases and controls groups. From the data observed in this study, methylation of the *EPB41L3* promoter region is more strongly associated with cancer than with age. Klutstein *et al.* (2016) suggest that age related DNA methylation might provide fertile ground for cancer initiation and progression. A study conducted on the association of DNA methylation in aging and cancer (Perez *et al.,* 2018), indicated that the relationship between age and DNA methylation is complex. However the *EPB41L3* promoter methylation changes observed in the current study

look less affected by age related epigenetic changes as the increased DNA methylation correlates more with cancer than with age of the women.

Comparing methylation level across cancer stage, gradual increase of methylation with severity of cervical pathology was found in the lower half of the stages followed by drop in methylation level as cancer worsens. This suggests *EPB41L3* promoter methylation or expression suppression is important early event in cervical cancer progression but less important in the later stages of the cancer. However, there is a strong overall positive correlation between the methylation levels and cancer stages with Spearman correlation coefficient r =0.68 (95% CI = 0.58 – 0.76, $p < 0.0001$). Correlation coefficient of r = 0.11 which was calculated for *EPB41L3* methylation and age was not statistically significant (p=0.21) and age and cancer stage were correlated with coefficient r= 0.28 (p=0.001) which implies age to be among risk factors for cervical cancer. Even though epidemiological data show that the odds is higher to develop cancer at older age than at younger age, it is believed that this is caused by increased exposures to other risk factors during the ages preceding the old age (Seppa *et al.*, 2015; White *et al.*, 2014).

Simple linear and multiple linear regression models for *EPB41L3* methylation and age with cancer score (a numerical proxy for cancer stages) showed *EPB41L3* methylation as a strong predictor of the early cancer stages than their age was. The increased methylation in *EPB41L3* promoter region was found to be a good predictor of cervical malignancy.

In conclusion CpG sites in *EPB41L3* promoter region were hypermethylated in cervical cancer. The differential methylation observed in cervical cells from healthy and cancerous tissues was associated with the process of carcinogenesis. Age as a factor that affects epigenetic markers was ruled out, because the association between methylation levels and cervical pathology status was very strong while there is no statistically significant association between methylation level and age. Increase in EBP41L3 promoter methylation was prominent only during the early stages of the cancer. *EPB41L3* expression suppression is an early epigenetic event in the process of cervical cancer development. This implies that pathways such as cell cycle regulation and cell motility and attachment, in which *EPB41L3* is involved, are the most likely altered pathways in the initial transformation of cervical tissue.

## 5.5. DNA methylation assays are good biomarkers to differentiate normal cervical tissue and CIN1 from CIN3 and worse cervical lesions

The two assays assessed in the current study (*EPB41L3* methylation and S5) had strong correlation because *EPB41L3* methylation is the mojor component of the S5 assay which was consistently measured in all the patients. Though *EPB41L3* methylation constitutes only ~31% of the S5 score, only 61.48% of the study participants were hrHPV positive from among which still only part had successfully measured methylation of HPV DNA to be included in the S5 assay. The strong correlation of the methylation scores with cancer stage is indicative of presence of underlying biological relationship. Non significance of correlation between methylation and age rules out the possibility of DNA methylation to be caused by age related epigenetic changes. Hence increased level of methylation of CpG sites in both the human *EPB41L3* gene promoter as well as HPV L1 and L2 genes in cancers than in healthy and precancerous lesions is biological phenomena associated with cancer progression. Hypermethylation of CpG sites in promoters of especially tumor suppressor genes are the potential drivers of the carcinogenesis processes (Chatterjee *et al.*, 2018). Dankai and colleagues (2019) demonstrated elevated methylation of *CADM1*, *FAM19A4*, and *MAL* genes in cervical cancer which could help detection of various high grade cervical lesions from normal and low grade lesions with better sensitivity than cytology and comparable specificity.

Lack of variation in methylation between healthy and CIN1 cells imply that DNA methylation in the studied genomic positions are key indicators or predictors of malignancy of lesions. There was clear variation between the CIN1 and CIN3+, indicating cellular transition from benign lesion to malignant. This is consistent with previous study where the *EPB41L3* (Vasiljević *et al.*, 2014a) and HPV L1 and L2 (Vasiljevic *et al.*, 2014b) genes methylation classified between CIN1 or less from CIN2 and CIN3 efficiently.

Higher correlation coefficient of the S5 with cancer stages compared with that of *EPB41L3* methylation alone is because of association of the HPV L1 and L2 gene mathylations with the process of cervical carcinogenesis. The diagnitic value that DNA methylation of CpG sites in the

HPV L1 and L2 genes was reported in previous works (Clarke *et al.*, 2018, Bryant *et al.*, 2014). Including methylation scores of the HPV genes in the assay has strengthened the diagnostic performance of the assay.

The methylation scores have clear pattern by the transformation status of the cells. This makes it amenable for use as diagnostic marker to differentiate between healthy cervical cells and those with high grade lesions. The *EPB41L3* methylation and S5 assays differentiate between cervical cells from normal or CIN1 and CIN3+ lesions with higher sensitivity (96%) and specificity (95%) at optimal cutoff points. Both S5 score and *EPB41L3* promoter methylation assays improved sensitivity and specificity of the hrHPV genotyping tests.

Selecting cutoff values using maximizing sum of specificity and sensitivity or Youden's index generated better cutoff values for both *EPB41L3* methylation (5.56) and S5 (2.44). At cutoff point 5.56, *EPB41L3* methylation had 94% sensitivity, 96% specificity and 0.97 AUC while S5 at cutoff 2.44 had 96% sensitivity, 95% specificity and 0.98 AUC. Minimizing the differences between specificity and sensitivity generated cut points 5.11 for *EPB41L3* methylation and 4.85 for S5. Sensitivity and specificity of *EPB41L3* methylation at at cutoff value 5.11 were 94% and 95% respectively while that of S5 at cutoff 4.85 were both 95%. The cutoff values selected both ways had similar AUC value. Other studies have also reported DNA methylation based tests to have better performance than cytology as well as HPV testing and genotyping tests. By screening from a genome wide methylation profile of cervical cancer specimens, Xu *et al.* (2019) identified four methylation markers that can differentiate between normal cervical tissue and tumor with sensitivity of 96.2%, specificity of 95.2% and an AUC greater than 94%. Two DNA methylation assays approved to be used for triage of ambiguous cervical screening test results, GynTect® (Oncgnostics) and QIAsure (Qiagen), have 61.2% and 70.5% sensitivity respectively of detecting CIN3 or worse lesions (Locke *et al.*, 2019). The current study showed much higher sensitivity, specificity, AUC and negative and positive predictive values.

S5 assay has consistently greater sensitivity, AUC and NPV than *EPB41L3* methylation at all the corresponding cutoff values while *EPB41L3* methylation had better specificity and PPV. AUC is considered the overall summary performance of a test. Hence S5 is only slightly better at

discriminating healthy cervix and CIN1 from high grade cervical lesions. It is likely that the performance of the S5 and *EPB41L3* tests in our study were quite similar because we had very few CIN3 and no CIN2. In previous study it has been observed that the improved performance of the S5 test versus *EPB41L3* was particularly evident in detecting the CIN2/3 lesions where S5 has a significantly higher sensitivity but a similar specificity as the *EPB41L3* test (Lorincz, 2016). S5 assay has shown a better sensitivity (86.1% versus 63.3%) and specificity (40.1% versus 29.1%) than HPV16/18 and cytology co-testing (Hernández-López *et al.*, 2019). Discripancies in the test parametrers such as sensitivity, specificity and AUC among different studies may arise from differences in protocols followed by the different researches.

DNA methylation changes in both human and HPV genes are important epigenetic factors in cervical cancer development. Diagnostic tests based on DNA methylation levels have good performance in detecting or predicting cervical cancer and high grade cervical lesions. S5 classifier, DNA methylation test that included methylation of both human and HPV genomic sites performed better than the human gene only methylation analysis. DNA methylation is promising marker to develop a diagnostic test with highest sensitivity and specificity. These assays should further be evaluated and enriched with more methylation data to be applied clinically.

# Chapter 6

# 6. Conclusions and Recommendations

## 6.1. Conclusions

Prevalence of hrHPV in Ethiopian women with abnormal cervical tissue (including CINs and invasive cervical cancer) is 80.2% and 15.4% in controls. HPV16 is the most prevalent genotype with prevalence of 69.8% followed by HPV45 (14.4%). HPV45 being the second most prevalent type in Ethiopian patients is noteworthy event.

Parity and age at first sexual intercourse are among socio-demographic factors identified to put women at risk of cervical cancer. Women with higher parity and earlier AAFSI are at increased risk of both being infected with hrHPV and developing cervical cancer. An increase in parity by one increases the odds of woman's risk of cervical cancer and hrHPV infection by 36% and 38% respectively. One year earlier AAFSI increases the odds for cervical cancer and hrHPV infection by 31% and 18% respectively.

Methylation level of CpG sites in the HPV16 L1 and L2 region is higher in cervical cancer than in hrHPV infected normal cervical tissue. Methylation pattern of CpG sites in the L1 region is more correlated with the cancer progression than those in the L2 region. Incremental pattern of HPV16 L1 and L2 methylation with cancer severity was observed at earlier stages of cancer but less so in advanced stages.

CpG sites in the human *EPB41L3* gene promoter region were also hypermethylated in cervical cancer cells compared with cervical cells from normal cervix and CIN1. The increase in *EPB41L3* promoter methylation follows the pathological severity for the first half of the progression and then drops at the most sever stages of the cancer. This implies that both HPV L1 and L2 gene and human *EPB41L3* promoter methylation to be early epigenetic event in the process of cervical carcinogenesis.

Methylation changes in both HPV and human genomic regions could be used to identify the pathological status or predict pathological consequences ahead of clinical manifestations. The methylation changes are good markers to differentiate between healthy and cancerous tissues. Levels of methylation of the human *EPB41L3* gene promoter and HPV16 L1 and L2 regions vary significantly between healthy and cancerous cervical tissue. Methylation pattern of CpG sites in human *EPB41L3* promoter region has strong correlation with cervical cancer stages. Evaluation of diagnostic performance of *EPB41L3* methylation alone as well as S5 classifier showed promising results. Both *EPB41L3* promoter methylation alone and S5 could classify normal cervical tissue from abnormal with better sensitivity, specificity and area under ROC curve (AUC) compared with cytology and hrHPV test.

## 6.2. Recommendations

From the results of the above five sections of this study, the following recommendations are forwarded.

- HPV based cervical cancer interventions planned in Ethiopia should consider the genotype distribution and prevalence and further epidemiological studies with larger sample size must be undertaken to confirm the prevalence before implementation.
- HPV45 must be given due attention in HPV tests and vaccinations in Ethiopia as it is the second most prevalent type in this and other previous study
- High parity women and women who have started sexual intercourse earlier should be given priority for cervical screening
- Adolescent education programs to raise awareness on family planning, early marriage and early sexual intercourse must be implemented considering the risks that parity and AAFSI bring withbregard to cervical cancer.
- Further research should be undertaken to clearly understand the epigenetic changes in both the HPV and human genomes that associate with cervical cancer since it may enhance our understanding of the natural history of the condition.

- DNA methylation as cervical cancer screening and triage may improve the efficacy of cervical cancer intervention if assessed, evaluated, improved further and utilized as triage tests especially with HPV testing.
- *EPB41L3* promoter methylation and S5 classifier has performed much better than the existing screening tests and hence should be considered to be utilized after being further evaluated with larger sample size.
- Epigenetic changes associated with cervical cancer progression should also be studied in depth for their potential use as treatment targets.

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# 8. Annexes

## Annex 1: Consent format and demographic and clinical history data sheet

A. Questionnaire

1. Introduction

   1.2 Study participant code_____ Sample code _____

   1.3 Date of interview_____

2. Age (in years)_____
3. Occupation _____ husband's occupation_____
4. Ethnicity _____
5. Permanent residence(where you lived for most of your age) ; Urban ☐ Rural ☐

   _____
6. Type of fuel used for cooking _____
7. History of other diseases (especially STI, HIV)
   a. _____
   b. _____
   c. _____
8. Number of children (if any)_____age of first child_____
9. Age at first parity_____
10. Age at first sexual intercourse _____
11. Contraceptive use yes ☐ No ☐

    If yes, mention type(s) of contraceptive and for how long

    a. _____

    b. _____
12. Number of sexual partners (any history of multiple sexual partners)_____
13. Do you or member of your family smoke cigarette? yes ☐ No ☐
14. Have you ever been on medication for long time? Specify _____
15. Is there family history of cancer?

    ☐ ☐

    a

1.Yes ☐ 2. No 3. Unknown

If yes which family member? (specify)_____

16. Type of staple food (diet) _____
17. Ca status and stage_____
    a. Clinical

    _____
    _____
    _____
    _____

    b. Histopathological

    _____
    _____
    _____
    _____

**B. Certificate of Consent**

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this research.

Name of Participant _____Sign._____ Date _____

**If illiterate**

*A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.*

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

*Name of witness*_____ *AND* *Thumb print of participant*

*Signature of witness* _____

Date _____

**Statement by the researcher/person taking consent**

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the purpose of the study and the processes and procedures to be undertaken.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been forced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

# በጥናት ለመሳተፍ የተሰጠ የስምምነት ውል

ከላይ በዝርዝር የተመለከተውን መረጃ አንብቤ ወይም ተነቦልኝ በሚገባ ተረድቻለሁ፡፡ ጥያቄ የመጠየቅ እድልም አግኝቼ ጥያቄዎቼ በተገቢ ሁኔታ ተመልሰውልኛል፡፡ ስለጥናቱ የተሰጠኝን መረጃ በትክክል ተረድቼ በጥናቱ ለመሳተፍ በፈቃደኝነት ተስማምቻለሁ፡፡

የተሳታፊዋ ስም_____ፊርማ_____ቀን_____

## ማንበብና መፃፍ ለማይችሉ

ስምምነቱ ማንበብና መፃፍ በምትችል (በሚችል) ምስክር አማካኝነት ይፈረማል፡፡ ከተቻለ ምስክሮች በተሳታፊዎች ቢመረጡ ወይም ከጥናት ቡድኑ ጋር ግንኙነት ባይኖራቸው ይመረጣል፡፡ ማንበብና መፃፍ የማይችሉ ተሳታፊዎች በጣታቸው አሻራ ይፈርማሉ፡፡ ምስክሮችም መረጃውን በትክክል ለተሳታፊዋ ስለማንበባቸው ይፈርማሉ፡፡

በስምምነት ቅፁ ላይ የተዘረዘረውን መረጃ ለታካሚዋ በትክክል ማንበቤን እንዲሁም ታካሚዋ ግልፅ ያልሆኑላቸውን ነጥቦች የመጠየቅ ዕድል አግኝተው ጠይቀው መረዳታቸውን እመሰክራለሁ፡፡ በተጨማሪም ታካሚዋ በጥናቱ ለመሳተፍ የተስማሙት በነፃ ፈቃዳቸው መሆኑን እመሰክራለሁ፡፡

*የምስክር ስም*_____ *የታካሚ/ተሳታፊ ጣት አሻራ*

*የምስክር ፊረማ*_____ቀን _____

## የአጥኚ ወይም የውል ተቀባይ ቃል

ለታካሚዋ/ተሳታፊዋ ስለጥናቱ የሚቻለውን ያህል በቂ መረጃ ሰጥቻለሁ፡፡ በተቻለ መጠንም ታካሚዋ ስለጥናቱ፣ አላማ አንዲሁም ጥናቱ ስለሚካሄድባቸው ዘዴዎችና በጥናቱ ወቅት ስለሚከናወኑ ድርጊቶች መረዳታቸውን አረጋግጫለሁ፡፡

ታካሚዋ/ተሳታፊዋ ስለጥናቱ ጥያቄ የመጠየቅ ዕድል ተሰጥቷቸው የጠየቋቸውን ጥያቄዎች ባለኝ ዕውቀት መጠን በትክክል አብራርቼ መልሻለሁ፡፡

ተሳታፊዋ ካለምንም ማስገደድ በነፃ ፈቃዳቸው በጥናቱ ለመሳተፍ መስማማታቸውን አረጋግጣለሁ፡፡

የዚህ ስምምነት ውል ቅጂ ለተሳታፊዋ ተሰጥቷል፡፡

የአጥኚ ወይም ውል ተቀባይ ስም_____ፊርማ_____ቀን_____

# Annex 2: IRB of Addis Ababa University protocol approval

ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES (IRB)
አዲስ አበባ ዩኒቨርስቲ ጤና ሳይንስ ኮሌጅ
Institutional Review Board

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 01/2018 Date: February 13, 2018
Protocol number: 016/14/Bioch

| | |
|---|---|
| **Protocol Title**: Polymorphism of the major Cancer predisposing genes, expression level changes and Human Papillomavirus (HPV) genome integration in Ethiopian cervical cancer patients | |
| Principal Investigator: | Birhanu Kumbi |
| 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: 03
2. Protocol Version Date: Jan 2018
3. Informed consent Version No. 03
4. Informed Consent Version Date: Jan 2018

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

III. TO NERC ☑

Institution Review Board (IRB) Approval: Period from: February 13, 2018 to February 12, 2019
Follow up report expected in: 3 Months ___6 Months __X__ 9 Months _____One year____

**Chairperson, IRB**
**Dr. Adamu Addissie**
**Signature** _____
**Date:** 15/2/2018

**Director of Research & Technology Transfer, CHS**
**Dr. Anetneh Belete**
**Signature** _____
**Date** _____

# Annex 3: DNA extraction protocol used in the study

## DNA isolation protocol from cotton swab and punch biopsy tissue

### I. Cotton swab

1. Immerse the dried cotton swab in 1.2 mL lysis solution (Lysis solution = 10 mM tris-HCL, 5 mM ethelyne diamine tetra acetate (EDTA) and 5% sodium dodisyl sulphate (SDS) at pH 8.0 containing 12 µL of 20mg/mL proteinase K
2. Incubate at 55$^{o}$C for 6 hours, (and keep it at room temperature overnight if necessary)
3. Remove the cotton swab
4. Add 650 µL cold solution of 8 M ammonium acetate with 1 mM EDTA and mix by gently vortexing
5. Chill on ice for about 10 minutes
6. Centrifuge at 6000 rpm for 20 minutes
7. Transfer 750 µL of the supernatant in to two clean 1.5 mL microtubes containing 750 µL cold isopropanol with wide bore pipette tips and mix by inverting gently several times (~20 times)
8. Centrifuge at 13000rpm for 10minutes and pour off the supernatant and invert each tube and leave to drain on clean absorbent paper
9. Add 1mL 70% ethanol and mix by inverting gently several times
10. Centrifuge at 13000 rpm for 10 minutes and pour off the ethanol and invert each tube and leave to drain on clean absorbent paper
11. Re-suspend the pellet in 100 µL 1XTE buffer

### II. Solid tissue

1. Mince the tissue as finely as possible on clean plate
2. Transfer it to 15 mL centrifuge tube containing 1.2 mL lysis solution with 12 µL of 20 mg/mL proteinase K
3. Incubate at 55$^{o}$C for 6 hours
4. Add 650 µL cold solution of 8 M ammonium acetate with 1 mM EDTA and mix by gently vortexing
5. Chill on ice for about 10 minutes
6. Centrifuge at 6000 rpm for 20 minutes

7. Transfer 750 µL of the supernatant in to two clean 1.5 mL microtubes containing 750 µL cold isopropanol with wide bore pipette tips and mix by inverting gently several times (~20 times)
8. Centrifuge at 13000 rpm for 10minutes and pour off the supernatant and invert each tube and leave to drain on clean absorbent paper
9. Add 1mL 70% ethanol and mix by inverting gently several times
10. Centrifuge at 13000 rpm for 10minutes and pour off the ethanol and invert each tube and leave to drain on clean absorbent paper
11. Re-suspend the pellet in 100 µL 1XTE buffer

# Annex 4. List of abbreviated cancer scores

| Disease Severity Grade | | Annotated Score |
|---|---|---|
| **Normal** | | 0 |
| **CIN1** | | 0.5 |
| **CIN3** | | 1 |
| **Cancer sages** | **IA** | 2 |
| | **IB** | 2.5 |
| | **IIA** | 3 |
| | **IIB** | 3.5 |
| | **IIIA** | 4 |
| | **IIIB** | 4.5 |
| | **IVA** | 5 |
| | **IVB** | 5.5 |

# Annex 5. Pair-wise comparison of mean parity among various stages of cervical cancer

| | .y. | group1 | group2 | p | p.adj | p.format | p.signif | method |
|---|---|---|---|---|---|---|---|---|
| 1 | Parity | Healthy | CIN1 | 0.096779 | 1 | 0.09678 | ns | Wilcoxon |
| 2 | Parity | Healthy | CIN3 | 0.058965 | 1 | 0.05897 | ns | Wilcoxon |
| 3 | Parity | Healthy | IB | 0.172774 | 1 | 0.17277 | ns | Wilcoxon |
| 4 | Parity | Healthy | IIA | 0.080665 | 1 | 0.08066 | ns | Wilcoxon |
| 5 | Parity | Healthy | IIB | 8.47E-07 | 3.80E-05 | 8.50E-07 | **** | Wilcoxon |
| 6 | Parity | Healthy | IIIA | 0.000132 | 0.0055 | 0.00013 | *** | Wilcoxon |
| 7 | Parity | Healthy | IIIB | 3.18E-06 | 0.00014 | 3.20E-06 | **** | Wilcoxon |
| 8 | Parity | Healthy | IVA | 3.67E-06 | 0.00016 | 3.70E-06 | **** | Wilcoxon |
| 9 | Parity | Healthy | IVB | 0.02469 | 0.91 | 0.02469 | * | Wilcoxon |
| 10 | Parity | CIN1 | CIN3 | 0.201137 | 1 | 0.20114 | ns | Wilcoxon |
| 11 | Parity | CIN1 | IB | 0.568508 | 1 | 0.56851 | ns | Wilcoxon |
| 12 | Parity | CIN1 | IIA | 0.519124 | 1 | 0.51912 | ns | Wilcoxon |
| 13 | Parity | CIN1 | IIB | 0.001734 | 0.071 | 0.00173 | ** | Wilcoxon |
| 14 | Parity | CIN1 | IIIA | 0.005224 | 0.2 | 0.00522 | ** | Wilcoxon |
| 15 | Parity | CIN1 | IIIB | 0.003259 | 0.13 | 0.00326 | ** | Wilcoxon |
| 16 | Parity | CIN1 | IVA | 0.005004 | 0.2 | 0.005 | ** | Wilcoxon |
| 17 | Parity | CIN1 | IVB | 0.127648 | 1 | 0.12765 | ns | Wilcoxon |
| 18 | Parity | CIN3 | IB | 1 | 1 | 1 | ns | Wilcoxon |
| 19 | Parity | CIN3 | IIA | 0.456612 | 1 | 0.45661 | ns | Wilcoxon |
| 20 | Parity | CIN3 | IIB | 0.394665 | 1 | 0.39467 | ns | Wilcoxon |
| 21 | Parity | CIN3 | IIIA | 0.219165 | 1 | 0.21917 | ns | Wilcoxon |
| 22 | Parity | CIN3 | IIIB | 0.527803 | 1 | 0.5278 | ns | Wilcoxon |
| 23 | Parity | CIN3 | IVA | 0.80823 | 1 | 0.80823 | ns | Wilcoxon |
| 24 | Parity | CIN3 | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 25 | Parity | IB | IIA | 1 | 1 | 1 | ns | Wilcoxon |
| 26 | Parity | IB | IIB | 0.585249 | 1 | 0.58525 | ns | Wilcoxon |
| 27 | Parity | IB | IIIA | 0.548006 | 1 | 0.54801 | ns | Wilcoxon |
| 28 | Parity | IB | IIIB | 0.430602 | 1 | 0.4306 | ns | Wilcoxon |
| 29 | Parity | IB | IVA | 0.627379 | 1 | 0.62738 | ns | Wilcoxon |
| 30 | Parity | IB | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 31 | Parity | IIA | IIB | 0.137259 | 1 | 0.13726 | ns | Wilcoxon |
| 32 | Parity | IIA | IIIA | 0.106303 | 1 | 0.1063 | ns | Wilcoxon |
| 33 | Parity | IIA | IIIB | 0.144022 | 1 | 0.14402 | ns | Wilcoxon |
| 34 | Parity | IIA | IVA | 0.195402 | 1 | 0.1954 | ns | Wilcoxon |
| 35 | Parity | IIA | IVB | 0.354539 | 1 | 0.35454 | ns | Wilcoxon |
| 36 | Parity | IIB | IIIA | 0.790881 | 1 | 0.79088 | ns | Wilcoxon |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 37 | Parity | IIB | IIIB | 0.712645 | 1 | 0.71265 | ns | Wilcoxon |
| 38 | Parity | IIB | IVA | 0.525551 | 1 | 0.52555 | ns | Wilcoxon |
| 39 | Parity | IIB | IVB | 0.120891 | 1 | 0.12089 | ns | Wilcoxon |
| 40 | Parity | IIIA | IIIB | 0.897708 | 1 | 0.89771 | ns | Wilcoxon |
| 41 | Parity | IIIA | IVA | 0.363854 | 1 | 0.36385 | ns | Wilcoxon |
| 42 | Parity | IIIA | IVB | 0.03489 | 1 | 0.03489 | * | Wilcoxon |
| 43 | Parity | IIIB | IVA | 0.414828 | 1 | 0.41483 | ns | Wilcoxon |
| 44 | Parity | IIIB | IVB | 0.400104 | 1 | 0.4001 | ns | Wilcoxon |
| 45 | Parity | IVA | IVB | 0.497029 | 1 | 0.49703 | ns | Wilcoxon |

# Annex 6. Pair-wise comparison of mean AAFSI by cancer stage

| | .y. | group1 | group2 | p | p.adj | p.format | p.signif | method |
|---|---|---|---|---|---|---|---|---|
| 1 | AAFSI | Healthy | CIN1 | 0.520245 | 1 | 0.52024 | ns | Wilcoxon |
| 2 | AAFSI | Healthy | CIN3 | 0.189923 | 1 | 0.18992 | ns | Wilcoxon |
| 3 | AAFSI | Healthy | IB | 0.376256 | 1 | 0.37626 | ns | Wilcoxon |
| 4 | AAFSI | Healthy | IIA | 0.010068 | 0.39 | 0.01007 | * | Wilcoxon |
| 5 | AAFSI | Healthy | IIB | 1.62E-04 | 6.80E-03 | 1.60E-04 | *** | Wilcoxon |
| 6 | AAFSI | Healthy | IIIA | 0.011436 | 0.43 | 0.01144 | * | Wilcoxon |
| 7 | AAFSI | Healthy | IIIB | 1.91E-05 | 0.00084 | 1.90E-05 | **** | Wilcoxon |
| 8 | AAFSI | Healthy | IVA | 2.38E-06 | 0.00011 | 2.40E-06 | **** | Wilcoxon |
| 9 | AAFSI | Healthy | IVB | 0.072757 | 1 | 0.07276 | ns | Wilcoxon |
| 10 | AAFSI | CIN1 | CIN3 | 0.180187 | 1 | 0.18019 | ns | Wilcoxon |
| 11 | AAFSI | CIN1 | IB | 0.396576 | 1 | 0.39658 | ns | Wilcoxon |
| 12 | AAFSI | CIN1 | IIA | 0.018913 | 0.7 | 0.01891 | * | Wilcoxon |
| 13 | AAFSI | CIN1 | IIB | 0.001884 | 0.075 | 0.00188 | ** | Wilcoxon |
| 14 | AAFSI | CIN1 | IIIA | 0.024519 | 0.88 | 0.02452 | * | Wilcoxon |
| 15 | AAFSI | CIN1 | IIIB | 0.000541 | 0.022 | 0.00054 | *** | Wilcoxon |
| 16 | AAFSI | CIN1 | IVA | 0.000122 | 0.0053 | 0.00012 | *** | Wilcoxon |
| 17 | AAFSI | CIN1 | IVB | 0.081618 | 1 | 0.08162 | ns | Wilcoxon |
| 18 | AAFSI | CIN3 | IB | 1 | 1 | 1 | ns | Wilcoxon |
| 19 | AAFSI | CIN3 | IIA | 0.65372 | 1 | 0.65372 | ns | Wilcoxon |
| 20 | AAFSI | CIN3 | IIB | 0.302781 | 1 | 0.30278 | ns | Wilcoxon |
| 21 | AAFSI | CIN3 | IIIA | 0.454661 | 1 | 0.45466 | ns | Wilcoxon |
| 22 | AAFSI | CIN3 | IIIB | 0.316834 | 1 | 0.31683 | ns | Wilcoxon |
| 23 | AAFSI | CIN3 | IVA | 0.1634 | 1 | 0.1634 | ns | Wilcoxon |
| 24 | AAFSI | CIN3 | IVB | 0.317311 | 1 | 0.31731 | ns | Wilcoxon |
| 25 | AAFSI | IB | IIA | 0.882146 | 1 | 0.88215 | ns | Wilcoxon |
| 26 | AAFSI | IB | IIB | 0.628634 | 1 | 0.62863 | ns | Wilcoxon |
| 27 | AAFSI | IB | IIIA | 1 | 1 | 1 | ns | Wilcoxon |
| 28 | AAFSI | IB | IIIB | 0.635093 | 1 | 0.63509 | ns | Wilcoxon |
| 29 | AAFSI | IB | IVA | 0.505192 | 1 | 0.50519 | ns | Wilcoxon |
| 30 | AAFSI | IB | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 31 | AAFSI | IIA | IIB | 0.681955 | 1 | 0.68195 | ns | Wilcoxon |
| 32 | AAFSI | IIA | IIIA | 0.844063 | 1 | 0.84406 | ns | Wilcoxon |
| 33 | AAFSI | IIA | IIIB | 0.836952 | 1 | 0.83695 | ns | Wilcoxon |
| 34 | AAFSI | IIA | IVA | 0.446067 | 1 | 0.44607 | ns | Wilcoxon |
| 35 | AAFSI | IIA | IVB | 0.73007 | 1 | 0.73007 | ns | Wilcoxon |
| 36 | AAFSI | IIB | IIIA | 0.462729 | 1 | 0.46273 | ns | Wilcoxon |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 37 | AAFSI | IIB | IIIB | 0.644452 | 1 | 0.64445 | ns | Wilcoxon |
| 38 | AAFSI | IIB | IVA | 0.700954 | 1 | 0.70095 | ns | Wilcoxon |
| 39 | AAFSI | IIB | IVB | 0.33402 | 1 | 0.33402 | ns | Wilcoxon |
| 40 | AAFSI | IIIA | IIIB | 0.570324 | 1 | 0.57032 | ns | Wilcoxon |
| 41 | AAFSI | IIIA | IVA | 0.302445 | 1 | 0.30245 | ns | Wilcoxon |
| 42 | AAFSI | IIIA | IVB | 0.483323 | 1 | 0.48332 | ns | Wilcoxon |
| 43 | AAFSI | IIIB | IVA | 0.390709 | 1 | 0.39071 | ns | Wilcoxon |
| 44 | AAFSI | IIIB | IVB | 0.422448 | 1 | 0.42245 | ns | Wilcoxon |
| 45 | AAFSI | IVA | IVB | 0.190381 | 1 | 0.19038 | ns | Wilcoxon |

# Annex 7. Pair-wise comparison of mean methylation of CpG sites in HPV16L1 region by cancer stage

| | .y. | group1 | group2 | p | p.adj | p.format | p.signif | method |
|---|---|---|---|---|---|---|---|---|
| 1 | HPV16L1.me | Healthy | IB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 2 | HPV16L1.me | Healthy | IIA | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 3 | HPV16L1.me | Healthy | IIB | 0.014706 | 0.41 | 0.015 | * | Wilcoxon |
| 4 | HPV16L1.me | Healthy | IIIA | 0.071429 | 1 | 0.071 | ns | Wilcoxon |
| 5 | HPV16L1.me | Healthy | IIIB | 0.025641 | 0.67 | 0.026 | * | Wilcoxon |
| 6 | HPV16L1.me | Healthy | IVA | 0.016667 | 0.45 | 0.017 | * | Wilcoxon |
| 7 | HPV16L1.me | Healthy | IVB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 8 | HPV16L1.me | IB | IIA | 1 | 1 | 1 | ns | Wilcoxon |
| 9 | HPV16L1.me | IB | IIB | 0.125 | 1 | 0.125 | ns | Wilcoxon |
| 10 | HPV16L1.me | IB | IIIA | 0.571429 | 1 | 0.571 | ns | Wilcoxon |
| 11 | HPV16L1.me | IB | IIIB | 0.333333 | 1 | 0.333 | ns | Wilcoxon |
| 12 | HPV16L1.me | IB | IVA | 0.266667 | 1 | 0.267 | ns | Wilcoxon |
| 13 | HPV16L1.me | IB | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 14 | HPV16L1.me | IIA | IIB | 0.75 | 1 | 0.75 | ns | Wilcoxon |
| 15 | HPV16L1.me | IIA | IIIA | 0.857143 | 1 | 0.857 | ns | Wilcoxon |
| 16 | HPV16L1.me | IIA | IIIB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 17 | HPV16L1.me | IIA | IVA | 0.933333 | 1 | 0.933 | ns | Wilcoxon |
| 18 | HPV16L1.me | IIA | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 19 | HPV16L1.me | IIB | IIIA | 0.969851 | 1 | 0.97 | ns | Wilcoxon |
| 20 | HPV16L1.me | IIB | IIIB | 0.609783 | 1 | 0.61 | ns | Wilcoxon |
| 21 | HPV16L1.me | IIB | IVA | 0.948623 | 1 | 0.949 | ns | Wilcoxon |
| 22 | HPV16L1.me | IIB | IVB | 0.5 | 1 | 0.5 | ns | Wilcoxon |
| 23 | HPV16L1.me | IIIA | IIIB | 0.732547 | 1 | 0.733 | ns | Wilcoxon |
| 24 | HPV16L1.me | IIIA | IVA | 0.658978 | 1 | 0.659 | ns | Wilcoxon |
| 25 | HPV16L1.me | IIIA | IVB | 0.285714 | 1 | 0.286 | ns | Wilcoxon |
| 26 | HPV16L1.me | IIIB | IVA | 0.767498 | 1 | 0.767 | ns | Wilcoxon |
| 27 | HPV16L1.me | IIIB | IVB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 28 | HPV16L1.me | IVA | IVB | 0.933333 | 1 | 0.933 | ns | Wilcoxon |

# Annex 8. Pair-wise comparison of mean methylation of CpG sites in HPV16L2 region by cancer stage

| | .y. | group1 | group2 | p | p.adj | p.format | p.signif | method |
|---|---|---|---|---|---|---|---|---|
| 1 | HPV16L2.me | Healthy | IB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 2 | HPV16L2.me | Healthy | IIA | 0.133333 | 1 | 0.133 | ns | Wilcoxon |
| 3 | HPV16L2.me | Healthy | IIB | 0.021978 | 0.62 | 0.022 | * | Wilcoxon |
| 4 | HPV16L2.me | Healthy | IIIA | 0.071429 | 1 | 0.071 | ns | Wilcoxon |
| 5 | HPV16L2.me | Healthy | IIIB | 0.021978 | 0.62 | 0.022 | * | Wilcoxon |
| 6 | HPV16L2.me | Healthy | IVA | 0.030303 | 0.79 | 0.03 | * | Wilcoxon |
| 7 | HPV16L2.me | Healthy | IVB | 0.666667 | 1 | 0.667 | ns | Wilcoxon |
| 8 | HPV16L2.me | IB | IIA | 0.8 | 1 | 0.8 | ns | Wilcoxon |
| 9 | HPV16L2.me | IB | IIB | 0.307692 | 1 | 0.308 | ns | Wilcoxon |
| 10 | HPV16L2.me | IB | IIIA | 0.857143 | 1 | 0.857 | ns | Wilcoxon |
| 11 | HPV16L2.me | IB | IIIB | 0.923077 | 1 | 0.923 | ns | Wilcoxon |
| 12 | HPV16L2.me | IB | IVA | 0.545455 | 1 | 0.545 | ns | Wilcoxon |
| 13 | HPV16L2.me | IB | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 14 | HPV16L2.me | IIA | IIB | 0.446154 | 1 | 0.446 | ns | Wilcoxon |
| 15 | HPV16L2.me | IIA | IIIA | 0.47619 | 1 | 0.476 | ns | Wilcoxon |
| 16 | HPV16L2.me | IIA | IIIB | 0.952747 | 1 | 0.953 | ns | Wilcoxon |
| 17 | HPV16L2.me | IIA | IVA | 0.635365 | 1 | 0.635 | ns | Wilcoxon |
| 18 | HPV16L2.me | IIA | IVB | 0.4 | 1 | 0.4 | ns | Wilcoxon |
| 19 | HPV16L2.me | IIB | IIIA | 0.553221 | 1 | 0.553 | ns | Wilcoxon |
| 20 | HPV16L2.me | IIB | IIIB | 0.291343 | 1 | 0.291 | ns | Wilcoxon |
| 21 | HPV16L2.me | IIB | IVA | 0.722343 | 1 | 0.722 | ns | Wilcoxon |
| 22 | HPV16L2.me | IIB | IVB | 0.307692 | 1 | 0.308 | ns | Wilcoxon |
| 23 | HPV16L2.me | IIIA | IIIB | 0.437083 | 1 | 0.437 | ns | Wilcoxon |
| 24 | HPV16L2.me | IIIA | IVA | 0.874875 | 1 | 0.875 | ns | Wilcoxon |
| 25 | HPV16L2.me | IIIA | IVB | 0.571429 | 1 | 0.571 | ns | Wilcoxon |
| 26 | HPV16L2.me | IIIB | IVA | 0.627738 | 1 | 0.628 | ns | Wilcoxon |
| 27 | HPV16L2.me | IIIB | IVB | 0.461538 | 1 | 0.462 | ns | Wilcoxon |
| 28 | HPV16L2.me | IVA | IVB | 0.545455 | 1 | 0.545 | ns | Wilcoxon |

# Annex 9. Pair-wise comparison of methylation level of CpG sites in *EPB41L3* promoter region by cancer stage

| | .y. | group1 | group2 | p | p.adj | p.format | p.signif | method |
|---|---|---|---|---|---|---|---|---|
| 1 | *EPB41L3*.me | Healthy | CIN1 | 0.852758 | 1 | 0.85276 | ns | Wilcoxon |
| 2 | *EPB41L3*.me | Healthy | CIN3 | 0.262105 | 1 | 0.2621 | ns | Wilcoxon |
| 3 | *EPB41L3*.me | Healthy | IB | 0.026929 | 0.89 | 0.02693 | * | Wilcoxon |
| 4 | *EPB41L3*.me | Healthy | IIA | 0.000541 | 0.02 | 0.00054 | *** | Wilcoxon |
| 5 | *EPB41L3*.me | Healthy | IIB | 1.17E-09 | 5.20E-08 | 1.20E-09 | **** | Wilcoxon |
| 6 | *EPB41L3*.me | Healthy | IIIA | 4.09E-05 | 0.0016 | 4.10E-05 | **** | Wilcoxon |
| 7 | *EPB41L3*.me | Healthy | IIIB | 1.01E-09 | 4.50E-08 | 1.00E-09 | **** | Wilcoxon |
| 8 | *EPB41L3*.me | Healthy | IVA | 1.92E-08 | 8.20E-07 | 1.90E-08 | **** | Wilcoxon |
| 9 | *EPB41L3*.me | Healthy | IVB | 0.021518 | 0.73 | 0.02152 | * | Wilcoxon |
| 10 | *EPB41L3*.me | CIN1 | CIN3 | 0.18177 | 1 | 0.18177 | ns | Wilcoxon |
| 11 | *EPB41L3*.me | CIN1 | IB | 0.057753 | 1 | 0.05775 | ns | Wilcoxon |
| 12 | *EPB41L3*.me | CIN1 | IIA | 0.002359 | 0.085 | 0.00236 | ** | Wilcoxon |
| 13 | *EPB41L3*.me | CIN1 | IIB | 8.76E-07 | 3.70E-05 | 8.80E-07 | **** | Wilcoxon |
| 14 | *EPB41L3*.me | CIN1 | IIIA | 0.00035 | 0.013 | 0.00035 | *** | Wilcoxon |
| 15 | *EPB41L3*.me | CIN1 | IIIB | 1.80E-06 | 7.40E-05 | 1.80E-06 | **** | Wilcoxon |
| 16 | *EPB41L3*.me | CIN1 | IVA | 7.22E-06 | 0.00029 | 7.20E-06 | **** | Wilcoxon |
| 17 | *EPB41L3*.me | CIN1 | IVB | 0.038521 | 1 | 0.03852 | * | Wilcoxon |
| 18 | *EPB41L3*.me | CIN3 | IB | 1 | 1 | 1 | ns | Wilcoxon |
| 19 | *EPB41L3*.me | CIN3 | IIA | 0.5 | 1 | 0.5 | ns | Wilcoxon |
| 20 | *EPB41L3*.me | CIN3 | IIB | 0.466667 | 1 | 0.46667 | ns | Wilcoxon |
| 21 | *EPB41L3*.me | CIN3 | IIIA | 0.5 | 1 | 0.5 | ns | Wilcoxon |
| 22 | *EPB41L3*.me | CIN3 | IIIB | 0.463768 | 1 | 0.46377 | ns | Wilcoxon |
| 23 | *EPB41L3*.me | CIN3 | IVA | 0.4 | 1 | 0.4 | ns | Wilcoxon |
| 24 | *EPB41L3*.me | CIN3 | IVB | 0.8 | 1 | 0.8 | ns | Wilcoxon |
| 25 | *EPB41L3*.me | IB | IIA | 0.666667 | 1 | 0.66667 | ns | Wilcoxon |
| 26 | *EPB41L3*.me | IB | IIB | 0.019048 | 0.67 | 0.01905 | * | Wilcoxon |
| 27 | *EPB41L3*.me | IB | IIIA | 0.055556 | 1 | 0.05556 | ns | Wilcoxon |
| 28 | *EPB41L3*.me | IB | IIIB | 0.043478 | 1 | 0.04348 | * | Wilcoxon |
| 29 | *EPB41L3*.me | IB | IVA | 0.114286 | 1 | 0.11429 | ns | Wilcoxon |
| 30 | *EPB41L3*.me | IB | IVB | 0.8 | 1 | 0.8 | ns | Wilcoxon |
| 31 | *EPB41L3*.me | IIA | IIB | 0.305786 | 1 | 0.30579 | ns | Wilcoxon |
| 32 | *EPB41L3*.me | IIA | IIIA | 0.317599 | 1 | 0.3176 | ns | Wilcoxon |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 33 | *EPB41L3*.me | IIA | IIIB | 0.444531 | 1 | 0.44453 | ns | Wilcoxon |
| 34 | *EPB41L3*.me | IIA | IVA | 0.427261 | 1 | 0.42726 | ns | Wilcoxon |
| 35 | *EPB41L3*.me | IIA | IVB | 1 | 1 | 1 | ns | Wilcoxon |
| 36 | *EPB41L3*.me | IIB | IIIA | 0.734719 | 1 | 0.73472 | ns | Wilcoxon |
| 37 | *EPB41L3*.me | IIB | IIIB | 0.414295 | 1 | 0.4143 | ns | Wilcoxon |
| 38 | *EPB41L3*.me | IIB | IVA | 0.629991 | 1 | 0.62999 | ns | Wilcoxon |
| 39 | *EPB41L3*.me | IIB | IVB | 0.523377 | 1 | 0.52338 | ns | Wilcoxon |
| 40 | *EPB41L3*.me | IIIA | IIIB | 0.409052 | 1 | 0.40905 | ns | Wilcoxon |
| 41 | *EPB41L3*.me | IIIA | IVA | 0.53356 | 1 | 0.53356 | ns | Wilcoxon |
| 42 | *EPB41L3*.me | IIIA | IVB | 0.516667 | 1 | 0.51667 | ns | Wilcoxon |
| 43 | *EPB41L3*.me | IIIB | IVA | 0.927902 | 1 | 0.9279 | ns | Wilcoxon |
| 44 | *EPB41L3*.me | IIIB | IVB | 0.663478 | 1 | 0.66348 | ns | Wilcoxon |
| 45 | *EPB41L3*.me | IVA | IVB | 0.463636 | 1 | 0.46364 | ns | Wilcoxon |

By lesion type

| | **.y.** | **group1** | **group2** | **p** | **p.adj** | **p.format** | **p.signif** | **method** |
|---|---|---|---|---|---|---|---|---|
| 1 | *EPB41L3*.me | normal | CIN1 | 0.852758 | 0.85 | 0.85 | ns | Wilcoxon |
| 2 | *EPB41L3*.me | normal | CIN3 | 0.262105 | 0.79 | 0.26 | ns | Wilcoxon |
| 3 | *EPB41L3*.me | normal | Cancer | 1.30E-16 | 7.80E-16 | < 2e-16 | **** | Wilcoxon |
| 4 | *EPB41L3*.me | CIN1 | CIN3 | 0.18177 | 0.73 | 0.18 | ns | Wilcoxon |
| 5 | *EPB41L3*.me | CIN1 | Cancer | 8.27E-09 | 4.10E-08 | 8.30E-09 | **** | Wilcoxon |
| 6 | *EPB41L3*.me | CIN3 | Cancer | 0.369289 | 0.79 | 0.37 | ns | Wilcoxon |