CLINICOPATHOLOGIC CHARACTERISATION OF VULVAR LESIONS AMONG HIV INFECTED WOMEN AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA, ZAMBIA

By

Fred Maate

A dissertation submitted to the University of Zambia in partial fulfilment of the requirements for the degree of Master of Medicine in Pathology

The University of Zambia

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DECLARATION OF ORIGINALITY

I, Dr Fred Maate, hereby declare that the submitted thesis entitled '*CLINICOPATHOLOGIC CHARACTERISATION OF VULVAR LESIONS AMONG HIV INFECTED WOMEN AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA, ZAMBIA*, ***is*** based on my original work except for duly acknowledged quotations and citations. I declare that this dissertation herein presented for the degree of Master of Medicine in Pathology has not been previously submitted either in whole or in part for any other degree at this or any other university, nor being currently submitted for any other degree.

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APPROVAL

This dissertation by Fred Maate has been approved in partial fulfilment of the requirements for the degree of Master of Medicine in Pathology at the University of Zambia.

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ABSTRACT

A wide spectrum of vulvar lesions has been reported. However, few studies have characterized vulvar lesions among HIV infected women. HIV infected women have a higher risk of vulvar neoplasia than those who are not HIV infected. Furthermore, a rise in the incidence of vulvar cancer has been reported particularly among young women attributed to high risk human papillomavirus infection (HR HPV). In Zambia, vulvar lesions including cancer have not been previously characterized. The study set out to describe the clinical and histopathologic features of vulvar lesions and to determine the prevalence of HR HPV among HIV infected women with vulvar neoplasia among HIV infected women at the University Teaching Hospital (UTH) in Lusaka.

The study enrolled 53 HIV infected women with vulvar lesions that were suspected to be cancer. The clinical features of the vulvar lesions were obtained using a questionnaire and focused clinical examination. Tissue for histologic evaluation of the lesions (Haematoxylin and eosin and immunohistochemistry- IHC), and determination of HR HPV (PCR) was collected by punch biopsy and fixed in 10% neutral buffered formalin and ATL buffer respectively. The histologic tissue samples were processed according to the standard protocol at the UTH and reported by two pathologists (including IHC for Ki67 and p53 for the invasive lesions). We analysed 38 samples histologically diagnosed as neoplastic, for HR HPV using X-pert® HPV after extraction of total deoxyribonucleic acid (DNA) using the NucliSens® easyMAG™ magnetic device. The data obtained was analysed using SPSS Version 22.

The mean age of all respondents was 40 years (range 23-63, SD 9.5**)**. The most frequent symptom reported was itching, the most frequent site involved was the labia majora and the most frequently reported lesion type was the warty type. Nine (17%) non- neoplastic lesions, three (5.7%) benign lesions, 20 (37.7%) vulvar intraepithelial lesions (VIN) and 21 (39.6%) invasive lesions were reported. Of the VINs, all were of the classic/ usual type. All the invasive lesions were of the SCC type. Of these, all were of the warty/ basaloid types. The prevalence of any type of HR HPV among all neoplastic lesions (in aggregate) and among invasive lesions was 81.6% and 88.9% respectively.

The HIV infected women with vulvar lesions were young (mean 40 years); the lesions most frequently presented as itchy warty labia majora lesions including nonneoplastic and benign lesions, Classic VIN and Warty/basaloid type SCC. The prevalence of any type of HR HPV among all neoplastic lesions (in aggregate) and among invasive lesions was 81.6% and 88.9% respectively.

Keywords: Vulvar lesions, vulvar intraepithelial neoplasm (VIN), Vulvar squamous cell carcinoma (SCC), Human Papillomavirus (HPV), Human immunodeficiency virus (HIV).

DEDICATION

This dissertation is dedicated to my dear wife, Vennah Beene Ng'andu-Maate, my three children Lubono, Lushomo Simutili, and Luyando Siabazundi Lutombi Maate, and my late parents Fred Friday Maate and Royce Mutinta Hamwanza-Maate.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
Ca	Invasive Cancer
CA	Condyloma Acuminata
CIN	Cervical intraepithelial neoplasia/ neoplasm
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HR	High-Risk
HR-HPV	High-Risk Human Papillomavirus
HSIL	High Grade Squamous Intraepithelial Neoplasm
LR	Low-Risk
LR-HPV	Low-Risk Human Papillomavirus
LSIL	Low Grade Squamous Intraepithelial Neoplasm
MOH	Ministry of Health
PCR	Polymerase Chain Reaction
SCC	Squamous Cell Carcinoma
SOM	School of Medicine
STI	Sexually Transmitted Infection
UNZA	University of Zambia
UJMT	University of North Carolina, John Hopkins University, Morehouse School of Medicine and Tulane University
UTH	University Teaching Hospital
VIN	Vulvar Intraepithelial Neoplasia
VIN 1	Vulvar Intraepithelial Neoplasia 1
VIN 2	Vulvar Intraepithelial Neoplasia 2
VIN 3	Vulvar Intraepithelial Neoplasia 3
SCC	Vulvar Squamous Cell Carcinoma
ZDHS	Zambia Demographic Health Survey
ZAMPHIA	Zambia Population Based Health Impact assessment

CHAPTER I

INTRODUCTION

1.1. Spectrum of vulvar lesions

A varied spectrum of lesions occurs in the vulva. They are classified as neoplastic or nonneoplastic (Ellenson and Pirog, 2015)(Arora *et al.*, 2014). The neoplastic lesions are classified further as benign, premalignant or malignant. In the study conducted by Arora *et al.* (2014) the most common nonneoplastic lesion was lichen *sclerosus et atrophicus*; the most common benign neoplastic lesion was *fibroepithelial* polyp; the only vulvar premalignant lesion was vulvar intraepithelial neoplasm (VIN); and among the malignant lesions, vulvar squamous cell carcinoma (SCC) was the only histologic type. As reported by others SCC is the most common histologic type of vulvar cancer. Other common malignant lesions include melanoma, sarcoma and basal cell carcinoma among others (Crum *et al.*, 2014).

1.2. Vulvar intraepithelial neoplasia

Vulvar intraepithelial neoplasia (VIN) are precursor lesions of invasive SCC, and they are of two types-differentiated VIN (also known as VIN simplex) and classical or usual VIN. Differentiated VIN occurs in older women while Classic VIN occurs in younger women; the latter is caused by HPV while the former is not. Classic VIN is also known as squamous intraepithelial lesion (SIL); there are two grades of Classic VIN: Low-grade SIL (LSIL) and high-grade SIL (HSIL) (Alkatout *et al.*, 2015; Allbritton, 2017).

1.3. Vulvar Cancer

Vulvar cancer is rare. It was reported to be one eighth as common as carcinoma of the cervix and accounted for four percent of gynaecologic cancers (Pirog, 2011). One study estimated that in the year 2018, there would be 44, 235 (0.2 percent) cases of and 15,222 (0.2 percent) deaths due to vulvar cancer in 185 countries in

contrast to 569,847 (3.2 percent) cases and 311, 365 (3.3 percent) deaths due to cancer of the cervix uteri. The age-standardized rates for vulvar and cervical cancer were 0.9 and 13.1, respectively (Bray *et al.*, 2018). In Zambia, a recent retrospective observational study nested on Zambia National Cancer Registry (ZNCR) histopathological and clinical data from 2007 to 2014, found that the prevalence of vulva/ vaginal cancer between 2007 and 2014 was 3.2/ 100,000 population (n= 21,512) representing 1.1 percent of all cancers. The standardized incidence rate (SIR) was reported to be 5.94 (CI 5.14-6,74) (Kalubula *et al.*, 2018).

SCC accounts for 95 percent of malignant tumourss of the vulva (Alkatout *et al.*, 2015; Crum *et al.*, 2014) and it is more common among elderly women above 60 years (Pirog, 2011); however, over the last two decades, a rise in its incidence has been observed particularly among young women attributed to infection with human papillomavirus (Rumbold *et al.*, 2012). There are two *etiologic-pathologic* forms of SCC-one that is associated with high-risk HPV (HR HPV) infection and another that is not. The HPV associated form is usually of the warty or basaloid type whereas the non-HR HPV associated form is usually of the Keratinizing type (Pino, Rodriguez-carunchio and Ordi, 2013). HR HPV associated SCC arises from vulvar intraepithelial neoplasia (VIN) of the usual/ classic type while the non-HR HPV associated vulvar carcinoma arises from the differentiated type of VIN and is associated with chronic inflammatory disorders such as *lichen sclerosus* and squamous cell hyperplasia (Pino, Rodriguez-carunchio and Ordi, 2013).

1.4. Vulvar neoplasia, HPV and HIV in Zambia

Studies have shown that HIV infected women have a higher risk of vulvar neoplasms than those who are not (Palmer, Vatish and Tidy, 2010; Reusser *et al.*, 2015). Other reported risk factors for vulvar cancer include smoking and HR HPV infection (Madeleine *et al.*, 1997). Zambia has a high HIV prevalence rate (15 per cent) among women aged 15 to 49 years (MOH, CSO, and ICF, 2014). Also, Kalubula et al. (2018) found that 23 percent of women with vulvar cancer were HIV infected. Therefore, HIV may be a significant factor in influencing the incidence of vulvar cancer in Zambia.

The burden of HR HPV related disease such as cervical cancer among HIV infected people in low to medium income countries (LMIC) is reported to be high (Patel *et al.*, 2018). Further, rates of HR HPV among African countries including Zambia are reported to be higher than in other regions of the world (Ogembo *et al.*, 2015). Therefore, the rate of HR HPV related vulvar neoplasms is expected to be high among HIV infected women in Zambia.

Studies demonstrate that vulvar lesions, particularly vulvar cancer, vary epidemiologically from country to country (Butt and Botha, 2017); Furthermore, few studies have characterized vulvar lesions among HIV infected women. In Zambia, there is no evidence in the literature on the characterization of vulvar lesions among HIV infected women.

This study sought to characterize vulvar lesions among HIV infected women that were clinically suspicious for malignancy at the University Teaching Hospital with a view to stimulate future multi-centre studies that could be used to design prevention strategies and treatment algorithms among HIV infected women in Zambia.

1.5. Statement of the Problem

Vulvar lesions including invasive cancer among HIV infected women have not been previously characterized in Zambia where the HIV prevalence rate among women of reproductive age and the burden of HPV related cervical cancer are high.

1.6. Justification of the Study

In view of the fact that there is variation in the distribution of vulvar lesions, particularly vulvar carcinoma, in different countries, it is essential to characterize them in Zambia. The results of this study may stimulate future population-based multicentre research, which may help to inform national policy regarding preventive measures for vulvar cancer such as HPV vaccination and *vulvar cancer* clinical care algorithms of HIV-infected women.

CHAPTER II

LITERATURE REVIEW

2.1 Clinical Features of Vulvar Lesions

2.1.1 Anatomy of the vulva

The vulva is the external female genitalia composed of the *mons pubis, clitoris, labia minora, labia majora, vulvar vestibule,* and *vestibulo-vaginal bulbs, urethral meatus, hymen, Bartholin* and *Skene glands* and *ducts*, and *vaginal introitus* (Blake, 2018). *The boundaries include the mons pubis anteriorly, the rectum posteriorly, and the genitocrural folds (thigh folds) laterally* (Thompson, 2019).

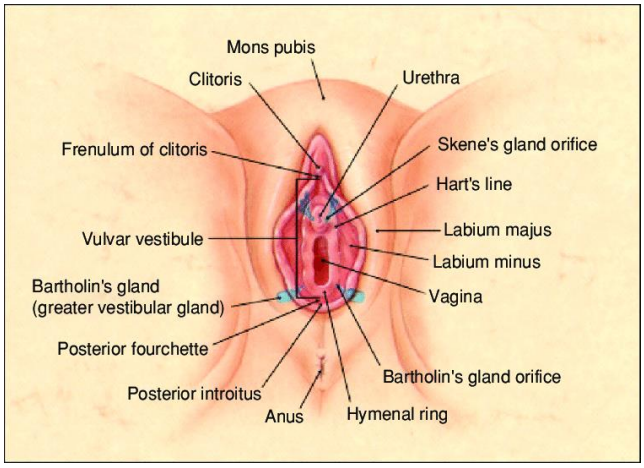

Figure 1: Anatomy of the vulva. Adapted from Research Gate-(Pukall, Smith and Chamberlain, 2009)

According to Thompson (2019) the functions of the vulva include the following:

1. *To act as sensory tissue during sexual intercourse*
2. *To assist in micturition, by directing the flow of urine*
3. *To defend the internal female reproductive tract from infection.*

Thompson described the components of the vulva as follows (Thompson, 2019):

i) **Mons pubis:** A fat pad at the anterior of the vulva, covered in pubic hair.

ii) **Labia majora:** Two hair-bearing outer folds embryologically derived from labio-scrotal swellings. They fuse posteriorly and extend anteriorly to the mons pubis.

iii) **Labia minora:** These are two hairless folds of skin embryologically derived from the urethral folds. They lie within the labia majora. They fuse anteriorly to form the prepuce (hood) of the clitoris and extend posteriorly on either side of the vaginal opening. They fuse again posterior to the vestibule, creating a fold of skin called the fourchette.

iv) **Vestibule:** This is the area between and around the labia. The external vaginal orifice (vaginal opening) and urethra open into the vestibule.

v) **Bartholin's Glands:** The Bartholin's glands are located on either side of the vaginal orifice. They secrete lubricating mucus from small ducts during sexual arousal.

vi) **Clitoris:** Located under the prepuce and embryologically derived from the genital tubercle. The clitoris is formed of erectile corpora cavernosa tissue, which becomes engorged with blood during sexual stimulation.

The innervation of the vulvar skin is mainly from the *ilioinguinal nerve* and the genital branch of the *genitofemoral* nerve. Lymphatic drainage is to the superficial inguinal nodes. Arterial blood supply is from the pudendal artery, and venous drainage is through the pudendal vein (Miranda, 2018; Thompson, 2019).

2.1.2 Clinical features of nonneoplastic vulvar lesions

The vulva is essentially skin, and most of the lesions that affect it are akin to those that affect the skin elsewhere on the body (University of Pennsylvania, 2019). Some of the nonneoplastic lesions of the vulva as mentioned by Clement and Young (2014) include viral infections, other types of infections (including syphilis), nonneoplastic epithelial disorders (including ***lichen sclerosus*** and ***squamous cell hyperplasia*** among others) and non-infectious inflammatory lesions, reactive lesions, and so-called other nonneoplastic lesions.

Viral infections of the vulva include human papillomavirus and herpes simplex (Clement and Young, 2014). The low oncogenic risk HPVs are the cause of condyloma acuminatum (described under neoplastic vulvar lesions). ***Herpes simplex*** infection clinically presents with vulvar pain, inguinal lymphadenopathy, malaise, fever, vesicles, pustules, and then painful ulcers appear sequentially. Of note, rare cases of chronic hypertrophic ***herpetic vulvitis*** may clinically simulate a neoplasm or harbour an invasive SCC. However, for most cases of herpetic vulvitis, the lesions persist for two to six weeks and regress spontaneously (Clement and Young, 2014).

Syphilitic lesions present as primary phase lesions characterized by superficial ulcers called chancre, and secondary phase lesions characterized by mucocutaneous rash and papules (condylomata lata) (Clement and Young, 2014).

Lichen sclerosus and *squamous cell hyperplasia* are two nonneoplastic disorders which are pathologically crucial due to their association with non-HPV related types of neoplastic lesions (differentiated VIN and Keratinizing type of SCC). These are not precursor lesions (Pino, Rodriguez-carunchio and Ordi, 2013). According to Clement and Young (2014), Lichen sclerosus can be asymptomatic but often causes *pruritus*, a burning sensation, and *dyspareunia*. They also present with irregular white patches on any portion of the vulva, and squamous cell hyperplasia presents with a white plaque-like thickening of the involved skin.

2.1.3 Clinical features of benign neoplastic vulvar lesions

According to the 2014 *World Health Organization Classification Of Tumours of The Vulva, benign lesions of the vulva include* ***benign squamous lesions*** *(including Condyloma acuminatum, seborrheic keratosis, keratoacanthoma, and vestibular papilloma),* ***benign glandular lesions*** *(papillary hidradenoma, mixed tumour, fibroadenoma, adenoma, adenofibroma and Bartholin gland cysts, among others),* ***benign soft tissue tumours*** *(such as lipoma, fibroepithelial stromal polyp,*

superficial angiomyxoma, aggressive angiomyxoma, and leiomyoma among others) and ***melanocytic lesions*** *(Crum et al., 2014).*

Condyloma acuminatum (CA, also known as *anogenital* warts) is a benign squamous lesion that is reported to be common in Africa (Chikandiwa *et al.*, 2018). CA is known to be caused by low *oncogenic* risk HPV types 6 and 11 and HPV infection is the most frequent sexually transmitted infection (Chikandiwa *et al.*, 2018). The prevalence of *anogenital* warts among HIV infected persons is said to be higher compared to those who are HIV negative (Low *et al.*, 2011). For this reason, among the benign lesions of the vulva, only the clinical presentation of *anogenital* warts is reviewed.

Because CA is a result of sexually transmitted infection, its prevalence peaks in the early sexually active years and patients complain of painless bumps and, less frequently, of *pruritus*, discharge, or bleeding (Léonard *et al.*, 2014).

According to Leonard *et al.* (2014):
"CA are soft, raised masses, with smooth, *verrucous*, or lobulated aspects that may appear as pearly, *filiform*, fungating, or plaque-like eruptions. The surface commonly shows finger-like projections, generally non- pigmented. They mainly occur in the moist areas of the labia *minora* and vaginal opening, but virtually, all genital regions may be affected (*fourchette*, labia *minora/majora*, pubis, clitoris, urethral *meatus*, perineum, perianal region, anal canal, introitus, vagina, and ectocervix)."

2.1.4 Clinical features of squamous intraepithelial lesions

Squamous intraepithelial lesions of the vulva include *low grade squamous intraepithelial lesion, high grade squamous intraepithelial lesion and differentiated-type vulvar intraepithelial neoplasia* (Crum *et al.*, 2014). In terms of aetiology these are classified into two categories:

1. HPV related squamous intraepithelial lesions so-called usual or classic vulvar intraepithelial neoplasm (VIN) and

2. Non-HPV related squamous intraepithelial lesions also called differentiated VIN (d VIN or VIN simplex).

In a manner analogous to the cervical intraepithelial neoplasm (CIN), there are two grades of Classic VIN: *Low grade squamous intraepithelial lesion (LSIL)* and *high grade squamous intraepithelial lesion (HSIL)*. LSIL is a squamous intraepithelial lesion characterized by dysplasia of the lower one-third of the squamous epithelium and HSIL is characterized by dysplasia involving the lower two-thirds of the squamous epithelium (VIN 2) or involves greater than two-thirds of the epithelium (VIN 3). On the other hand, differentiated VIN is considered to be equivalent to VIN 3 (Léonard *et al.,* 2014; Reich *et al.,* 2015).

LSIL by definition denotes a low-risk of concurrent or future invasive cancer. Synonyms include VIN 1 of usual type, mild squamous dysplasia, flat condyloma, koilocytic atypia, and koilocytosis; On the other hand, HSIL carries a high-risk of progression to cancer (Crum *et al.*, 2014). Differentiated VIN has a higher risk of progression to invasive cancer than the classic HSIL (Blake, 2018).

Classic VIN can either be warty, basaloid or have both warty and basaloid features. The warty type is verruciform; hence its clinical features are similar to those of condyloma acuminatum (Crum *et al.,* 2014). Generally, classic VIN is reported to occur in young women with a peak in incidence at 45–49 years of age (Léonard *et al.*, 2014). This view is supported by Blake (2014) who reports that vulvar cancer incidence increases in premenopausal women. Both Blake (2014) and Crum *et al.* (2014) report that classic VIN is associated with HPV. Smoking and immunosuppression are risk factors. Further, these lesions are frequently multifocal (Blake, 2018; Crum *et al.,* 2014 and Pino. 2013). According to Pino (2013), the clinical presentation of Classic VIN can be diverse but usually presents as a distinct lesion with sharp margins which can be flat, raised or ulcerated.

On the other hand, differentiated VIN affected older women, was unifocal and unicentric, was not associated with HPV infection but was associated with vulvar dermatoses mainly lichen sclerosus and had the potential to progress to invasive carcinoma (Léonard *et al.*, 2014).

Vulvar intraepithelial neoplasia (VIN) were asymptomatic in 50% of cases. If symptomatic, the main symptoms included itching, pruritus, pain and dyspareunia with varied clinical appearance from unifocal to multifocal lesions. Therefore, complete gynaecological evaluation was necessary to exclude multicentric lesions that affected the cervix, vagina and the anal region. Diagnosis of VIN could be subtle, and hence to avoid delay the "physician must exercise a high degree of suspicion, and vulvar biopsy should be used liberally. Furthermore, it is impossible to rule out cancer by clinical examination of a vulvar lesion. Therefore, histopathology is the gold standard of diagnosis" (Léonard *et al.*, 2014).

2.1.5 Clinical features of invasive carcinoma

a. Epidemiology of vulvar cancer

Carcinoma of the vulva is rare with an estimated age-standardised (world standard) incidence rate up to 1.5 women per 100,000 women per year (Buttmann-schweiger *et al.*, 2015); and it accounts for four percent of all female genital cancers. It is the fourth most common gynaecologic malignancy and it occurs mainly in women aged over 60 years. Squamous cell carcinoma is the most common type (86 percent) of vulvar cancer (Prat and Mutch, 2018). In the United States, vulvar cancer accounts for approximately six percent of cancers of the female genital tract and 0.7 percent of all cancers in women. Women have a 1 in 333 chance of developing vulvar cancer at some point during their life (American Cancer Society., 2019).

According to a recent retrospective observational study nested on Zambia National Cancer Registry (ZNCR) histopathological and clinical data from 2007 to 2014, Kalubula *et al.*, (2018) found that the prevalence of vulva and vaginal cancer was 3.2/ 100,000 population (n= 21,512) representing 1.1 percent of all cancers. The standardized incidence rate (SIR) was reported to be 5.94 (CI 5.14- 6.74). These findings are higher than that found in other African countries such as Uganda, Zimbabwe, and Malawi where age-standardized incidence rates are 0.6, 1.1, and 1.0 per 100,000 women per year, respectively (Kroeber *et al.*, 2018).

According to Kalubula *et al.* (2018), the age-specific incidence rate per 100,000 was reported to be highest in the age group 40 to 49 (1.61) followed by 60 to 69 (1.23) and 50 to 59 (1.22) suggesting that in Zambia, vulvar cancer was more common among younger women, compared to other countries such as Tunisia where vulvar cancer is reported to be more common among older women. The median age of women with vulvar cancer in Tunisia was reported to be 65.4 years and 86.9 per cent of the patients were older than 55 years (Kehila *et al.*, 2017).

According to the study by Kalubula *et al.*, (2018), 24 percent(n= 246) patients with vulvar/ vaginal cancer were HIV infected. This finding is similar to findings in South Africa where 23.7 percent(n=169) of patients with vulvar cancer were HIV infected (Butt and Botha, 2017). However, these findings were much lower than in a study from Burkina Faso where HIV infected vulvar cancer patients amounted to 28.6 percent(n= 21) (Zongo *et al.*, 2016).

The African epidemiologic studies seem to correlate with an American study which demonstrated that there was a difference in the distribution of vulvar disease between white and African-Americans. African-Americans with vulvar cancer were relatively younger (mean 57 years) than white Americans (mean 67 years) (Rauh-Hain *et al.*, 2013).

b. Symptoms and signs of vulvar cancer

According to Crum *et al.* (2014), "vulvar cancer can present as an ulcer, nodule, macule or *pedunculated* mass. In more advanced cases symptoms include discharge, bleeding, and pain. Odour or self-palpation of a mass may bring the patient to the physician." Zongo *et al.*, (2016) in their case series study in Burkina Faso, found that most of the patients presented late and pain and ulceration were the main reasons for consultation. These reports are in agreement with Alkatout *et al.*, (2015) who reported that pruritus was the most common and long-lasting reported symptom of vulvar cancer, followed by vulvar bleeding, discharge, dysuria, and pain. Hacker (2004), rendered the following description for the clinical features of vulvar cancer similar to Nagarajan, (2019)

“The most frequently reported symptom of vulvar cancer is a long history of pruritus. Less common presenting symptoms include vulvar bleeding, discharge, dysuria, and pain. The most common presenting sign of vulvar cancer is a vulvar lump or mass. Rarely, patients present with a large, fungating mass. On physical examination, the vulvar lesion is usually raised and may be fleshy, ulcerated, leukoplakic, or warty in appearance. Most squamous cell carcinomas are unifocal and occur on the labia majora. Approximately 5 percent of cases are multifocal, and the labia minora, clitoris, or perineum may be involved as primary sites. It is also reported that vulvar cancer can also present with a warty tumour, a long-standing ulcer, groin mass, a plaque or rarely as an erythematous rash. It may be asymptomatic, painful or itchy, associated with burning, soreness, bleeding or discharge.”

2.2. Histopathologic Features of Vulvar Lesions

2.2.1. Histopathologic features of selected nonneoplastic vulvar lesion- Herpes simplex infection

Herpes simplex virus (HSV) is the most common cause of genital ulceration. It presents with multiple small papules and macules followed by vesicles that rupture and cause painful ulceration. The histologic hallmarks are of multinucleated giant cells with ground glass nuclei due to the intranuclear virus. These viral cytopathic effects are prominent at the junction of the ulcerated and non-ulcerated areas. Intraepithelial vesicles contain rounded acantholytic keratinocytes. The keratinocytes show viral cytopathic changes of ground glass nuclei, nuclear moulding and multinucleated giant epithelial cells (Chaux, Alcides, and Cubilla, 2019).

2.2.2. Histopathologic features of benign neoplastic vulvar lesions: condyloma acuminatum

a. **Gross Findings:** Condyloma acuminatum presents as an exophytic mass that can range from discrete papillary excrescences to extensive and coalescent "cauliflower-like" masses (Léonard *et al.,* 2014).

Microscopic Findings: Characteristic histologic features of condyloma acuminatum include papillae lined by acanthotic squamous epithelium with fibrovascular cores. Other features include hyperkeratosis, parakeratosis and hypergranulosis (Clement and Young, 2014).

2.2.3. Histopathological features of vulvar intraepithelial neoplasia

a. Classification of vulvar intraepithelial neoplasia:

There are two forms of vulvar intraepithelial neoplasia (VIN): Classic or usual type VIN is caused by HR HPV. It is also known as Squamous intraepithelial lesion (SIL). There are two grades of SIL, that is, Low-grade SIL (LSIL) which corresponds to low-grade VIN (VIN- 1) and high grade SIL (HSIL) which corresponds to VIN-2 or VIN-3 analogous to CIN-2 and CIN-3 of the cervix (Léonard *et al.*, 2014; Reich *et al.*, 2015; Crum *et al.*, 2014).

VIN of the classic type has traditionally been defined as follows Vulvar intraepithelial neoplasia grade 1 (VIN 1) if the dysplastic cells involve the lower third of the epithelium; VIN 2 (moderate grade dysplasia) when the dysplastic cells are present in the lower two-thirds of the epithelium; and VIN 3 (high-grade dysplasia) if there is greater than two-thirds involvement of the epithelium by the dysplastic cells (Leonard et al., 2014). LSIL implies a low-risk of concurrent and future cancer. Synonyms for LSIL include VIN 1 of the usual type, mild squamous dysplasia, flat *condyloma, koilocytic atypia, and koilocytosis.* On the other hand, HSIL carries a significant clinical risk of invasive cancer development if not treated. Synonyms for HSIL include VIN 2 and VIN 3 of the usual type, moderate squamous dysplasia, carcinoma in situ, Bowen disease and Bowenoid dysplasia (Crum *et al.*, 2014).

Another form of the vulvar squamous intraepithelial lesion is the so-called differentiated VIN or VIN simplex. This form is not associated with HPV. It is equivalent to VIN 3 due to its associated high-risk to progress into invasive SCC.

VIN simplex is said to be frequently associated with lesions such as lichen *sclerosus* lichen simplex *chronicus*

b. Gross findings of vulvar intraepithelial neoplasia:

According to Léonard *et al.*, (2014) in their review article**, LSIL(Low-grade VIN, VIN1)** appears as pale areas, whereas HSIL (High-grade VIN, VIN 2/ VIN 3) lesions appear as white or erythematous papules or macules that frequently coalesce or show a *verrucous* growth. Approximately 10-15 percent of the lesions are hyper-pigmented. Approximately two-thirds of VIN lesions are multifocal.

c. Microscopic Findings of Vulvar intraepithelial neoplasia:

i) **Usual/Classic VIN (UVIN):** Usual or classic VIN is characterized by features similar to those seen in other HPV- associated intraepithelial lesions such as cervical intraepithelial neoplasia (CIN). These pre-neoplastic lesions are characterized by acanthosis, hyperkeratosis and parakeratosis. The epithelium is characterized by dysplastic squamous cells with increased nuclear to cytoplasmic rations, nuclear pleomorphism and hyperchromasia. Usual VIN is divided into warty ("condylomatous") and basaloid types**,** mainly based on the architecture and appearance of the intraepithelial lesions (Clement and Young, 2014; Léonard *et al.*, 2014).

The warty type shows a papillary pattern, acanthosis, with cytological signs of viral infection (koilocytic changes, multinucleation, and coarse granules). The basaloid type presents as a flat-surfaced lesion composed of a homogeneous population of small atypical parabasal type cells on nearly the whole thickness of the epidermis. The epithelium lacks cellular maturation and koilocytic changes which are rarely seen. (Léonard *et al.*, 2014).

ii) Differentiated VIN (DVIN):

Differentiated VIN is characterised by among others, the following features: acanthotic epithelium demonstrating atypia of the basal layer with a high degree of epithelial maturation and differentiation in the superficial layers, keratin pearls within rete ridges, inter-anastomosis of the rete ridges (Leonard et al., 2014).

2.2.4. Histopathologic features of invasive cancer

a. Classification of vulvar cancer:

Squamous cell carcinoma (SCC) accounts for 95 percent of all forms of vulvar cancer. There are two types of SCC: Keratinizing squamous cell carcinomas and basaloid and warty types. Keratinizing squamous cell carcinoma comprises 70 percent of vulvar SCC, is not associated with Human Papillomavirus (HPV) infection and is said to arise from precursor lesions, i.e., differentiated vulvar intraepithelial lesion (differentiated VIN or VIN simplex). VIN simplex is reported to arise from longstanding lichen sclerosus (chronic atrophic vulvitis) or vulvar epithelial hyperplasia (Crum *et al.*, 2014).

Basaloid and warty types of vulvar carcinoma comprise 30 percent of cases of vulvar SCC, are associated with high-risk HPV (HR HPV) infection and arise from precursor lesions: usual or classic vulvar intraepithelial lesion (Classic VIN) (Crum *et al.*, 2014).

The College of American Pathologists (2018) summarized the two etiologic types of vulvar cancer as shown in Table 1:

Table 1: Etiopathologic classification of SCC

	Keratinizing Squamous Carcinoma	**Basaloid and warty Squamous carcinoma**
Prevalence	More common (approximately 80%)	Less common (approximately 20%)
Age	Older females	Younger females
Distribution	Usually unifocal; may be multifocal	Often multifocal
Association with multifocal lower genital tract neoplasia	Rare	Common
Morphology	Keratinizing	Warty & basaloid
Associated vulvar intraepithelial neoplasia (VIN)	Uncommon: differentiated type	Common: classic type
Association with high-risk human papillomavirus (HPV)	No	Yes Type 16>18
Association with vulvar dystrophy	Common	Rare
Immunohistochemistry	p53: Some cases positive p16: Negative or focally positive at stromal interface	p53: Negative p16: Positive

Table 1: Histologic forms of vulvar cancer

b. Etiopathogenesis of vulvar squamous cell carcinoma:

High-risk types of human papillomaviruses express oncogenic proteins that inactivate tumour suppressors, activate cyclins, inhibit apoptosis, and combat cellular senescence, steps which lead to lack of inhibition in cell proliferation, induction of cell cycle progression and lack of death of non-lethally injured cells. The viral proteins, E6 and E7, play a vital role in the malignant transformation. HPV DNA integration into the host genome

results in interruption of the viral DNA within the E1/E2 open reading frame, leading to loss of the E2 viral repressor and overexpression of the oncoproteins E6 and E7 (Ellenson and Pirog, 2015; Blake, 2018)

Overexpressed E6 binds to and mediates the degradation of the tumour suppressor protein p53 and also stimulates the expression of TERT, the catalytic subunit of telomerase thereby immortalising the infected cell (Ellenson and Pirog, 2015).

E7 binds to the retinoblastoma (RB) protein and displaces the E2F transcription factors that are usually sequestered by RB, thereby promoting progression of the cell through the cell cycle and also in overexpression of cyclin-dependent kinase inhibitor (CDKI) p16 (CDKN2A), a vital CDKI for the G1/S phase checkpoint of the cell cycle. Normally, RB inhibits transcription of p16/ INK4a gene (Ellenson and Pirog, 2015). The overexpression of p16, therefore, can be used as a surrogate marker for increased expression of viral oncogenes in dysplastic and cancerous cells (Ellweg, Chmidt, and Oeberitz, 2001).

By definition, HR HPVs are those which are associated with cervical and anogenital cancers (including vulvar cancer) (Léonard et al., 2014). The International Agency for Research on Cancer (IARC) in 2005 defined 13 genotypes of HPV as high-risk/ carcinogenic: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (International Agency for Research on Cancer, 2007). Other forms not related to cancer are said to be low-risk (LR HPV). HPV 6 and 11 are examples and these are known to cause benign exophytic lesion such as condyloma acuminatum (Ellenson and Pirog, 2015).

Infection with HR HPV alone is not sufficient for carcinogenesis. Genetic and environmental cofactors influence full malignant transformation. (Ellenson and Pirog, 2015). HR HPV environmental cofactors for carcinogenesis include smoking, immunosuppression, and long-term use of oral contraceptives (Léonard et al., 2014).

In contrast to the HPV dependent pathway, the HPV-independent pathway of vulvar SCC and DVIN reportedly is not well known (Léonard et al., 2014). Chronic epithelial irritation in lichen sclerosus or squamous cell hyperplasia may contribute to gradual transformation to a malignancy (Ellenson and Pirog, 2015). Frequently detected genetic mutations in DVIN and SCC including mutations of TP53 or PTEN and epigenetic alterations may contribute to vulvar carcinogenesis (Ellenson and Pirog, 2015).

c. Gross Findings of SCC:

Grossly, vulvar cancer will appear as a warty tumour, an ulcer, a plaque or rarely an erythematous rash (Nagarajan, 2019). On the other hand, according to Ellenson and Pirog, (2015) SCC presents as an exophytic or endophytic ulcerated lesion which may involve the labia majora and minora. The majority of the lesions are solitary while only ten percent are multifocal.

d. Histological Findings of vulvar SCC:

According to the World Health Organization, the histologic forms of SCC include Keratinizing, non-Keratinizing, basaloid, warty, and verrucous (Crum *et al.*, 2014).

i) ***Keratinizing SCC:*** Keratinizing squamous cell carcinomas are variably mature with keratin pearls, and the immature keratinocytes are strongly p53 positive. Most of these neoplasms tend to be well differentiated, and the surface often shows minimal cytologic atypia even when deeply invasive. As a result, superficial biopsies may not be diagnostic of invasive carcinoma. Although these tumours may be associated with HPV, they are more often HPV and p16 negative and differentiated type VIN may be present in the adjacent surface epithelium. Lichen sclerosus and lichen planus may also be present (Crum *et al.*, 2014). keratinizing squamous cell carcinoma is said to be the most common histological subtype of SCC and occurs most frequently in women older than 65 years. The tumour cells have abundant eosinophilic cytoplasm with low nuclear to cytoplasmic ratio (Nagarajan, 2019; Léonard *et al.*, 2014)in addition to prominent central

keratin pearls). The nuclei are atypical and enlarged, and have prominent nucleoli.

ii) ***Non-keratinizing SCC*:** Non-keratinizing SCC is not associated with HPV and is characterized by little or no keratinization and a total lack of keratin pearl (Léonard *et al.*, 2014).

iii) ***Basaloid SCC:*** Basaloid SCC is associated with HPV. It lacks keratin pearls and is composed of cohesive nests of immature basaloid type squamous cells that resemble HSIL (VIN 2/3) (Crum *et al.*, 2014).The cells are ovoid, relatively uniform in size, and the nuclei show evenly distributed granular chromatin with no evident nucleoli. (Léonard *et al.*, 2014).

iv) ***Warty (condylomatous) SCC:*** Warty SCC is associated with HPV. It is characterised by papillary architecture, and rare keratin pearls in invasive tumour nests and superficial HPV associated koilocytic atypia (Crum *et al.*, 2014; Léonard *et al.*, 2014).

v) ***Verrucous SCC (Verrucous carcinoma):*** Verrucous carcinoma is associated with low-risk HPV types 6 and 11. Clinically, it is warty. Histologically, it is highly differentiated, variably keratinised and invades in the form of bulbous pegs with a pushing border (Crum *et al.*, 2014).

There are pitfalls regarding differentiation between keratinizing type and the basaloid and warty types of SCC. Sometimes there is an overlap of the features of the keratinizing SCC with those of the HPV driven SCC types. In such cases, invasive tumour architecture and nuclear features, as well as the type of VIN in the epithelium can be utilized to define the type of SCC (Pirog, 2011) (Table 2).

Table 2: Histologic features for differentiating between keratinizing and warty/basaloid SCC

	SCC Types	
	Keratinizing type	Basaloid and warty types
Adjacent in situ lesion	Differentiated VIN	Usual VIN
Nuclear features	Pale, vesicular chromatin and prominent nucleolus	Dark course granular chromatin and inconspicuous nucleoli

Adapted table (Pirog, 2011).

It is crucial to accurately diagnose the type of SCC as the overall risk for recurrence is higher in keratinizing carcinoma (Pirog, 2011).

e. Staging and treatment of vulvar cancer

The most widely used staging system is the *International Federation of Gynaecology and Obstetrics (FIGO)* staging system; the latest version is the 2018 version. Also used is that of the American Joint Committee (AJCC 8th Edition) on cancer staging's Tumour, nodes and metastases (TNM) system. Tumour size and number of lymph nodes are important in appropriately staging of the tumour. Staging is done on excision specimens. Both versions are highlighted in appendix 8.

Early-stage vulvar carcinoma is treated by wide local excision. The procedure involves radical/ modified radical vulvectomy en bloc with bilateral inguinofemoral lymphadenectomy or at least sentinel node sampling. Pelvic exenteration is done only in locally advanced disease. Primary radiotherapy is used for unresectable disease. Adjuvant radiation therapy is used for high-risk primary tumours (tumour size greater than four cm, lymph vascular invasion, close or positive resection margins and metastasis to lymph nodes). Primary chemotherapy is administered for unresectable tumours to avoid significant postoperative morbidity. (Nagarajan, 2019).

2.3. Prevalence of Human papillomavirus among vulvar lesions

HPV is known to be the most frequent sexually transmitted infection. It is known to cause both benign and malignant vulvar lesions (Reusser *et al.,* 2015; Nelson and Stockdale, 2013). Low oncogenic risk HPVs such as HPV 6 and 11 are known to cause condyloma acuminatum and are also associated with verrucous carcinoma. Indeed, verrucous carcinoma is known to mimic condyloma acuminatum clinically (Allbritton, 2017). While studies have been done relating to the prevalence of HPV in vulvar neoplasia in many countries, no such studies have been done in Zambia.

2.3.1. Prevalence of HPV in VIN

Studies have shown that the prevalence of HPV in VIN is generally high. For example, one study found that the global prevalence of VIN was 86.7 percent (N= 2296) (De Sanjosé *et al.,* 2013) . Based on systematic literature search of PubMed, Embase, and Cochrane Library databases another study found that HPV prevalence was 76.5 (Faber *et al.,* 2017)

2.3.2. Prevalence of HPV in vulvar cancer

De Sanjosé *et al.* (2013) in a global study (N= 2296) reported that HPV prevalence in invasive cancer (IVC) was 28.6 percent; 25.1 percent were both HPV-DNA and p16INK4a positive. In this study, the cases of IVC cases were largely Keratinizing squamous cell carcinoma (KSCC) (N = 1234). The overall prevalence of HPV related IVC cases was highest in younger women for any histological subtype. SCC with warty or basaloid features (SCC-WB) (N = 326) were more likely to be HPV and p16 INK4a positive (AP = 69.5%, CI = 63.6-74.8) versus KSCC (AP = 11.5%, CI = 9.7-13.5). HPV 16 was the commonest type (72.5%) followed by HPV 33 (6.5%) and HPV 18 (4.6%). The view of De Sanjose et al. was that the prevalence of HPV in vulvar cancer had been overestimated in previous studies and therefore they suggested the use of both HPV DNA detection and p16INK4a in determining the prevalence HPV (De Sanjosé *et al.,* 2013).

An Austrian study revealed that 23 percent (N= 188) of Austrian women vulvar cancer were positive for HPV. Overall, 77% of all cases suffering from warty or basaloid SCC were HPV positive, compared to 10% of the keratinizing SCC cases ($p < 0.001$). The most common type was HPV 16 (31/38), followed by HPV 31, 33, and 44 (2/38 each). In contrast to the global results, there was no case related to HPV 18 or 45 observed in the Austrian series (Pils *et al.*, 2017). An earlier study by Hartwig et al (2015) reported that in Europe, the overall prevalence of HPV in vulvar cancer was 19.3% (95% CI: 16.7– 22.0) and based on this the relative contribution of HPV16/18 and HPV16/18/31/33/45/52/58 were estimated at 73.6% (95% CI: 66.4–79.9) and 84.0% (95% CI: 77.6–89.0), respectively

While there is no study conducted on the prevalence of HPV among vulvar lesions in Zambia, there are other studies which studied prevalence of HPV among women with other conditions. In order to add context to this dissertation, the next two sections review the epidemiology of HPV and that Of HIV in Zambia as well as the methods of detection of HPV.

2.4. Epidemiology of HPV and HIV in Zambia and the methods of detection of HPV

2.4.1. Epidemiology of HPV in Zambia

The published studies on the epidemiology of human papillomavirus in Zambia were based on cervical smears or vaginal lavage. There were no studies which had investigated prevalent HPV types among women with vulvar neoplasia in Zambia. A study based on vaginal lavage samples, collected between 1998-1999 from a total of 70 Zambian women showed that HIV infected patients were twice as likely to have an HR HPV as HIV negative individuals, while the distribution of LR HPVs was unaffected by HIV status. The study found that the most common HPV genotypes detected among both HIV infected and uninfected women were types 16 and 18 (21.6% each), which was approximately three-fold higher than the rates for HPV16, and ten-fold higher than the rates for HPV18 in the United States

(Ng'andwe *et al.*, 2007). Another study based on cervical cytology smears of only HIV-infected women (N= 145) showed a different pattern of distribution, where the most common types of HR HPV included HPV 52 (37.2%), 58 (24.1%) and 53 (20.7%) compared to HPV 16 (17.2%) and 18 (13.1%) in women with high-grade squamous intraepithelial lesions or squamous cell carcinoma (SCC). In this study $CD4^+$ cell count and grade of cervical pathology were important factors-high-risk HPV was more common among women with counts $<200\,\mu l^{-1}$ (odds ratios (OR): 4.9, 95% confidence intervals (CI): 1.4–16.7, P=0.01) and in women with high-grade or severe cervical cytological abnormalities (OR: 8.0, 95% CI: 1.7–37.4, P=0.008) (Sahasrabuddhe *et al*., 2007). The study by Ngandwe et al. (2007) are consistent with findings from other parts of the world such as the United States of America (USA). A meta-analysis based on Pub Med database revealed that HPV 16 and 18 were the primary types observed in cervical and vaginal cancer; HPV 16 was the most common type among vulvar cancers and HPV 31 the next most frequent type among U.S. cervical cancers (Insinga *et al.*, 2008). Also, similar to other previous reviews, HPV prevalence varied by grade of disease whereby, HPV 16, 18, 31, 51, 52 and 66 were each prevalent in greater than five percent of CIN 1 cases compared with only HPV 16 and 18 observed in excess of five percent among invasive cervical cancers (Insinga *et al.*, 2008).

2.4.2. Methods of detection of human papillomavirus

Human papillomavirus infection can be detected from formalin fixed, and paraffin embedded infected tissue by antibody detection and in situ hybridization (Davy and Doorbar, 2005).

HPV can also be detected by polymerase chain reaction (PCR). Various methods have been developed that detect different components of HPV: Generic or consensus primers amplify all HPV types and include MYO9/MY011, PMYO9/11 GP5+/GP6+(Davy and Doorbar, 2005) and CP1 and CPII (Chisanga et al., 2015). These primers amplify a region of the major viral capsid L1 gene that is highly conserved (Davy and Doorbar, 2005). After use of these consensus primers, one

would need to conduct genotyping using type-specific primers. Other methods include probe-based methods and multiplex PCR.

There are several targets for the detection of HPV using different techniques (Kaufmann, 2007). These include GP5+/GP6+ targeted using generic polymerase chain reaction (GP-PCR) systems, E1 to E5 regulatory genes (detected using multiplex PCR systems), E6/E7 detected using multiplex PCR Mrna oncoproteins systems (Figure 2).

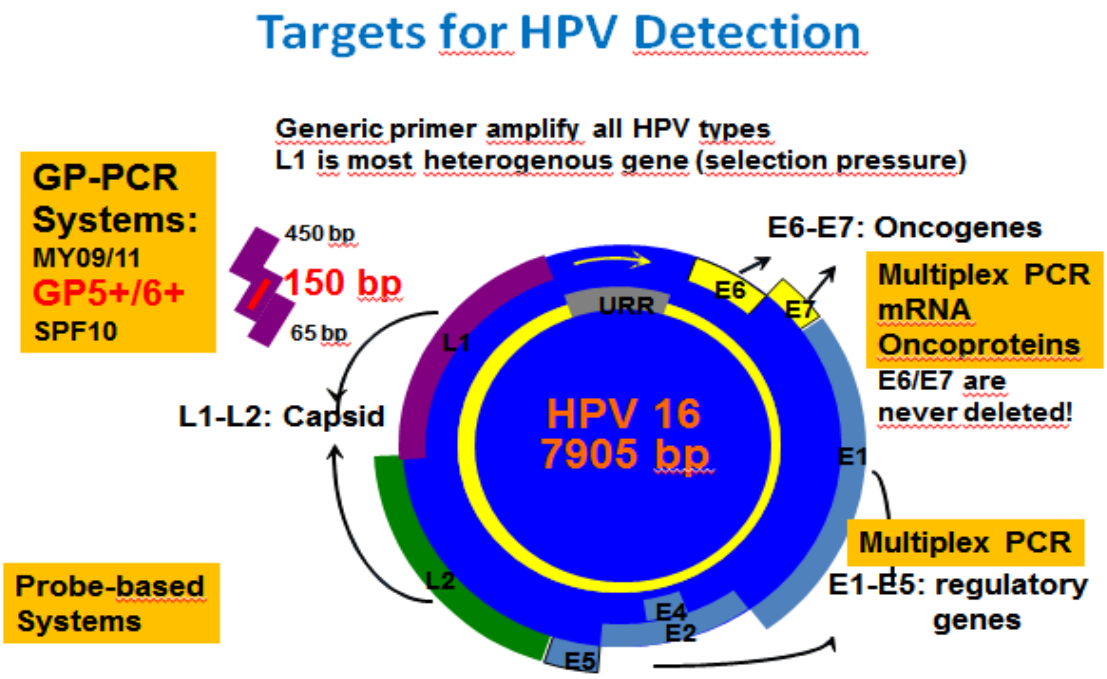

Figure 2: Targets for HPV detection

Some ot the commoercially available methods which have been used widely in the detection of cervical HPV infection include hybrid capture 2 (HC-2, Quiagen 2) and Xpert HPV among others. There are over There are over 220 commercially available methods for HPV detection (Kaufmann, 2017) (Table 3).

Table 3: Examples of Commercially available HPV test kits

	TEST SYSTEM	TYPE OF TEST
1	Cervista HPV 16/18	Probe
2	Cervista HPV HR	
3	HC2 HPV DNA Test	
4	HC2 high risk HPV-DNA Test	
5	Digene HPV Genotyping P5 Test	
6	HPV Easy typing kit	DNA PCR
7	f-HPV testing	
8	AMPICOR HPV test	
9	Cobas HPV test	
10	Real time HR HPV	
11	**Xpert HPV**	
12	BD Onclarity HPV test	
13	ProDect Chip HPV typing	
14	HPV Genotype 14 Real-TMQuant HPV	
15	PapilloCheck	
16	Anyplex TM 11HPV28Detection	
17	HPV Direct flow chip	
18	INNO-LIPA HPV Genotype Extra	
19	CLART HPV 2	
20	Linear Array HPV Genotype	
21	Infinit HPV Genotyping Assay	
22	Nuclisens EasyQ HPV	RNA
23	APTIMA HPV Test	
24	APTIMA HPV 16 18/45 genotype	
25	OncoE6TM Cervical Test	Protein

Studies have evaluated the comparative performance of hybrid capture 2 (HC-2, Quiagen) and X-pert HPV (Cepheid). The latter detects 14 HR-HPV types while the former detects 13. X- pert HPV has a higher sensitivity than HC-2. This study utilised X-pert HPV because a Cepheid-validated X-pert machine was readily available in the UNC Laboratory within UTH. The UNC laboratory was best suited as it had periodic external quality control by the College of American Pathologists (CAP).

2.4.3. Vaccines against human papillomavirus infection

Vaccination against human papillomavirus infection has proved to be effective in preventing HPV-related cancer. Vaccination efforts have already resulted in

positive outcomes in countries such as Scotland. A recently published paper demonstrated that there were statistically significant reductions in all grades of cervical intraepithelial neoplasia (CIN), equating to vaccine effectiveness estimates of 80% or higher after routine immunization at age 12-13 years in Scotland (Palmer *et al.*, 2019). Different types of vaccines are available including the quadrivalent HPV vaccine, Gardasil® 9 (Sanofi Pasteur MSD)/ Silgard® (Merck Sharp & Dohme), and the bivalent HPV vaccine, Cervarix® (GlaxoSmithKline Biologicals). In Europe these vaccines were indicated for the prevention of cervical, vulvar and vaginal premalignant lesions and cervical cancer related to HPV16/18. The quadrivalent HPV vaccine was also indicated against premalignant anal lesions and anal cancer and protected against low-risk HPV6/11, which are responsible for about 90% of genital warts (Hartwig et al., 2015).

2.4.4. Epidemiology of HIV in Zambia

The recently released *Zambia Population-based HIV Impact Assessment (ZAMPHIA) 2016 final report* showed that the prevalence of HIV among adults aged 15-59 years in Zambia was 12.0% (14.6% among females and 9.3% among males). This corresponded to approximately 960,000 people aged 15-59 years living with HIV in Zambia. Among those aged 15-49 years, the prevalence was higher among females (14.3%) than among males (8.3%), as well as among those residing in urban areas (14.4%) compared to those living in rural areas (8.7%). Lusaka had an HIV prevalence rate of 15.7 percent second only to the western province which had a prevalence rate of 15.9 percent (Ministry of Health, 2019). Among women aged 15- 59 years, HIV prevalence peaked in the age group 40-49 years as shown in Figure 3.

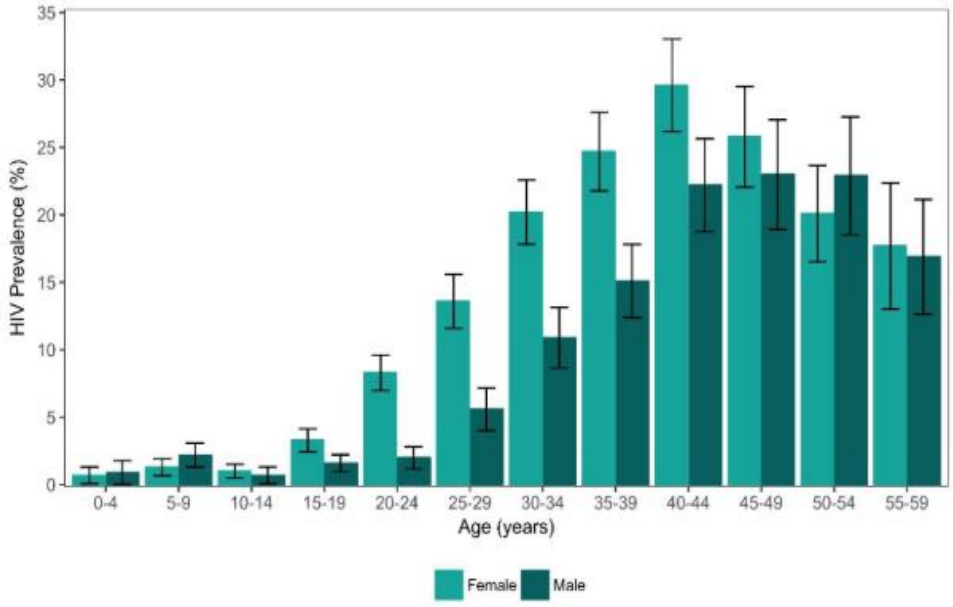

Figure 3: HIV prevalence by age and sex, ZAMPHIA 2016

2.5. Research Questions

What are the clinical and pathological characteristics of vulvar lesions among HIV infected women presenting to the University Teaching Hospital (UTH) in Lusaka, Zambia for care? What is the prevalence of high risk human papillomavirus infection among women with neoplastic lesions?

2.6. Objectives

2.6.1. General Objective

To characterize vulvar lesions clinically and pathologically among HIV infected women, presenting to the UTH in Lusaka, Zambia for care.

2.6.2. Specific Objectives

a. To describe the clinical features of vulvar lesions among HIV infected women presenting to the UTH for care.

b. To describe the histopathologic features of vulvar lesions among HIV infected women receiving care at the UTH.

c. To determine the prevalence of high oncogenic risk human papillomavirus (HR HPV) infection among women with neoplastic lesions of the vulva at the UTH.

CHAPTER III

MATERIALS AND METHODS

3.1. Study Design

The study was a cross-sectional hospital-based clinical and laboratory study.

3.2.Study Site

The study site was the UTH, the country's only tertiary referral hospital**.** The hospital is located in the capital city of Zambia next to the country's only cancer treatment centre (Cancer Diseases Hospital-CDH) and receives patients from all the ten provinces of Zambia. The provincial hospitals refer patients requiring gynaecologic and oncologic intervention directly to UTH and CDH through UTH. Study nurses enrolled participants in this study during the routine gynaecology clinic in the hospital. Specimen collection procedures did not require for a patient to be admitted to a hospital. The UTH had enough space to afford privacy and confidentiality for the participants for enrolment, administration of a questionnaire and collection of samples.

Laboratory technicians and scientists processed biopsy tissue for haematoxylin and eosin (H&E) histology slides, and immunohistochemistry at the UTH's Department of Pathology and Microbiology, Histopathology section. The Centres for Infectious Disease Research in Zambia (CIDRZ) Kalingalinga laboratory processed blood samples for CD4 count. The University of North Carolina (UNC) laboratory at UTH evaluated extracted total DNA for HR HPV.

The target population for this study was that of women presenting to the department of obstetrics and gynaecology and had a gynaecologic disease. The study population consisted of patients with a gynaecologic disease who had lesions suspicious for vulvar cancer, were 18 years of age or older and were infected with HIV.

3.3. Sampling Frame

3.3.1. Sampling Methods

Study participants were selected from the gynaecology clinic by purposive sampling. Study nurses identified and enrolled participants during routinely planned gynaecologic clinics in the Department of Obstetrics and Gynaecology. Target clients were approached for possible recruitment by study nurses, and those eligible enrolled in the study. Written informed consent was obtained using a consent form and participants allocated unique identification numbers. There was no identifying information collected.

3.3.2. Sample Size

At the time of study formulation, there had been no study that had any published data on the incidence or prevalence of vulvar cancer among gynaecology patients at the UTH. According to Zyaambo *et al*, based on the National Cancer Registry (a register for all cancers in Zambia) for the years 1990-2009, vulva cancer accounted for only 0.5 percent (n= 35) of total number of cancers (n= 7501) which were notified by sex in the specified time period (9). According to a hospital-based study in Nigeria, vulva cancer accounted for 3.6 percent of gynaecologic cases (Omotoso *et al.*, 2016). Seeing that this study was a hospital-based study, a prevalence of 3.6 percent at UTH as in the Nigerian study was used.

Fifty-three (53) clients were recruited based on the formula:

$$\textbf{Sample size} = \frac{(\text{Z score})^2 \times \text{prevalence} \times (1-\text{prevalence})}{(\text{Margin of error})^2}$$

The calculations were based on the assumptions of Z score of 1.96, a margin of error of +/-five percent and estimated prevalence rate of 3.6 percent.

Therefore, the sample size was = $\frac{(1.96)^2 \times 0.036 \times (1-0.036)}{(0.05)^2}$;

$= \frac{3.8416 \times 0.036 \times (0.964)}{0.0025}$;

$= 0.138298 \times 385.6$

$= 53.32755$

~= <u>53 participants</u>

3.3.3. Inclusion Criteria

Women presenting to the Department of Obstetrics and Gynaecology were enrolled into the study if they were HIV infected, 18 years of age or older and had vulvar lesions suspicious for malignancy and agreed to give informed written consent.

3.3.4. Exclusion Criteria

A woman was excluded from the study if they had a bleeding disorder, or were too ill to withstand a bedside punch biopsy procedure. Women who had previously been diagnosed with vulvar cancer were also excluded from the study.

3.3.5. Summary of Study Workflow

A total of 56 participants were approached. Two of them were not eligible: one was younger than 18 years of age, while the other was an older woman (70 years of age) who was not HIV infected. For one participant, CD4 count results were unavailable at the time of recruitment, and the participant refused to have blood drawn; she promised to avail the results but was lost to follow up. Six participants refused to have a second punch biopsy to be done despite having consented earlier. Of the 47 punch biopsies collected for molecular tests, DNA was extracted for all of them, but X-pert HPV PCR was only conducted on the 38 neoplastic ones. Of the 53 histologic biopsies, immunohistochemistry was conducted on the 21 cases of invasive carcinoma. Figure 4 shows the flow chart of the research.

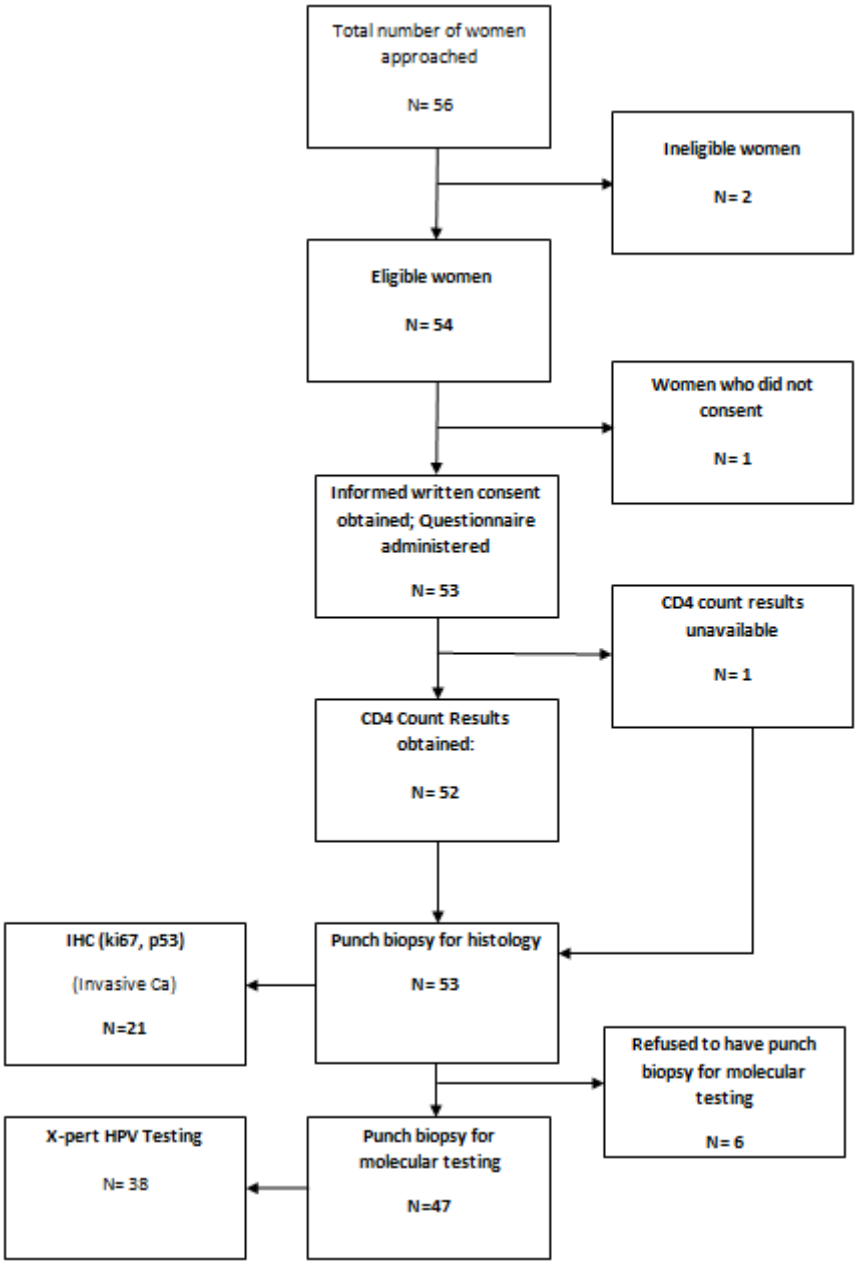

Figure 4: Summary of study workflow.

3.4. Methodology for the clinical Characterization of Vulvar Lesions

Demographic characteristics and clinical history of vulvar disease among the participants were obtained using a structured questionnaire administered by a study nurse. A clinical examination was conducted by a medical doctor to determine the site, size (whether less or greater than 2 cm), number and type of vulvar lesion and associated presence or absence of lymph node enlargement. The nurses and

attending doctors reviewed the patients' records for CD4 count results. For participants without recent CD4 count results (within three months) blood was drawn and taken to the CIDRZ laboratory for analysis.

3.5. Methodology for the Histopathologic characterization of vulvar lesions

Tissue for histopathologic evaluation was collected using a Keyes punch biopsy into an appropriately sized container of 10 percent neutral buffered formalin and fixed for 24 hours.

3.5.1. Procedure for punch biopsy

The Keyes punch biopsy can be used to obtain a portion of tissue in an area that is diffuse, large, multicentre, or suspected to be benign in origin. On the other hand, an excisional biopsy is often useful in situations where the lesion is small or where both diagnosis and therapy can be achieved at the same time (Wells, 2009). This study utilized the punch biopsy.

The clinician explained the procedure to the patient and proceeded to conduct the punch biopsy as described in the appendix.

3.5.2. Protocol for Pathologic Evaluation of Punch Biopsy

Gross evaluation of the punch biopsy was based on a grossing template for skin punch biopsy (Lester, 2010).

3.5.3. Histological Sample Processing

Samples for histology were processed according to histopathology laboratory Standard Operating Procedures (SOPs) attached as an appendix.

3.5.4. Immunohistochemistry

Slides for immunohistochemistry were processed according to the SOPs currently in use in the UTH histopathology laboratory. Immunohistochemistry was ordered after routine H&E histology. If H&E showed cancer, appropriate immunohistochemistry was ordered. This study proposed to conduct immunohistochemistry for $p16^{INK4A}$/ CDKN2A, p53(Product of TP53 gene), and Ki67 (MIB1). P16 was meant to be used as a surrogate marker for high-risk HPV; TP53 tumour suppressor gene is the most critical gatekeeper of the genome. Mutations in TP53 have been reported to be an underlying genomic alteration that is important in the pathogenesis of non-HPV driven vulvar carcinoma. Ki67 is a marker of proliferation; it was used utilized for the assessment of the aggressiveness of a tumour. The obtained antibody for p16 failed in both cases and controls and was therefore non-informative in this study. Immunohistochemistry for P53 and ki67 was successfully conducted.

Most of the reagents for immunohistochemistry including antibodies for p53 and ki67 were procured from Cell Marque Corporation (6600 Sierra College Blvd Rocklin CA) including. The antibody P16 (ab54210 Anti-CDKN2A/p16INK4a) was procured from Abcam. Immunohistochemistry was conducted using *HiDef polymer-based protocol attached in the appendix*.

3.6. Methodology for determination of HPV prevalence among neoplastic lesions

The sample for PCR was collected using a Keyes punch biopsy at the same time for collection of the sample for histopathologic evaluation. The sample was collected into ATL buffer (in 1.5ml microcentrifuge tube) and stored in a refrigerator at -80^0 Celsius. Once enough samples were collected, the next step was that of DNA extraction as explained below.

3.6.1. Total deoxyribonucleic Acid (DNA) Extraction

The stored samples were first allowed to defrost to room temperature; they were then mixed with 20 microliters of proteinase K, incubated and vortexed to ensure complete mixing. Total DNA extraction from pre-lysed samples was conducted using the NUCLISENS® EASYMAG™ automated extraction machine (housed within the Department of Pathology and Microbiology at UTH) following the manufacturer's protocol with an elution volume of 100 micro-litres. The extraction of DNA using the NUCLISENS® EASYMAG™ follows four basic steps depicted in figure 5.

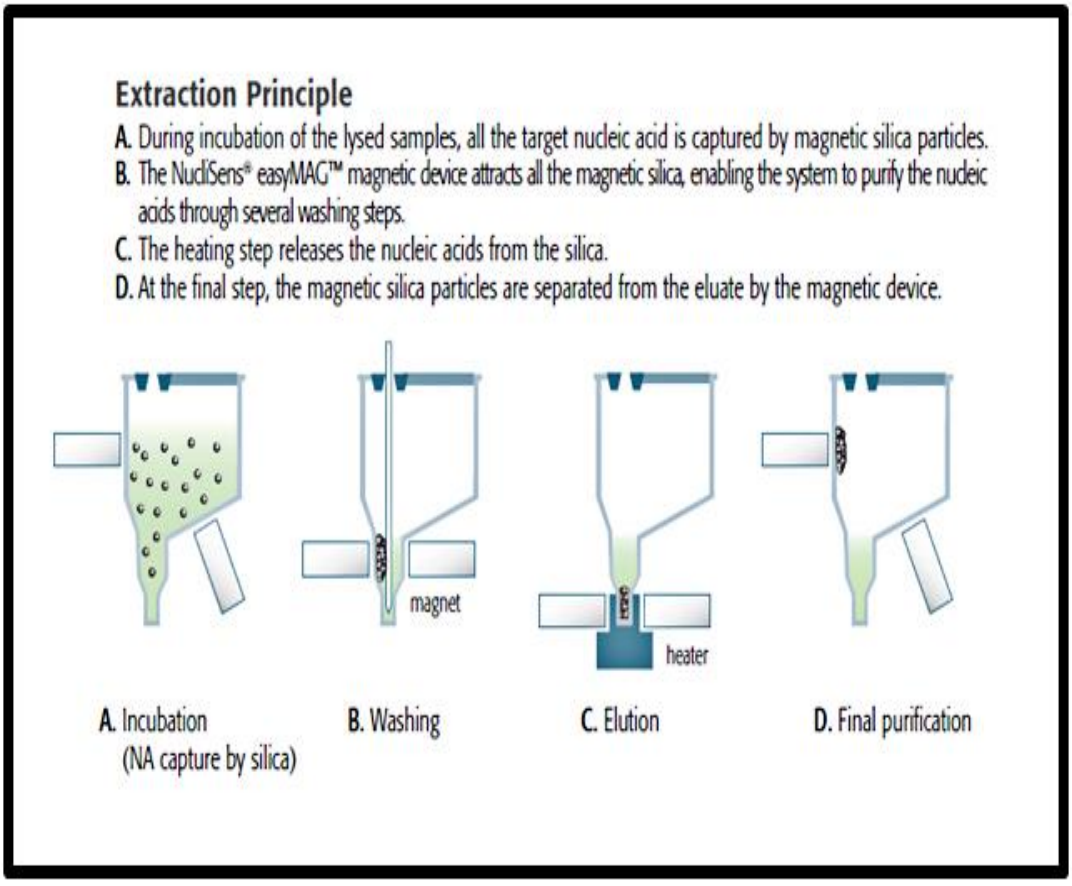

Figure 5: NUCLISENS® EASYMA(R) Extraction Principle.

The EASYMAG™ uses a technology known as BOOM technology which involves binding of the nucleic acid to silica, which is ultimately separated at the final step of elution of DNA. (figure 6).

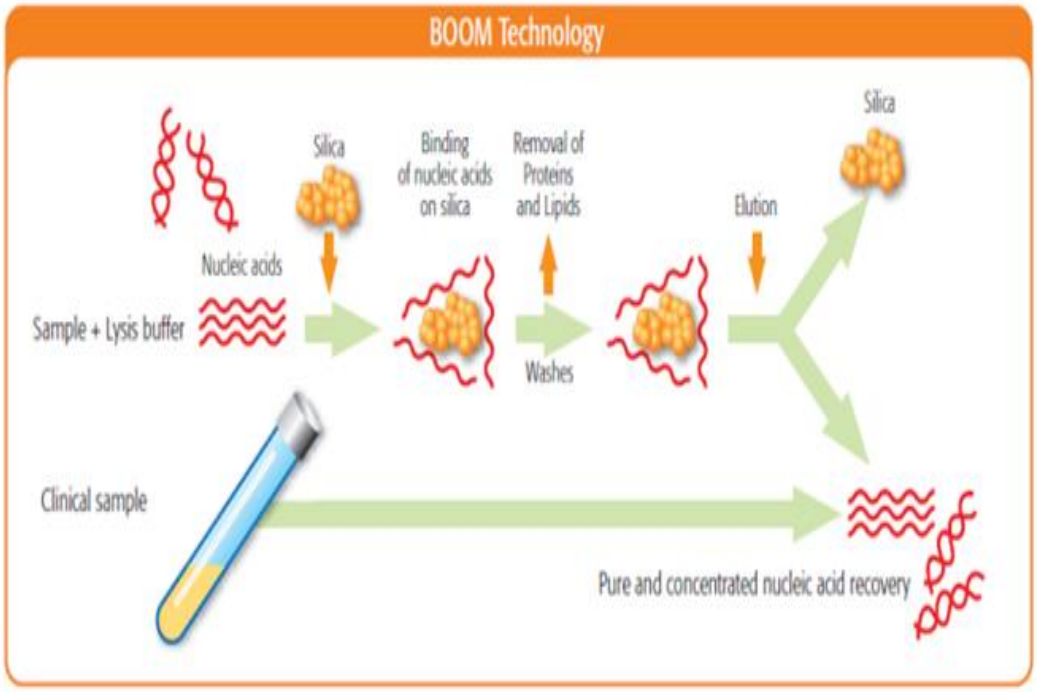

Figure 6: Boom technology of the NUCLISENS® EASYMAG.

There are four steps for processing the tissue using NUCLISENS® EASYMAG™ including:

- Step 1: Data entry
    - Enter sample set data: set sample matrix, set sample size and choose elution volume
    - Define order of sample set to create run

- Step 2: Sample loading
    - Load samples into 8-wel sample vessels
    - System is compatible with primary or pre-lysed samples
    - Convenient sample vessel carrier supplied with system
    -

- Step 3: System loading
    - Insert aspiration tip sets
    - Insert sample vessel strips and scan barcode

    - easyMAG automatically verifies
    - Sample and tip set insertion
    - On-board reagents

- Step 4: Start Run
    - For pre-lysed samples add silica
    - Start run
    - For primary samples add silica 10 minutes into run
    - Fast throughput for up to 24 specimens/ run; 1 hour for 24 primary samples

3.6.2. Polymerase Chain Reaction for the Detection of High-Risk Human Papillomavirus Infection Using HPV Expert

The 100 microliters of each sample extracted were allowed to thaw and were then mixed with *Thin Prep* solution (Hologic, USA) to 1ml. The solution was then allowed to homogenize after which it was placed in the cartridge. The cartridge was then loaded in the Gene Expert machine and processed according to manufacturer's instructions.

3.7. Quality Control

We ensured that quality control was conducted in all stages including in the pre-analytical, analytical and post-analytical stages.

3.7.1. Pre-analytical:

Manufacturers' instruction for storage and preparation of reagents were adhered to.

3.7.2. Analytical:

All tests were conducted according to standard protocols attached as appendices. A procedural control is included in the tests for histology and immunohistochemistry.

The Cepheid Xpert HPV test kit has an inbuilt control, called sample adequacy control which ensures the reliability of the results.

3.7.3. Post-analytical:

The obtained data was coded and entered into an excel sheet and transferred to SPSS version 22 for analysis. The histology slides were examined by the primary investigator and by two pathologists, and the final diagnosis was based on consensus agreement.

3.8.Study Outcomes

The primary outcome in this study was histologically confirmed vulvar lesion. The secondary outcome was the finding of HR HPV in the neoplastic samples.

3.9. Study variables

3.9.1. Dependent Variables:

The dependent variables were the results of the questionnaire and clinical examination, laboratory results such as routine haematoxylin and eosin (H&E) histology, immunohistochemistry and the results of human papillomavirus genotyping.

a. Clinical Examination Findings:

Clinical dependent variables included the vulvar symptoms and the clinical examination findings; the latter included the site, size type of vulvar lesion and the presence of enlarged inguinal lymph nodes. Other clinical variables included CD4 count and whether the patient was on antiretroviral therapy or not.

b. Histologic findings:

The histologic results were reported as nonneoplastic, benign, vulvar intraepithelial neoplasia and invasive cancer. The vulvar neoplasia was classified according to the

2014 WHO/IARC Classification of Tumours of Female Reproductive Organs. Squamous Neoplastic Lesions were classified as either vulvar squamous intraepithelial lesion or invasive squamous cell carcinomas. Vulvar intraepithelial lesions were classified as low grade squamous intraepithelial lesion (LSIL= VIN 1) or high grade squamous intraepithelial lesion (HSIL= VIN 2 and 3). Invasive squamous cell carcinoma was classified as either Keratinizing, warty or basaloid. In the evaluation of vulvar histological specimen, the American College of Pathologists Protocol for the examination of specimen from the patients with carcinoma of the vulva was used (attached in the appendix).

i. Histologic diagnostic criteria for the classification of squamous cell histologic subtypes:

In addition to features described in the literature review, classification of a SCC was based upon nuclear features and the type of VIN present. If VIN was of the usual type, the tumour was classified as either warty or *basaloid*. If the VIN were of the differentiated type, the tumour would be classified as *keratinizing* type all other features described before are present. The presence of nuclei without prominent nucleoli would make the tumours to be classified as either warty or *basaloid*. However, tumour cells with the prominent single nucleolus and vesicular chromatin would render a diagnosis of keratinizing squamous cell carcinoma. Basaloid SCC diagnosis was based on small uniform cells with increased nuclear to cytoplasm ratio. Diagnosis of warty SCC was based on large, pleomorphic and occasionally giant neoplastic squamous cells. Diagnosis of keratinizing SCC would be based on the presence of central keratinization among cells with keratin pearl formation and onion pattern of keratin.

c. HPV Status:

Results for HPV were reported as either positive or negative for HR HPV. Further, genotypes were reported as HPV 16, HPV 18_45 or HPV P3, HPV P4, and HPV P5.

3.9.2. Independent variables

Independent variables included sociodemographic, lifestyle, sexual behaviour, and gynaecologic history. *Sociodemographic variables* included age (in years), marital status, employment status and level of education; *Lifestyle variables* included: current smoker, ever smoked, current alcohol use, history of birth control use and type of birth control; *Sexual behaviour variables* included age at first sexual intercourse, number of lifetime sexual partners and number of lifetime sexual partners of spouse; *Gynaecologic History variables included* currently pregnant and self-reported STI history.

3.10. Data Analysis

Data obtained in this research was analysed using quantitative methods. The study variables were coded and entered in statistical package SPSS version 22. The results were reported through display into graphs, charts amongst other ways. Descriptive statistics such as the mean or median and the corresponding measures of spread were made, that is, standard deviation or interquartile range for continuous variables either following a normal distribution or not. For categorical variables, numbers and percentages were documented. In determining statistical significance, *0.05 level of significance and 95 percent* confidence levels were used.

1.10.1. Objective 1: Clinical Characterization of Vulvar lesions

The clinical features were tabulated according to the histologic diagnoses and presented as frequencies:

a. Symptoms
b. Signs-Clinical examination findings including the type of lesion, site, size and the presence of inguinal lymph node enlargement

Levene's t test was used to compare if there was a significant difference between the mean age of participants with invasive cancer and for all other diagnostic categories in aggregate. The Chi square test for independence was used to compare if there was a significant difference in the frequency of pain, painful sex, bleeding

and lesion discharge among those with invasive cancer compared to those without invasive cancer.

1.10.2. Objective 2: Histopathological characterization of vulvar lesions

The types of the lesions were presented as frequencies and percentages. The histologic diagnoses and subtypes were tabulated as proportions/ percentages. The histologic categories are outlined in figure 7.

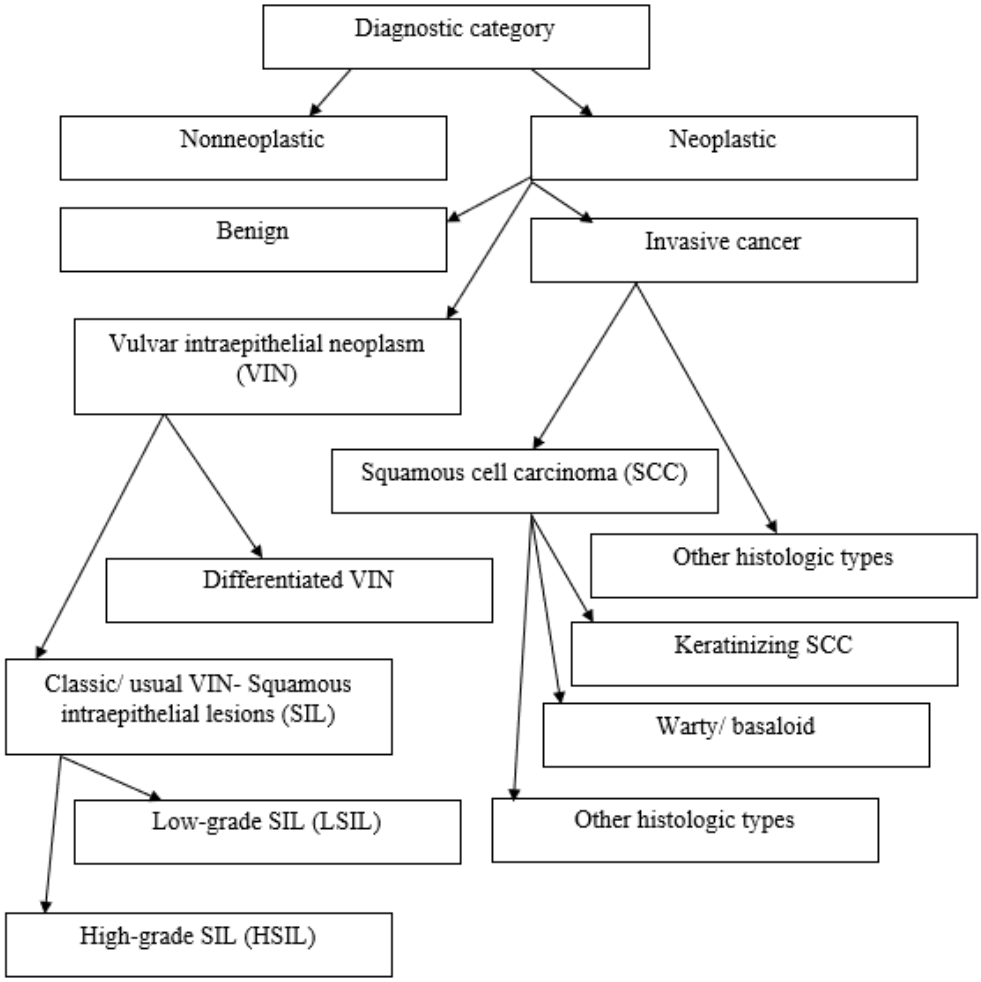

Figure 7: Histologic diagnostic categories

1.10.3. Objective 3: The prevalence of HPV among vulvar neoplasia

The prevalence of HR HPV was tabulated for all the neoplastic lesions (in aggregate) tested and disaggregated for each diagnostic category and specific diagnosis. The prevalence was tabulated both as a count and percentage.

3.11. Ethical Considerations

Ethical approval was sought from the University of Zambia Biomedical Research Ethics Committee (UNZABREC), National Research Ethics Authority (NHRA), from the University of North Carolina at Chapel Hill. Permission to conduct the study was obtained from the UTH Management. Informed written consent was sought from participants. Clients were free to decline participation in the study without any bearing on how they would be treated. Further, the clients were free to withdraw from participation at any time without obligation to give explanations. Throughout the process of recruitment, enrolment, and collection of information, participants' privacy was guaranteed.

The decision regarding which patients required a biopsy based on clinical suspicion of cancer was determined by the attending clinician. The Principal investigator had no role in this decision.

Only authorized individuals had access to the participants and study protocols and other documents. All collected data were coded and kept under lock and key and only accessible to authorized persons (the Principal investigator and supervisors). Discomfort and pain were minimized by ensuring the application of local anaesthetic and administration of analgesics. Strict confidentiality was upheld by all study assistants. Only qualified nurses and doctors were present throughout the stages of recruitment/enrolment, clinical examination and the biopsy procedure.

3.12. Study limitations

There were a number of limitations to this study. This study was cross sectional and there was no follow up of patients to document the treatment and survival of the patients. Also, one participant could not be followed up for CD4 count.

Further, because the instrument used for histologic diagnosis (the Keyes punch biopsy needle) was purely for diagnosis, pathologic staging of the invasive lesions could not be done. This study only targeted women who had come for the very first visit; patients who had been previously diagnosed and already had histologic diagnosis were not included. Knowledge of the characteristics of the lesions in these women would have provided more insight into vulvar lesions.

The information collected through the questionnaire may have been affected by recall bias.

The antibody for p16/ INK4a could not function as expected. p16/INK4a is used as surrogate marker for high-risk HPV and when overexpressed indicated viral DNA integration into host cells. Due to the failure to successfully conduct this test, this test did not prove viral DNA integration into the host DNA. The results of HR HPV using gene expert simply show that HR HPV is found on the “scene of the crime” but does not prove causality.

The fact that some of the HPV Xpert HR HPV results are pooled, we may therefore not know the exact genotypes.

CHAPTER IV

RESULTS

4.2. Summary of characteristics of all participants

Out of the total number of 53 participants with vulvar lesions, suspicious for malignancy that were recruited, the mean age was 40 years (range 23-63 years, standard deviation 9.5). Most of the participants were on antiretroviral (ARV) medication (96.2 percent). The most frequent CD4 count range was 200-499u/ul. The most commonly involved site on the vulvar was the labia majora: lesions in 45 (84.9 percent) participants involved the labia majora. Invasive cancer accounted for 39.6 percent of the cases (21/53) compared to other diagnoses which accounted for 60.4 percent in aggregate. Among the vulvar intraepithelial lesions, there was no case of classic VIN seen. Also, there was no case of keratinizing squamous cell carcinoma seen among SCC. Among the 38 neoplastic lesions analysed for HR HPV, 81.1 percent were positive (Table 4).

Table 4: Summary of the characteristics of all participants (N=53)

		n	%
Social-demographic characteristics and risk factors			
Age: Mean [Range (SD)]	40 [23-63 (9.5)]		
Number on ART	On ART	51	96.2
CD4 Count (N=52) *	199 or less	11	21.2
	200-499	25	48.1
	500-999	16	30.8
Positive smoking history	Smoker	3	5.7
Positive history of alcohol use	Yes	17	32.1
Positive history of Oral contraceptive use	Yes	22	41.5
Age (years) at first sex: Mean [Range (SD)]	17.9[14-25(2.6)]		
Lifetime sexual partners: Mean [Range (SD)]	4.9[1-20(4.4)]		
Objective 1: Clinical features of vulvar lesions			
Most frequent symptom experienced on the vulva	Itching	46	88.5
Least frequent symptom experienced on the vulva	Painful sex	5	9.6

Frequency of Sites involved on the vulva	*Labia majora*	45	84.9
	Labia minora	39	73.6
	Clitoris	14	26.4
Frequency of lesions greater than 2cm		44	83
Most frequent type of vulvar lesion	Warty	19	35.8
Enlarged inguinal lymph nodes		25	47.2

Objective 2: Histopathologic features of vulvar lesions			
Non- neoplastic		9	17
Benign		3	5.7
VIN	*Classic/Usual*	20	37.7
	Differentiated	0	
Invasive cancer-SCC	*Basaloid/ warty*	21	39.6
	Keratinizing	0	

Objective 3: Prevalence of HR HPV among neoplastic lesions		
Overall prevalence of HR HPV (N=38)	31	81.6
Prevalence in VIN (N=17)	13	76.5
Prevalence in SCC (N=18)	16	88.9

4.2.1. Age groups and other background characteristics of all participants

Table 5 shows that most of the participants were in the age group 30 to 39 years. The majority were residents of Lusaka and urban areas. Most of them were married, had a history of being employed and had a primary level of education.

Table 5: Background characteristics of all participants

	(N=53)	
	n	%
Age, years		
20-29	8	15.1
30-39	20	37.7
40-49	13	24.5
50-59	11	20.8
60-69	1	1.9
Place of origin		
Non-Lusaka	13	24.5
Lusaka	40	75.5

Urban vs. rural		
Urban	50	94.3
Rural	3	5.7
Marital status		
Single	8	15.1
Married	21	39.6
Divorced/ separated	13	24.5
Widowed	11	20.8
Employment history		
Never employed	19	35.8
Employed	34	64.2
Level of education		
None	4	7.5
Primary	23	43.4
Secondary	20	37.7
Tertiary	6	11.3

The population was normally distributed according to age. The most frequent age group was 30 to 39 years (Figure 8).

Figure 8: Histogram of age frequency with normality curve (N=53)

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The participants with invasive cancer (Ca) were older than all other diagnostic categories (Figure 9).

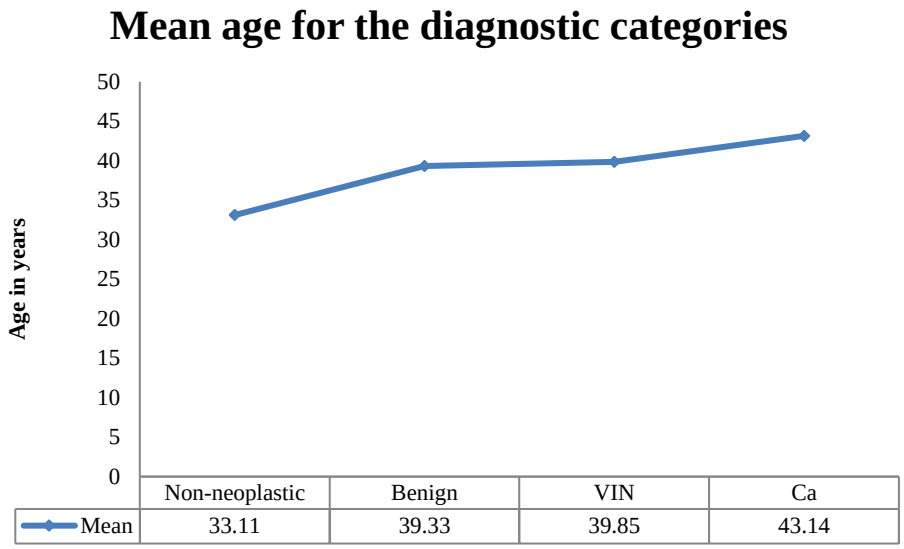

Figure 9: Mean age for the diagnostic categories

Participants with invasive cancer were significantly older than the rest (Figure 10)

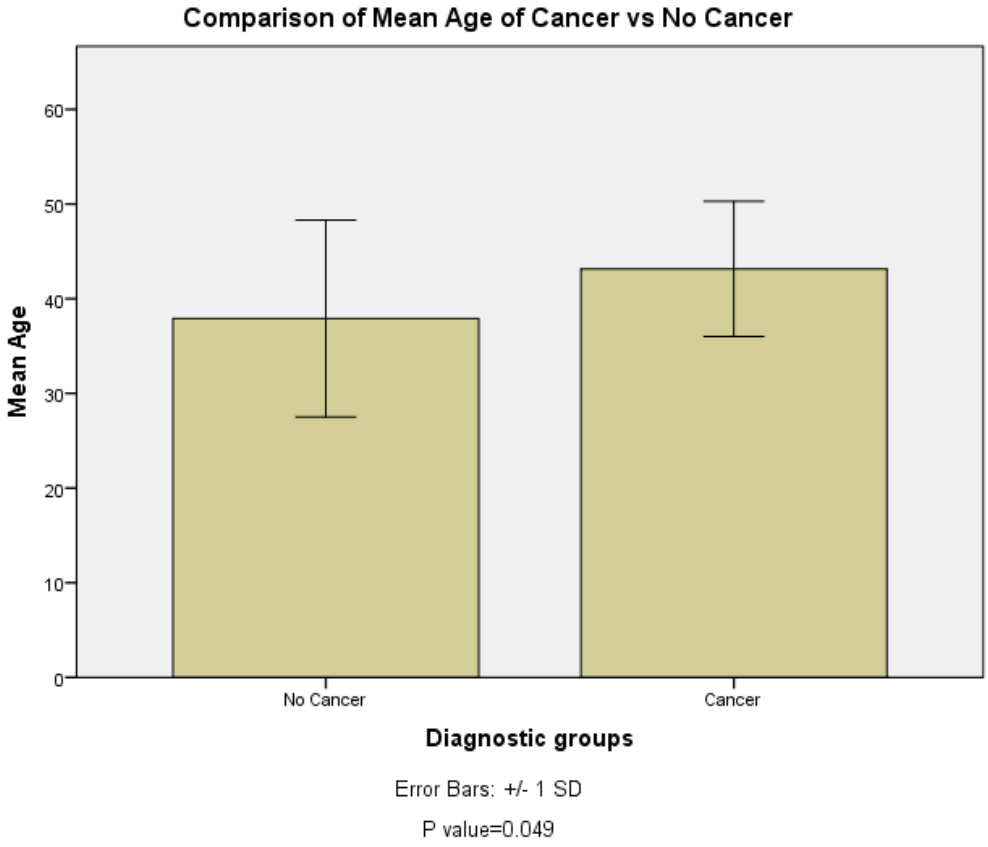

Figure 10: Comparison of mean age for invasive cancer vs. other diagnoses

4.2.2. Risk factors among women with VIN and invasive cancer

Table 6 shows the frequencies of various risk factors in VIN and invasive cancer. Cigarette smoking and alcohol use was low among participants with invasive cancer.

Table 6: Risk factors in VIN and invasive cancer

	VIN (N=20)		Ca (N=21)	
	n	**%**	**n**	**%**
History of cigarette smoking				
No	19	95	20	95.2
Yes	1	5	1	4.8
History of alcohol intake				
No	13	65	14	66.7
Yes	7	35	7	33.3
Age at first sexual intercourse				
Mean (Range)	17.3 (14-22)		17.8 (15-25)	
SD	2.1		2.8	
Number of lifetime sexual partners				
Mean (range)	5.5(2-20)		5.6 (1-15)	
SD	5.2		4.4	
The frequency of respondents' use of oral contraceptives				
Oral	9	45	7	33.3

4.2.3. HIV Characteristics of all participants

Among all participants, the majority were on ARV treatment (96.2 percent). Of those on ARV treatment, the mean duration on ARV treatment was 5.9 years. With regards CD4 count, the majority had a CD4 count in the range 200-499u/ul (48 percent) (Table 7).

Table 7: ARV Treatment status and duration among all participants

	All	
On ARV Treatment? (N=53)	n	%
No	2	3.8
Yes	51	96.2

Duration of ART (N=51)	
Mean (years)	5.9
Range	0-17

The majority of respondents had been on ARVs. Duration less than one year was reported as 0 years. All respondents with benign lesions, VIN and invasive cancer had been on ART while two among those with nonneoplastic lesions had not been on ART. The mean duration on ART for benign lesions was 8.3 years (range 0 to 15 years), 5.6 years (range 0 to 17) in VIN and 6.71 years (range 0 to 14 years) among invasive lesions.

The Majority of the participants had a CD4 count in the range 200-499 u/ul. Among those with benign lesions (condyloma acuminatum) the CD4 count results were 26u/ul, 368u/ul and 719u/ul (Figure 11).

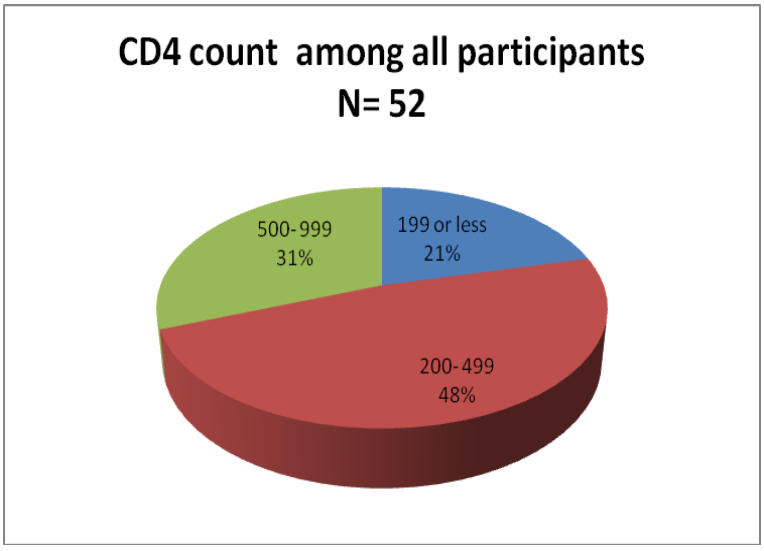

Figure 11: CD4 Count ranges among all participants.

The majority of the participants with VIN had CD4 count in the range 200-499u/ul (Figure 12).

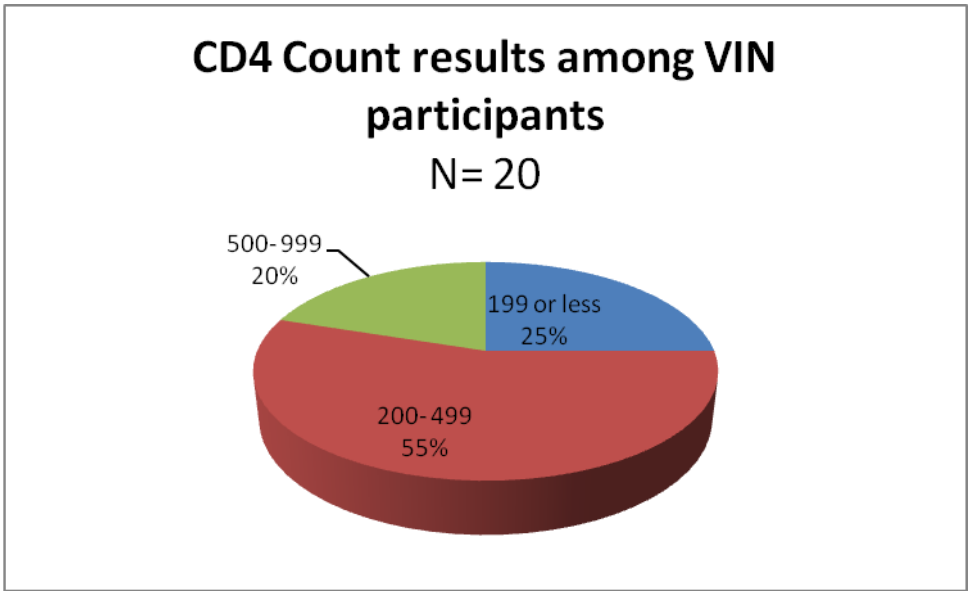

Figure 12: CD4 Count in VIN

The CD4 count distribution among those with invasive cancer is shown in figure 13. The majority of participants with invasive cancer had CD4 Count in the range 200-499u/ul (50%). Only 15% had CD4 count of 199u/ulor less.

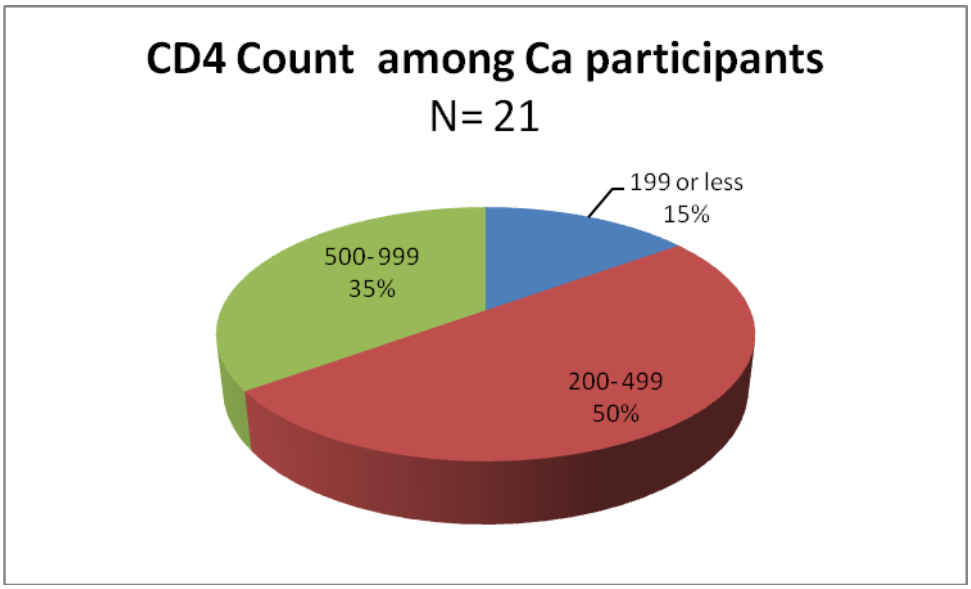

Figure 13: CD4 count in invasive cancer.

4.2.4. History of cervical cancer screening and diagnosis among all participants

Table 8 shows that 33 (62.3 percent) of the 53 participants had been previously screened for cervical cancer and 2 (3.8 percent) had been diagnosed with cancer of the cervix.

Table 8: Cervical cancer screening and diagnosis among all participants

	(N= 53)	
	n	**%**
History of cervical cancer screening		
No	20	37.7
Yes	33	62.3
Diagnosed with cervical cancer		
No	51	76.2
Yes	2	3.8

Among those with VIN and those with invasive cancer, one of each group had been diagnosed with cervical cancer (Table 9).

Table 9: Cervical cancer screening and diagnosis history among participants with VIN and Invasive Cancer (Ca).

	VIN (N=20)		**Ca (N=21)**	
	n	**%**	**n**	**%**
History of cervical cancer screening				
No	7	35	8	38.1
Yes	13	65	13	61.9
Diagnosed with cervical cancer				
No	19	95	20	95.2
Yes	1	5	1	4.8

4.3. Clinical characteristics of Vulvar lesions

4.3.1. Clinical characteristics among all participants

a. Symptoms among all participants

i. The frequency of duration of symptoms on the vulva among all participants

The most common duration of vulvar symptoms among all participants was less than one year, followed by one year as shown in figure 14. The mean duration for all participants and those with nonneoplastic lesions was 3.7 years. among all participants. Duration less than one year was designated as zero years. The figure shows that 14 respondents had symptoms for less than one year.

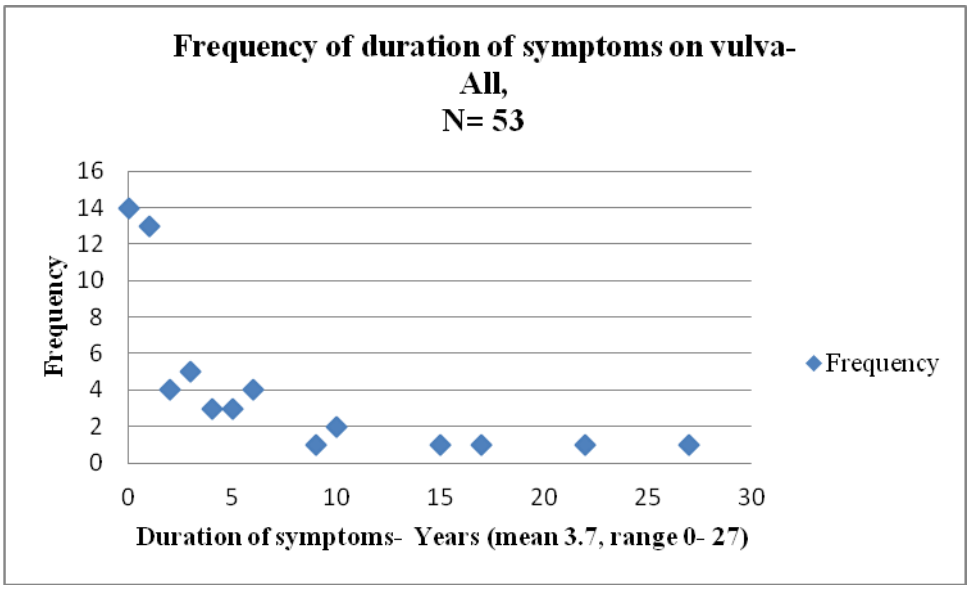

Figure 14: Frequency of duration of symptoms on the vulvar (N=53)

ii. The first symptom experienced on the vulva among all participants

The most commonly experienced first symptom was itching (45.3 percent) (Table 10).

Table 10: The First symptom experienced among all participants

	All	N= 53
Symptom	N	%
Itching	24	45.3
Pain and itching	1	1.9
Pain	3	5.7
Blister	1	1.9
Painful sex	1	1.9
Bleeding	1	1.9
Black Patches	1	1.9
Boil	2	3.8
Growth	8	15.1
Pimple	10	18.9
Warts	1	1.9

iii. The frequency of pain, painful sex, bleeding and lesion discharge among all participants from onset to time of enrolment

Itching was the most frequent symptom *ever experienced* (88.5 percent). The least commonly experienced symptom was painful sex (9.6 percent) (Table 11)

Table 11: The frequency of pain, painful sex, bleeding and lesion discharge among all participants

	All (N= 53)	
Symptom	n	%
Itching	46	88.5
Pain	41	78.8
Painful sex	5	9.6
Bleeding	19	35.6
Lesion discharge	35	67.3

b. Clinical examination findings among all participants

Table 12 shows that the most commonly involved site was the *labia majora* (45 percent) followed by the *labia minora* (39 percent). Majority of lesions were multifocal and larger than two centimetres. Most of the lesions were *warty*. Lymph node enlargement was found in 47.2 percent of cases.

Table 12: Clinical examination findings for all participants

	n	**%**
The site of vulvar lesion		
Labia Majora	45	84.9
Labia Minora	39	73.6
Clitoris	14	26.4
Other sites involved by lesions		
Vagina	3	5.7
Perianal skin	4	7.5
Anus	1	1.9
Gluteal skin	1	1.9
Number of vulvar lesions		
Single	10	18.9
Multiple	43	81.1
Size of the lesion (of the largest, if multiple)		
≤ 2cm	9	17
> 2cm	44	83
Type of vulvar lesion		
Smooth exophytic	10	18.9
Warty	19	35.8
Fungating	13	24.5
Flat	3	5.7
Ulcerated	8	15.1
Enlarged inguinal lymph nodes		
No	28	52.8
Yes	25	47.2

4.3.2. Clinical characteristics of participants with nonneoplastic vulvar lesions

a) Symptoms among participants with nonneoplastic lesions

i. The frequency of duration of symptoms on the vulva among participants with nonneoplastic lesions

The most common duration of vulvar symptoms among all participants was less than one year, followed by a duration of one year. The mean duration of symptoms was 2.2 years (Figure 15).

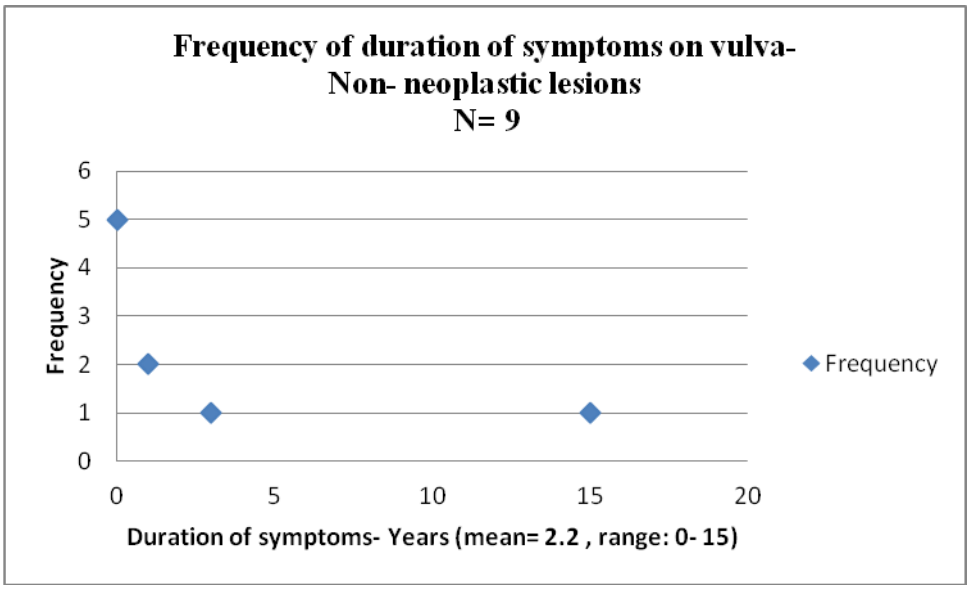

Figure 15: Frequency of duration of symptoms among participants with nonneoplastic lesions

ii. The first symptom experienced among participants with nonneoplastic lesions

The most commonly experienced first symptom was itching (44.4 percent).

iii. The frequency of pain, painful sex, bleeding and lesion discharge among participants with nonneoplastic lesions from onset to time of enrolment.

Itching was the most common symptom ever experienced (100 percent), and painful sex was not experienced at all.

b. Clinical examination findings of nonneoplastic lesions

The most frequent site involved by nonneoplastic lesions was the labia majora (100 percent of cases) followed by the labia minora (6 cases, 66.7 percent). Three cases involved the clitoris (33.3 percent) and one extended to the gluteal skin as well. All the lesions were multifocal, and 8 (88.9 percent) were 2cm or more in size. More commonly, the lesions were either warty or ulcerated (3 cases for each, 33.3 percent). Two cases (22.2 percent were smooth exophytic lesions) and one was fungating (11.1 percent)

4.3.3. Clinical characteristics of participants with benign lesions

a. Symptoms of among participants with benign lesions

i. The frequency of duration of symptoms on the vulvar among participants with benign lesions

The duration of symptoms among the participants with benign lesions was *zero, one* and *ten years*.

ii. First symptom experienced by participants with benign lesions

Two of the participants reported itching as the first symptom. The other participant reported growth as a first symptom.

iii. The frequency of itching, pain, painful sex, bleeding and lesion discharge among participants with benign lesions

The frequency of itching, pain, lesion discharge was two (66.7 percent for each) while bleeding was only reported in one (33.3 percent). Painful sex was not reported by any of the three participants with benign.

b. Clinical examination findings of benign vulvar lesions

Of the three benign vulvar lesions, all three involved the labia majora and labia minor, in one case as a single lesion and in two cases, multiple. Two of the three were greater than 2cm in size. Two lesions were warty, and one was fungating. Enlarged inguinal lymph nodes were found in only one case.

4.3.3. Clinical characteristics of vulvar intraepithelial neoplasia (VIN)

a. Symptoms in VIN

i. The frequency of duration of symptoms on the vulvar among participants with VIN

The majority among those with VIN had vulvar symptoms for five years or less (80 percent); Eight (40 percent) had symptoms for one year or less (Figure 16).

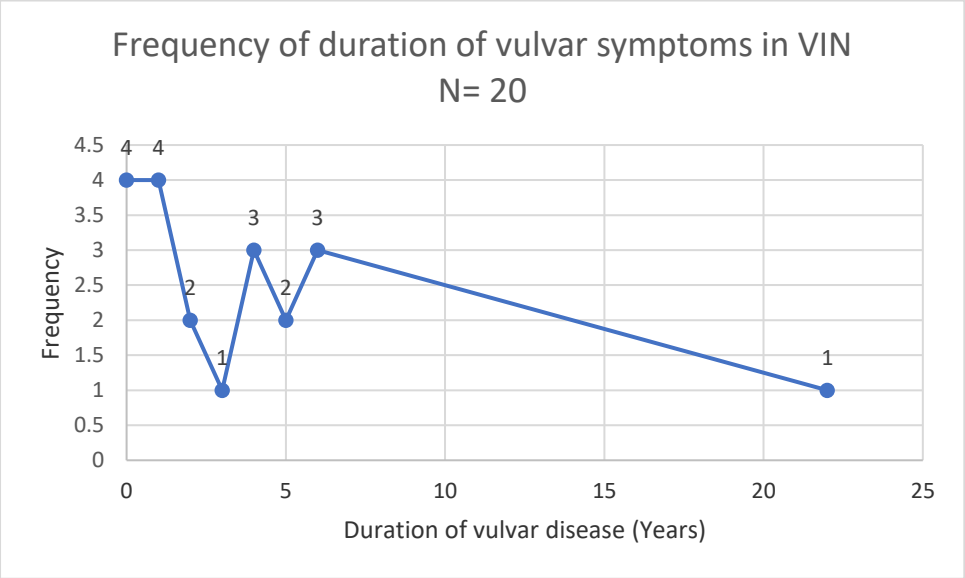

Figure 16: Frequency of duration of vulvar symptoms in VIN.

ii. First symptom experienced by participants with VIN

The most frequently reported first vulvar symptom in VIN was itching (nine cases-45 percent), followed by pimple (6 cases-30 percent) (Figure 17).

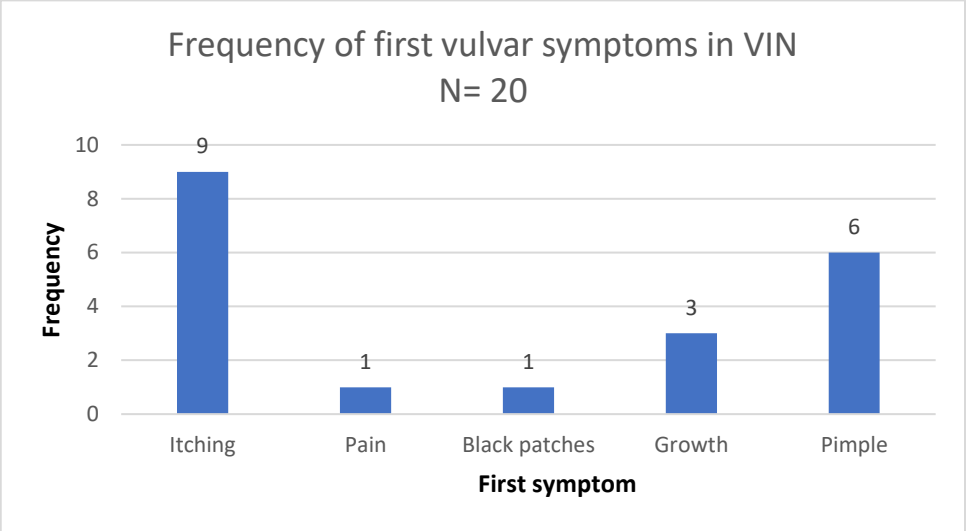

Figure 17: Frequency of first vulvar symptom in VIN

iii. The frequency of itching, pain, painful sex, bleeding and lesion discharge among participants VIN

Itching was experienced by 18 (94.7 percent), pain in 13 (68.4 percent), lesion discharge in 9 (47.8 percent), bleeding in 3 (15.8 percent) and painful sex in 2 (10.5 percent) of participants with VIN (Table 13).

Table 13: Itching, pain, painful sex, bleeding and lesion discharge in VIN and invasive Cancer (Ca)

	VIN(N=20)		Ca(N=21)	
Symptom	n	%	n	%
Itching	18	94.7	17	81
Pain	13	68.4	20	95.2
Painful sex	2	10.5	3	14.3
Bleeding	3	15.8	13	61.9
Lesion discharge	9	47.4	18	85.7

b. Clinical examination findings of VIN

Fourteen (70 percent), fourteen (70 percent), four (20 percent) and 2 (10 percent) of cases with VIN involved the labia majora, labia minora, clitoris, and perianal skin respectively. 16 cases (80 percent) were multiple compared to single lesions (4 cases 16 percent). The lesion was warty in 11 cases (55 percent), flat in 3 cases (15 percent), and fungating, smooth exophytic and ulcerated in 2 cases each (10 percent). In nine cases (45 percent) there was associated inguinal lymph node enlargement (Table 14).

Table 14: Clinical examination findings of VIN and invasive cancer.

	VIN (N=20)		Ca (N=21)	
	n	**%**	**n**	**%**
The site of vulvar lesion				
Labia Majora	14	70	19	90.5
Labia Minora	14	70	16	76.2
Clitoris	4	20	7	33.3
Other sites involved by lesions				
Vagina			3	14.3
Perianal skin	2	10	2	9.5
Anus			1	4.8
Number of vulvar lesions				
Single	4	20	5	23.8
Multiple	16	80	16	76.2
Size of the lesion (of the largest, if multiple)				
≤ 2cm	5	25	2	9.5
> 2cm	15	75	19	90.5
Type of vulvar lesion				
Smooth exophytic	2	10	6	28.6
Warty	11	55	3	14.3
Fungating	2	10	9	42.9
Flat	3	15		
Ulcerated	2	10	3	14.3
Enlarged inguinal lymph nodes				
No	11	55	9	42.9
Yes	9	45	12	57.1

4.3.4. Clinical characteristics of invasive cancer

a) Symptoms among participants with invasive cancer

i. The frequency of duration of symptoms on the vulvar among participants with invasive cancer

Seven (33.3 percent) participants of those with invasive cancer had the shortest duration of symptoms for one year followed by four (19 percent) participants with duration of less than a year. In aggregate 16 (76 percent) participants had symptoms for five years or less, and 13 (62 percent) had symptoms for one year or less (figure 18). Sixteen (76 %) participants with invasive cancer had symptoms for five years or less and 13 (62%) had symptoms for one year or less.

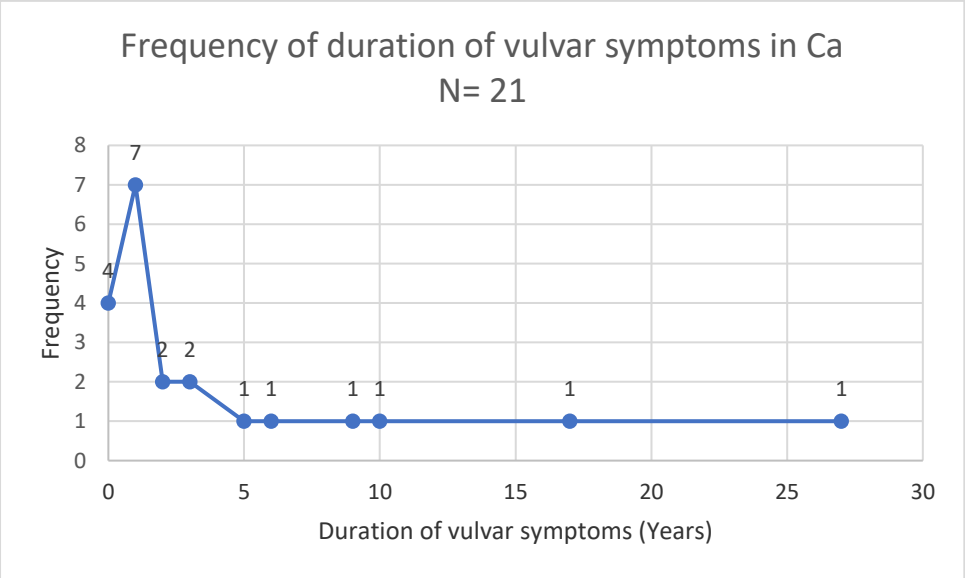

Figure 18: Frequency of duration of vulvar symptoms in invasive cancer

ii. First symptom experienced by participants with invasive cancer

Nine (42.9 percent) had itching as a first symptom, while four (19 percent) reported a pimple as a first symptom. A growth and pain were each reported in two (10 percent for each) cases while warts, a boil, bleeding and pain, and itching were reported in one (4.8 percent for each) case.

The symptom itching was the most frequently reported in 9 (49 %) of participants with invasive cancer followed by *a pimple* (4 cases= 19 %) (Figure 19)

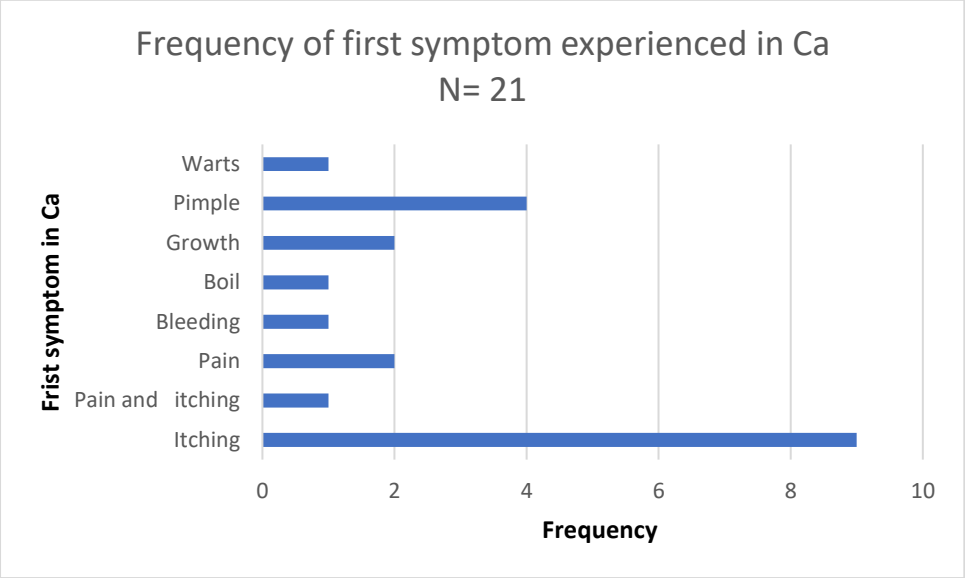

Figure 19: Frequency of the first symptom experienced in invasive cancer

iii. The frequency of itching, pain, painful sex, bleeding and lesion discharge among participants with invasive cancer

The symptom pain, was reported in 20 (95.2 percent) participants with invasive cancer, itching in 17 (81 percent), bleeding in 13 (61.9 percent) and painful sex in three (14.3 percent) (Table 13)]. Participants with invasive cancer were more likely to have pain, bleeding and discharge than those without invasive cancer (P value 0.012, 0.001 and 0.014 respectively). There was no statistical significance in difference presence of itching and painful sex between those with invasive cancer and those without (p 0.309 and 0.328 respectively).

b) Clinical examination findings of invasive cancer

With regards site, 19 cases (90.5 percent) involved the labia majora, 16 (76.2 percent) involved the labia minora, 7 (33.3 percent) the clitoris, three (14.3 percent)

the vagina, two (9.5 percent) the perianal skin and one (4.8 percent) the anus. In terms of the number of lesions, five (23.8 percent) were single while 16 (76.2 percent) were multiple lesions. Regarding size, two (9.5 percent) were two centimetres or less while 19 (90.5 percent) were more than two centimetres. Regarding the type of lesion, nine (42. 9 percent) were fungating lesions, six (28.6 percent) were smooth exophytic lesions, warty and ulcerated lesions were each seen in 3 participants. In terms of lymph node enlargement, 12 (57.1 percent) cases had associated lymph node enlargement while 9 (42.9 percent) had no lymph node enlargement.

4.4. Histopathologic Features of Vulvar Lesions

4.4.1. Histopathologic features of all participants

Among all lesions clinically suspicious for cancer, 83 percrent of them were neoplastic lesions and rest (19 percent) were nonneoplastic. Of those that were neoplastic, invasive cancer accounted for 46 percent of the cases, vulvar intraepithelial neoplasms accounted for 46 percent and seven percent by bening lesions.

The differentiated type of VIN and keratinizing squamous cell carcinoma were not seen in this study.

Figure 20 shows a summary of the frequencies for the diagnostic categories:

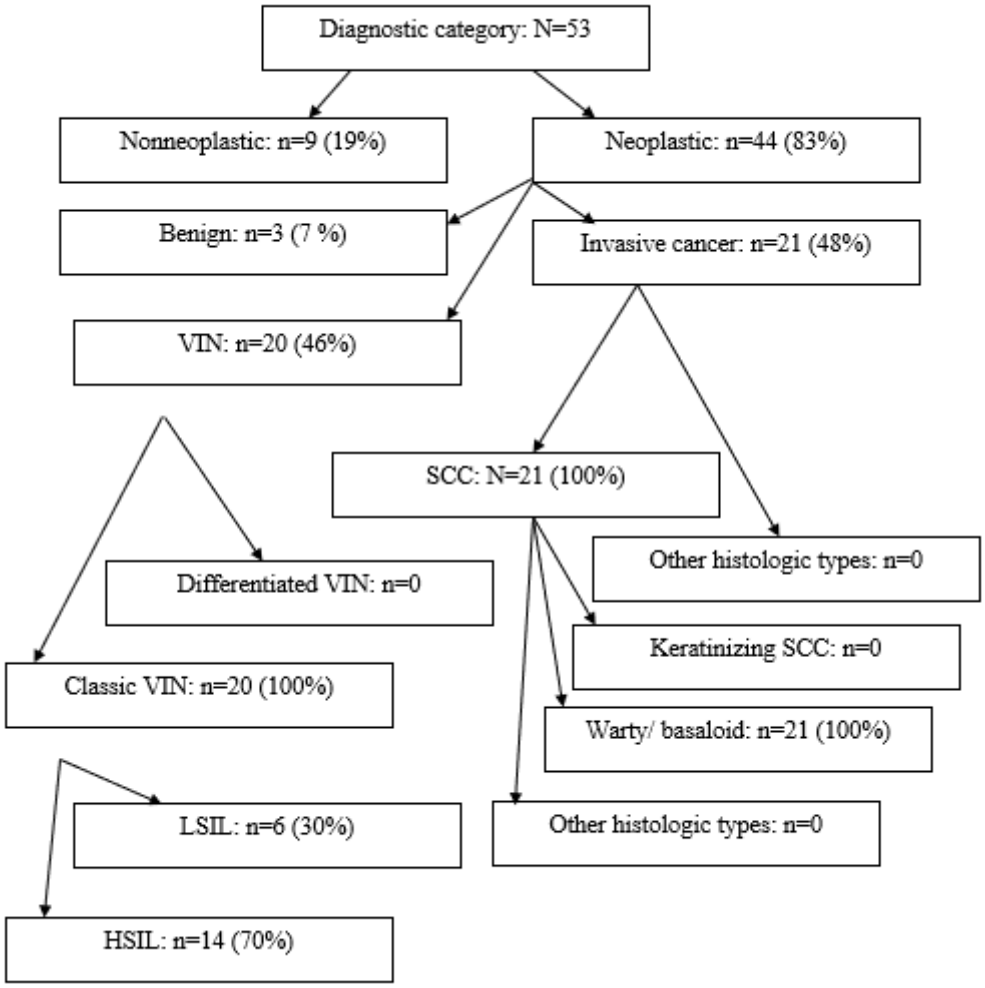

Figure 20: Histologic diagnostic categories

In table 15, the histologic diagnoses are presented as percentage frequencies of all cases (N=53)..

Table 15: Specific histologic diagnoses for all participants (N= 53)

	n	%
Nonneoplastic		
Acute non-specific inflammation	1	1.9
Chronic non-specific inflammation	7	13.2
Herpes simplex ulcer	1	1.9

Neoplastic		
Benign		
Condyloma acuminata	3	5.7
Vulvar intraepithelial neoplasia (VIN)		
Low grade squamous intraepithelial lesion (LSIL)	6	11.3
High grade squamous intraepithelial lesion (HSIL)	14	26.4
Invasive carcinoma		
Squamous cell carcinoma	21	39.6

4.4.2. Histopathologic features of nonneoplastic vulvar lesions

Among nonneoplastic lesions, chronic non-specific inflammation was the most common histologic diagnosis accounting for 13.2 percent (7 cases) of all cases. One case each of acute non-specific inflammation and herpes simplex ulcer accounted for the other nonneoplastic lesions. The histologic section of herpes simples showed skin with focal ulceration and multinucleated keratinocyte, with margination of chromatin and moulding of nuclei (Figures 21 and 22).

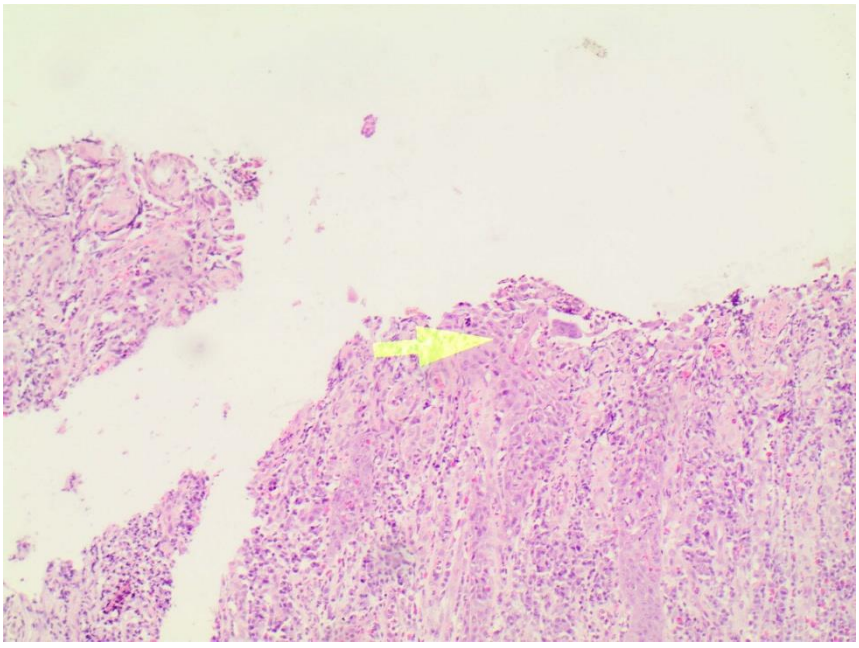

Figure 21: Herpes simplex ulcer x100 magnification. H&E stain.

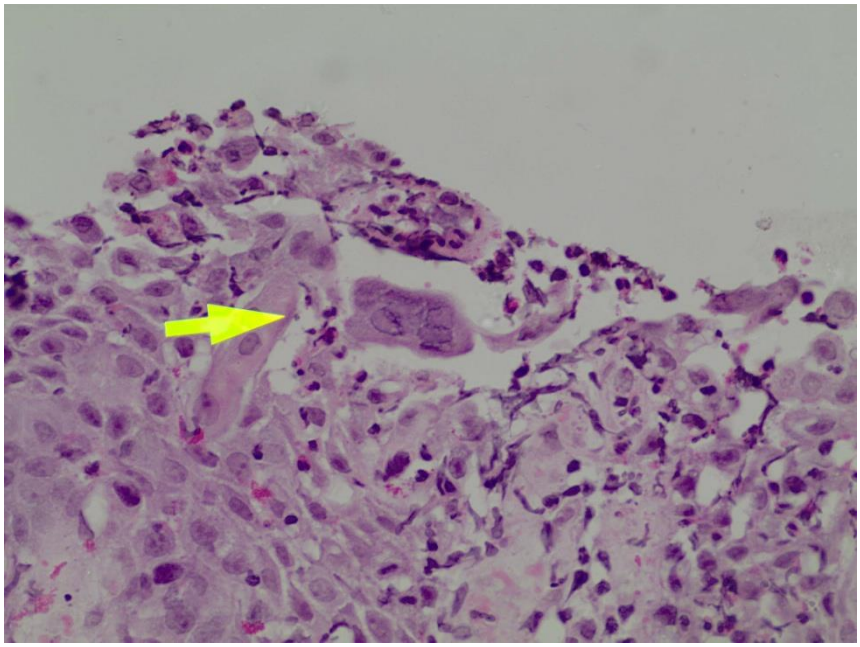

Figure 22: Herpes simplex ulcer x400. H&E stain.

4.4.3. Histopathologic features of benign lesions

Of the benign lesions, only **condyloma acuminatum** was seen. Sections showed characteristic features of hyperkeratosis, papillomatosis, koilocytic atypia and hypergranulosis in the granular cell layer. There was no atypia in the lining epithelium. There was no evidence of invasive malignancy (Figure 23).

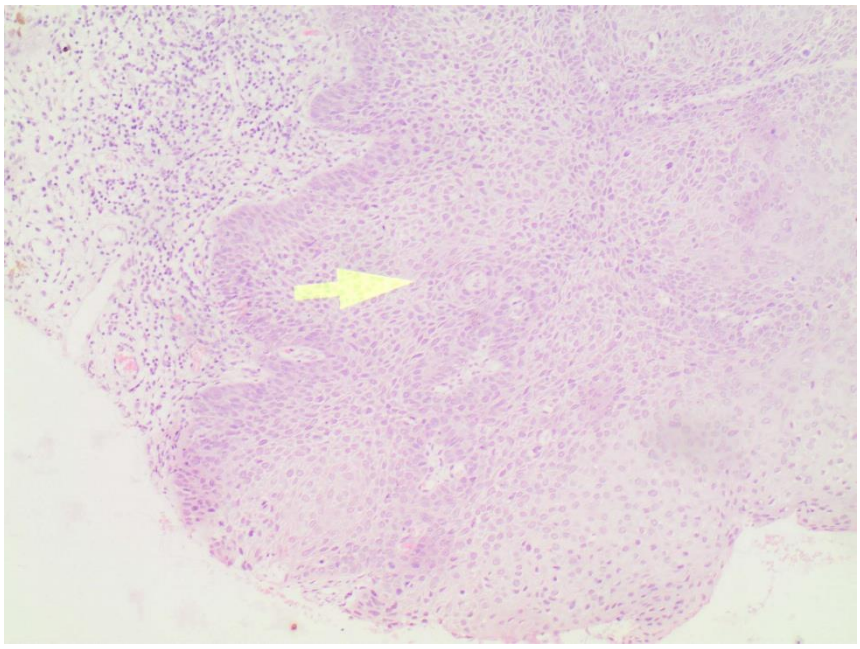

Figure 23: Condyloma acuminatum (x100 magnification). H&E stain.

4.4.4. Histopathologic features of vulvar intraepithelial neoplasia

All the vulvar intraepithelial lesions observed were of the classic/ usual type. Six cases of LSIL (11.3 percent of all cases) and 14 cases of HSIL (26.4 percent of all cases) were seen. Figure 26 and 27 show classic VIN (HSIL) of the warty type. The epithelium shows dysplasia of more than two-thirds (arrow head); the surface shows keratinisation (arrow).

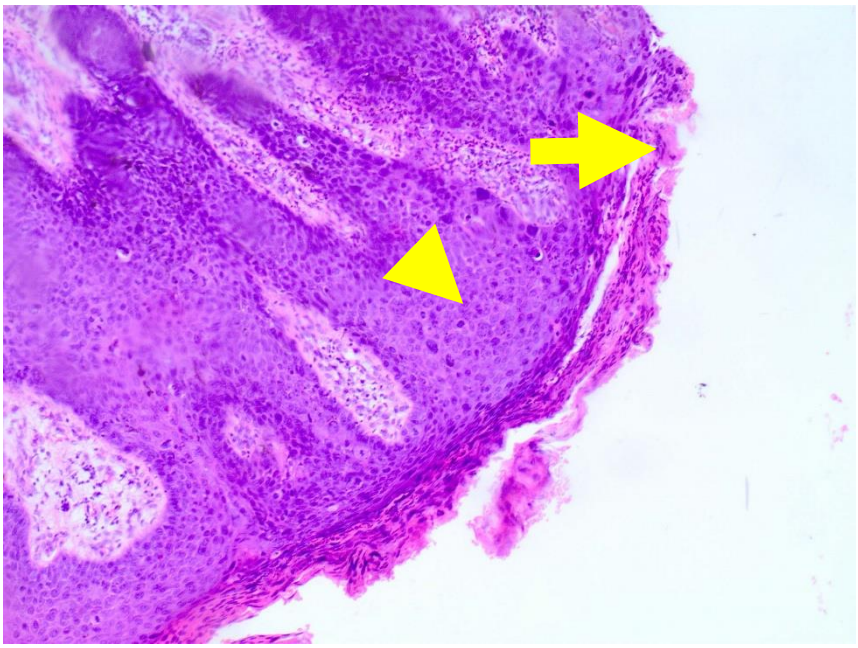

Figure 24: Classic VIN-HSIL x 100 magnification. H&E stain

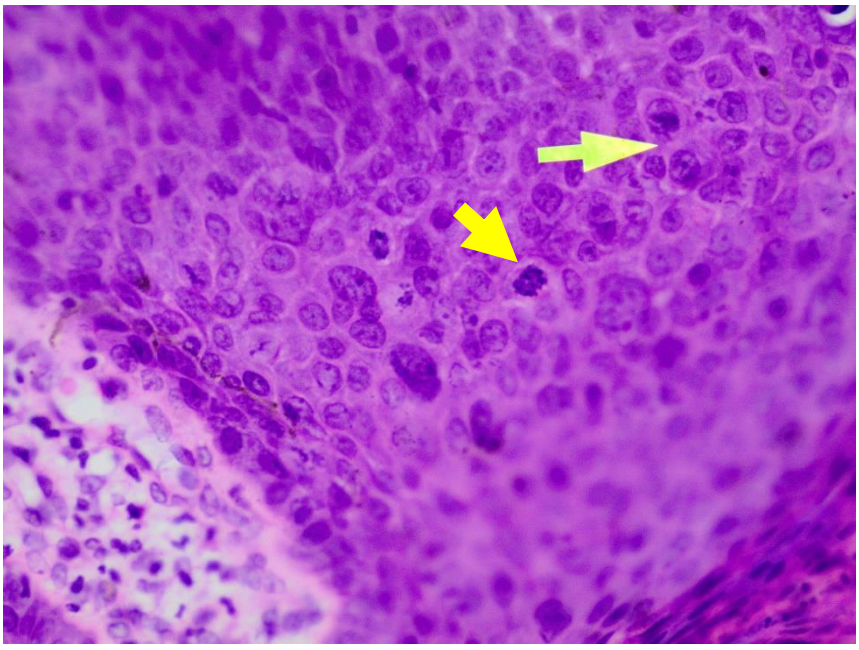

Figure 25: Classic VIN (x400magnification, H&E stain)

4.4.5. Histologic features of invasive carcinoma

All the cases of invasive cancer were squamous cell carcinomas (SCC).

a) Histologic subtypes of SCC

All the cases of SCC were of the warty or basaloid types. None were of the keratinizing type. Between the warty and basaloid types, Figure 26 shows that the majority of cases were warty types of SCC.

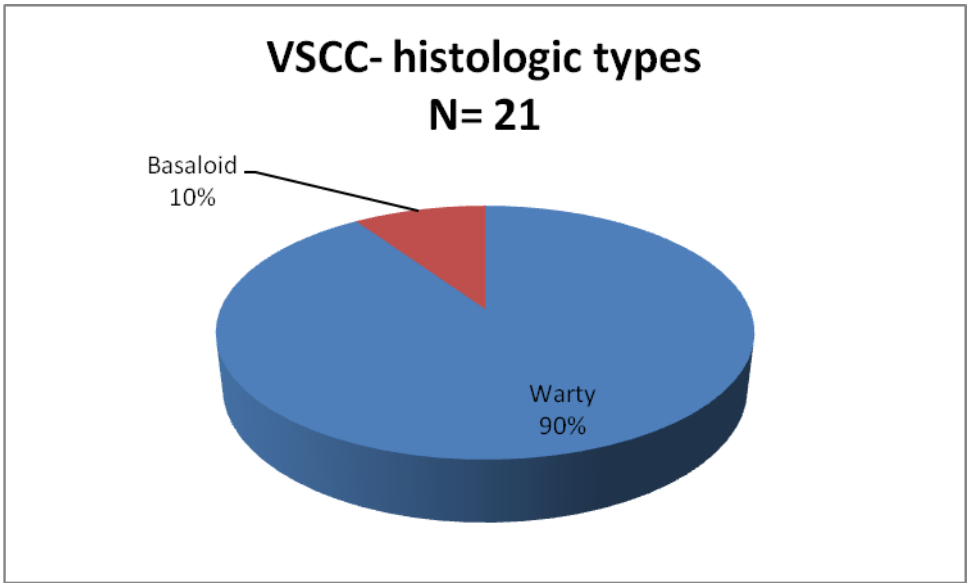

Figure 26: Histologic subtypes of SCC

Basaloid squamous cell carcinoma was identified by cells with high nuclear to cytoplasmic ratios, coarse chromatin, atypical mitoses and incospicous nucleoli.

The Figures 29, 30 and 31 show photomicrographs of basaloid SCC. The arrow in Figure 28 and Figure 29 is pointing at the focus of invasive basaloid squamous cell carcinoma.

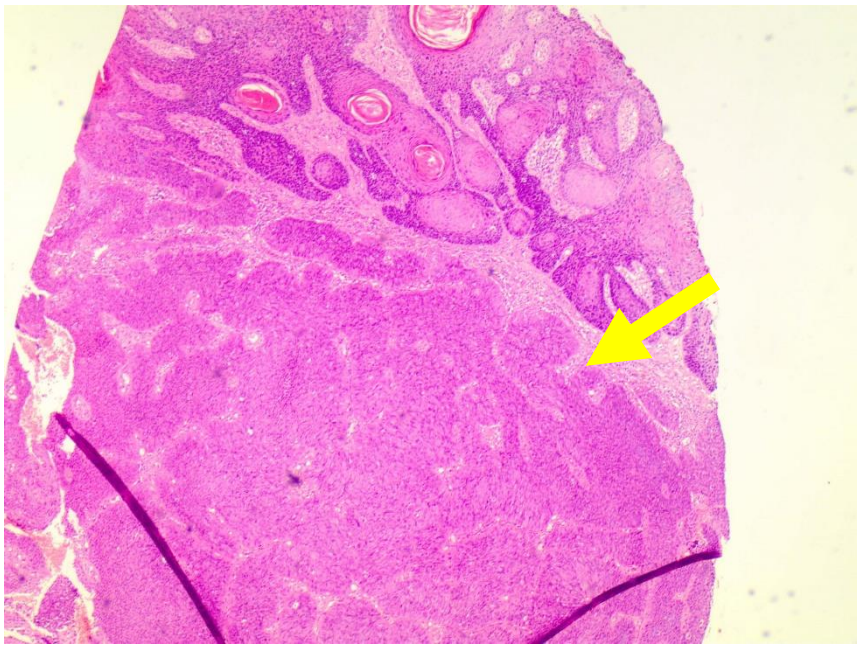

Figure 27: Basaloid SCC x40 magnification. H&E stain.

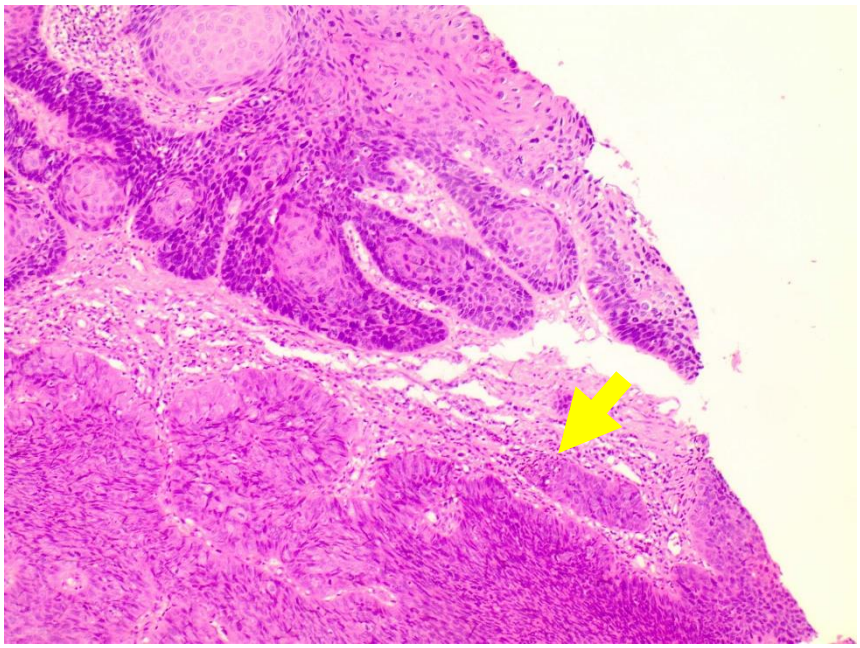

Figure 28: The same lesion as in Figure 29 of Basaloid SCC (arrow) at x100. H&E stain.

The arrow in Figure 29 is showing an atypical mitosis in basaloid squamous cell carcinoma.

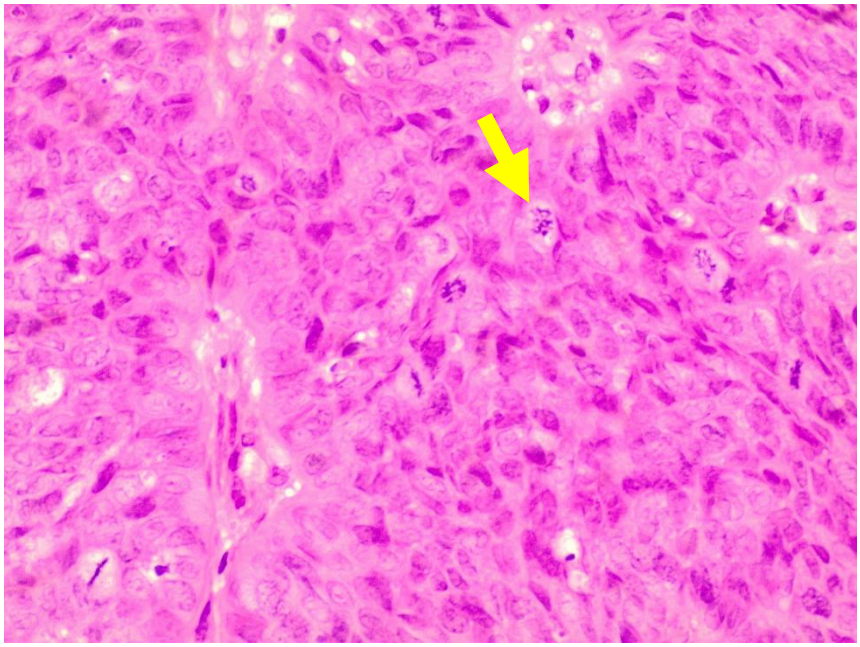

Figure 29: The same lesion as in Figure 28 and 29 at x400 magnification. H&E stain.

Figures 32, 33, and 34 show a warty type of SCC characterized by warty surface epithelium and infiltrative islands and tongues of squamous cells without keratin pearl formation. Warty invasive SCC in Figure 32 (arrow) was characterised by overlying warty epithelium (arrow head).

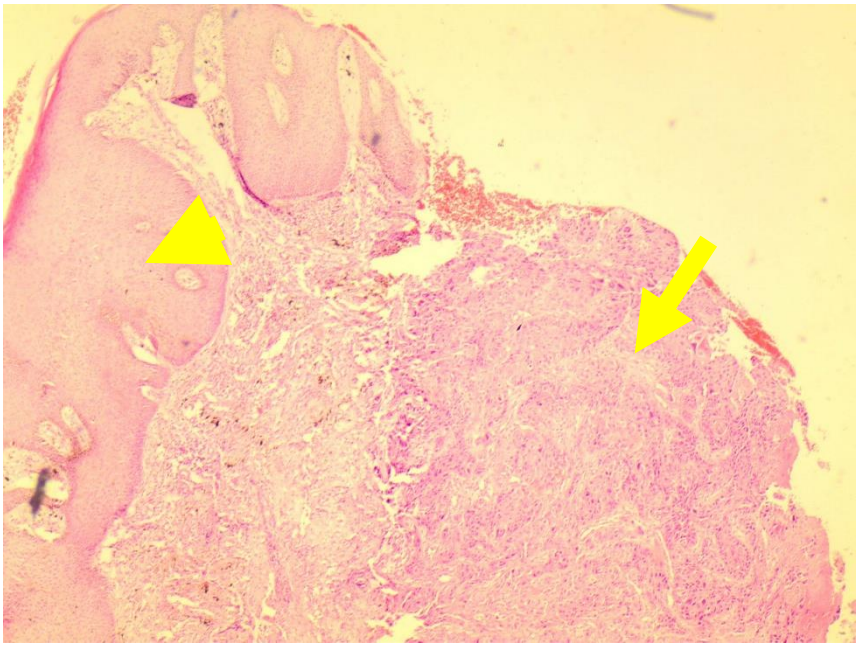

Figure 30: Warty SCC at x40 magnification. H&E stain.

Figure 32 shows invasive warty SCC (arrow) characterised by nests of malignant squamous cells without keratin pearls

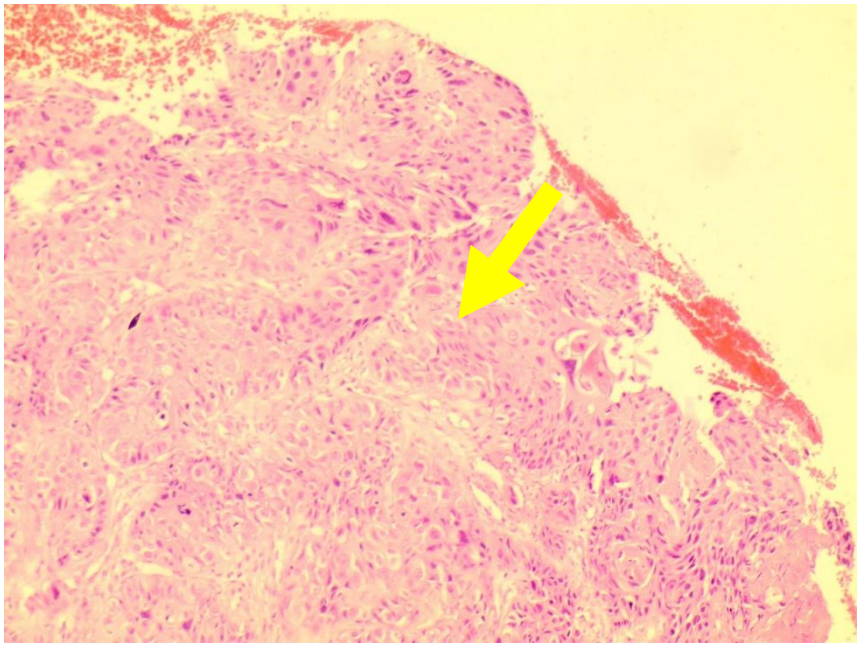

Figure 31: Warty SCC at x100 magnification. H&E stain.

Figure 33 shows warty SCC characterised by hyperchromatic nuclei with eosinophilic cytoplasm (arrow). Nucleoli are not prominent.

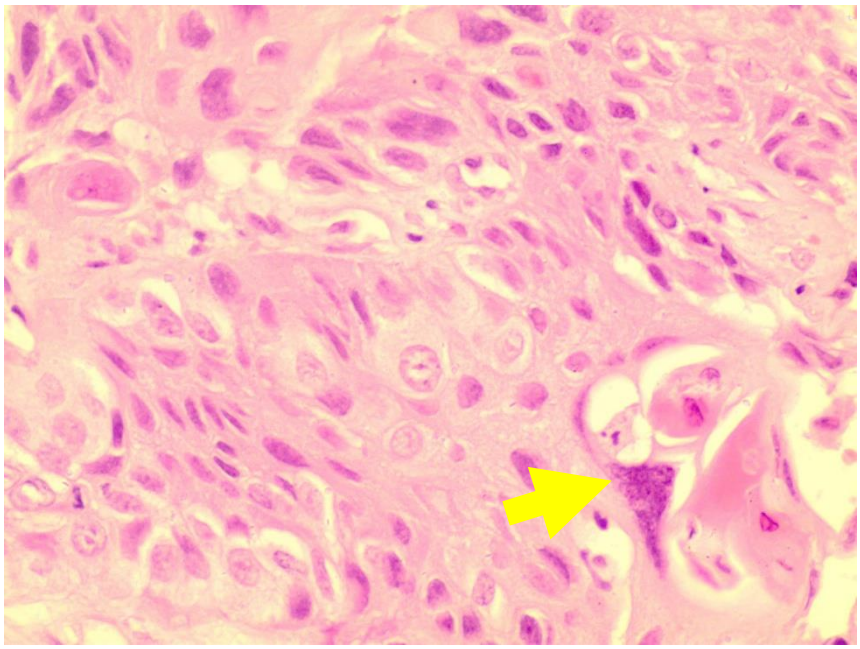

Figure 32: Warty SCC at x400 magnification. H&E stain.

b) Histologic grade of SCC

SCC of the warty and basaloid types are not graded (Kurmanm, Toki and Schiffman, 1993). Therefore, all the SCC cases were not graded.

c) Lymph vascular invasion

Only one case (5 percent) had lymph vascular invasion compared to 20 that did not have (95 percent). Figure 33 shows lympho-vascular invasion (arrow) in warty SCC.

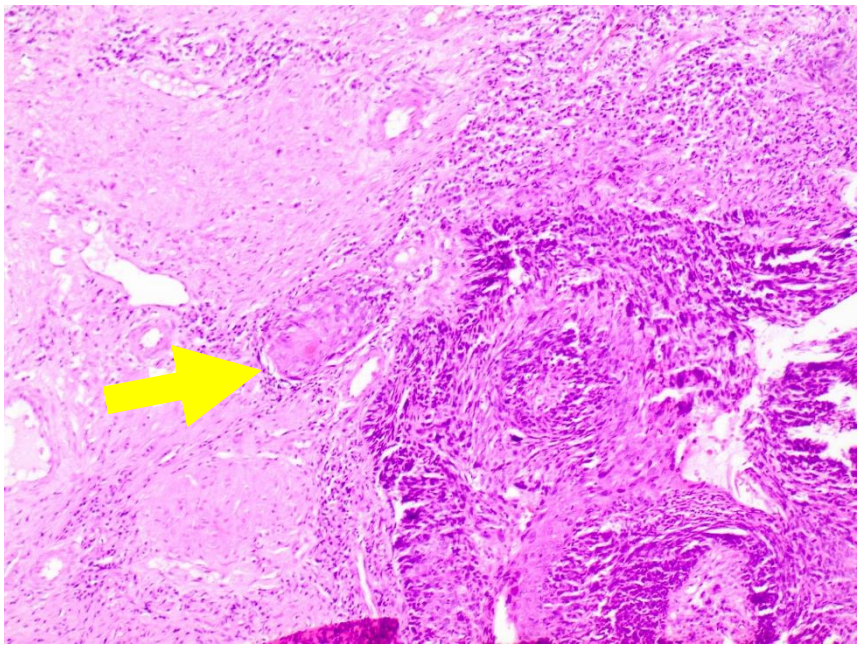

Figure 33: Lymphovascular invasion (LVI) in a warty SCC (x100 magnifications). H&E stain.

The majority of SCC had a high proliferation index (ki67). Fourteen (66.7 percent) cases had a proliferation index of 70 percent or more. Virtually all SCCs had high proliferation index.

Figure 35 shows ki67 proliferation index for squamous cell carcinoma.

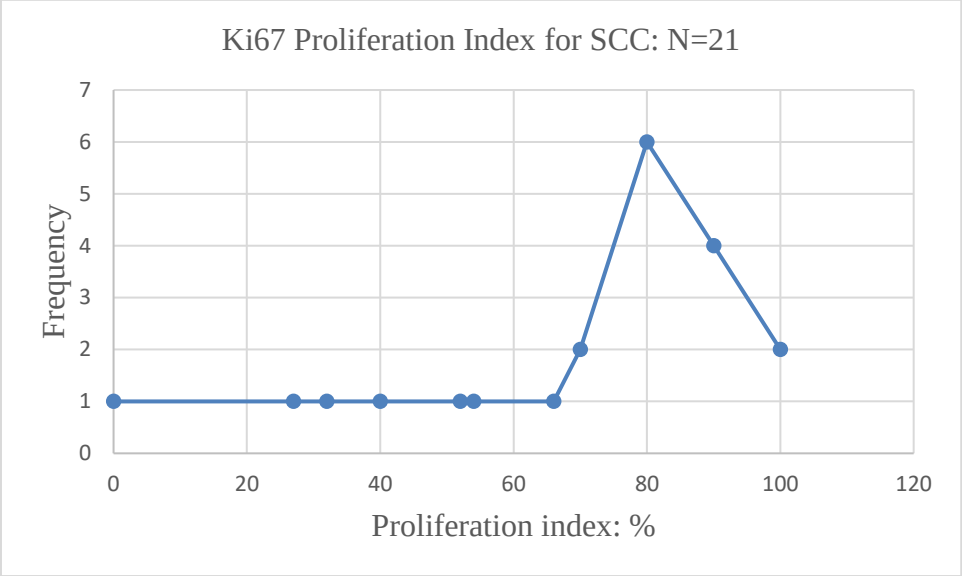

Figure 34: Ki67 proliferation index for SCC

Figure 35 shows diffusely positive (100%) Ki67 in warty SCC.

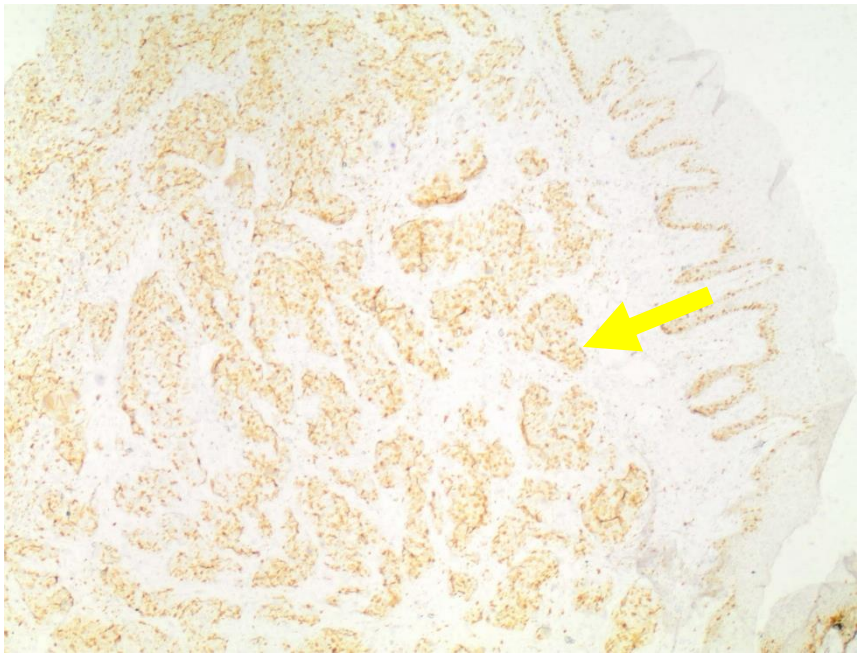

Figure 35: Ki67 proliferation index x40 magnification in SCC

Figure 36 (same SCC as in Figure 35 shows nuclear pattern of staining of Ki67 (arrow).

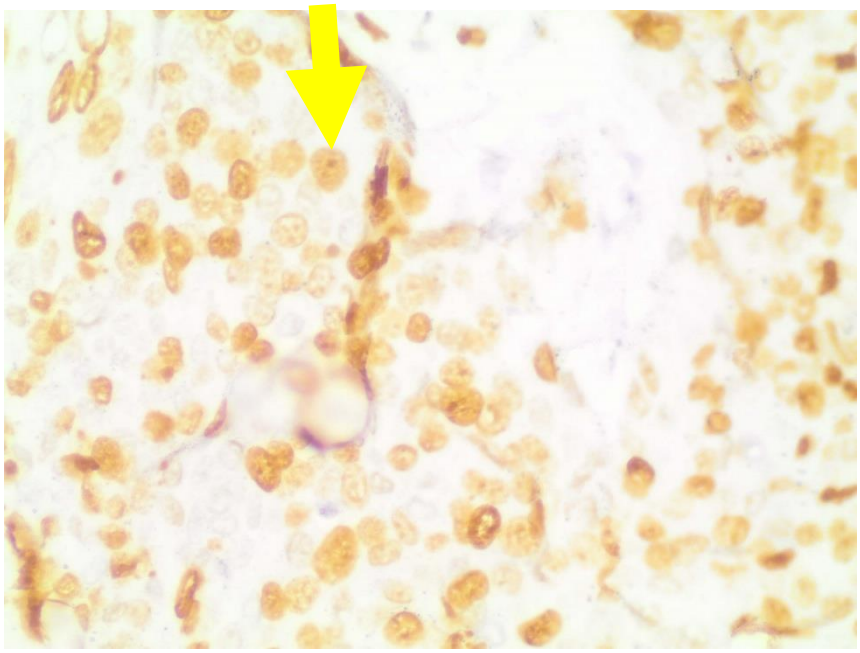

Figure 36: Ki67 proliferation index x400 magnification in SCC

d) P53 immunostaining in SCC

Only one case of SCC (warty type) was positive for p53 (Figures 37 and 38).

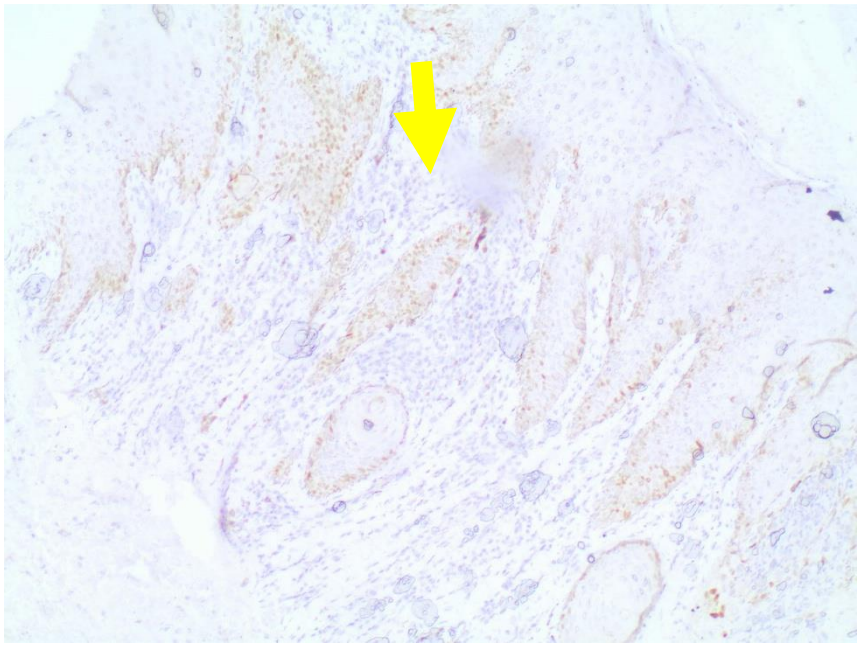

Figure 37: P53 IHC in SCC x40 magnification.

Figure 38 shows nuclear pattern of staining of P53 (arrow).

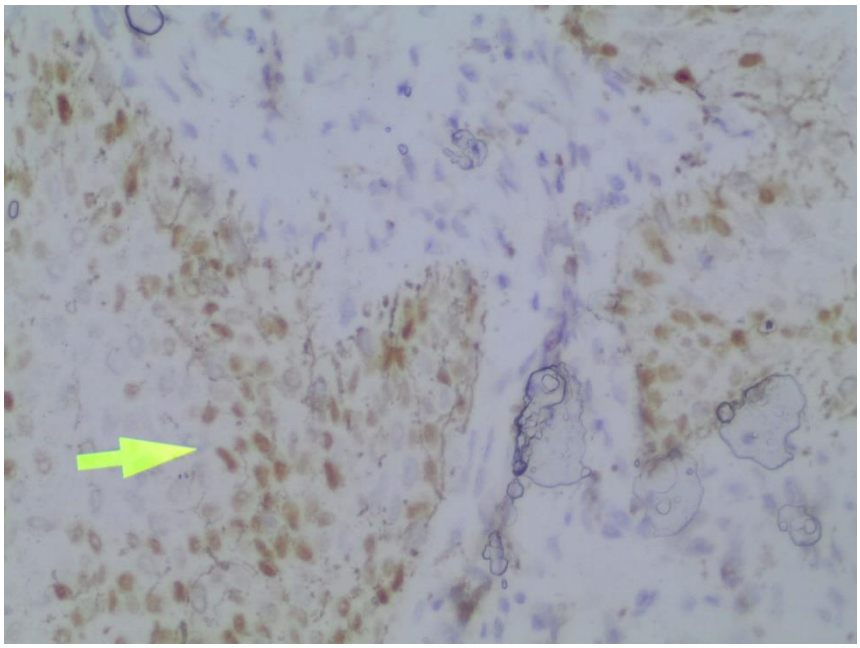

Figure 38: P53 IHC in SCC x400 magnification

e) P16 immunostaining

None of the controls and case samples stained positive for p16.

4.4.6. Prevalence of HR HPV in Vulvar Lesions

a) Prevalence of any type of HR HPV among all neoplastic lesions

Of the 38 samples of extracted DNA, 31 (81.6 percent) were positive for any type of HR HPV (Table 16).

Table 16: Prevalence of any HR HPV in vulvar neoplastic lesions (Xpert® HPV)

	Histologic diagnosis									
	ALL (N=38)		C/A (N= 3)		LSIL (N=4)		HSIL (N=13)		SCC (N= 18)	
Any HR HPV	n	%	n	%	n	%	n	%	n	%
Neg.	7	18.4	1	33.3	1	25	3	23.1	2	11.1
Pos.	31	81.6	2	66.7	3	75	10	76.9	16	88.9

C/A= Condyloma acuminatum, SIL= Squamous intraepithelial lesion, LSIL= low grade squamous intraepithelial lesion, HSIL= High grade squamous intraepithelial lesion, Ca= invasive carcinoma, SCC= Squamous cell carcinoma

b) Prevalence of specific HR HPV among all neoplastic lesions

Of the 31 neoplastic lesions which tested positive for HR HPV, 19 (61.3 percent) were positive for HPV 16. The second most common type was the group P3 (31, 33, 35, 52 & 58) -15 cases (48.4 percent). HPV 18_45 was the least common being positive in only one case (3.2 percent) (Figure 17).

Table 17: Prevalence of specific types of HR HPV among vulvar neoplastic lesions (Xpert® HPV)

	All (N= 31)		C/A (N= 3)		LSIL (N= 3)		HSIL (N= 13)		SCC (N= 16)	
HPV	n	%	n	%	n	%	n	%	n	%
16	19	61.3			3	100	8	61.5	8	50.0
18_45	1	3.2							1	6.3
P3	15	48.4	2	66.7			5	38.5	8	50.0
P4	2	6.5	1	33.3					1	6.3
P5	4	12.9	1	33.3			2	15.4	1	6.3

C/A= Condyloma acuminatum, SIL= Squamous intraepithelial lesion, LSIL= low grade squamous intraepithelial lesion, HSIL= High grade squamous intraepithelial lesion, Ca= invasive carcinoma, SCC= Squamous cell carcinoma

P3, P4 and P5 are pooled results where: P3 includes types 31, 33, 35, 52 & 58; P4 includes 51 & 59 and P5 includes 39, 56, 66 & 68; HPV 18 and 45 are reported together.

c) The frequency of HR HPV according to respondents/ patterns of HR HPV infection among all neoplastic lesions

Of the 31 cases which tested positive for any HR HPV two of them had more than one type of HR HPV infection (6.5 percent) compared to those with only one type of HR HPV. One of them had HPV 16 and P5 (39, 56, 66 & 68); the other had HP 16, P3 (31, 33, 35, 52 & 58) and P4 (51 & 59). P4 and P5 were seen only in combination with other HR types. HPV 16 was present in all combined infections. P3 was present in only one mixed infection. HPV 16, P3 and HPV 18_45 occurred as single infections type and group infections respectively (Figure 39).

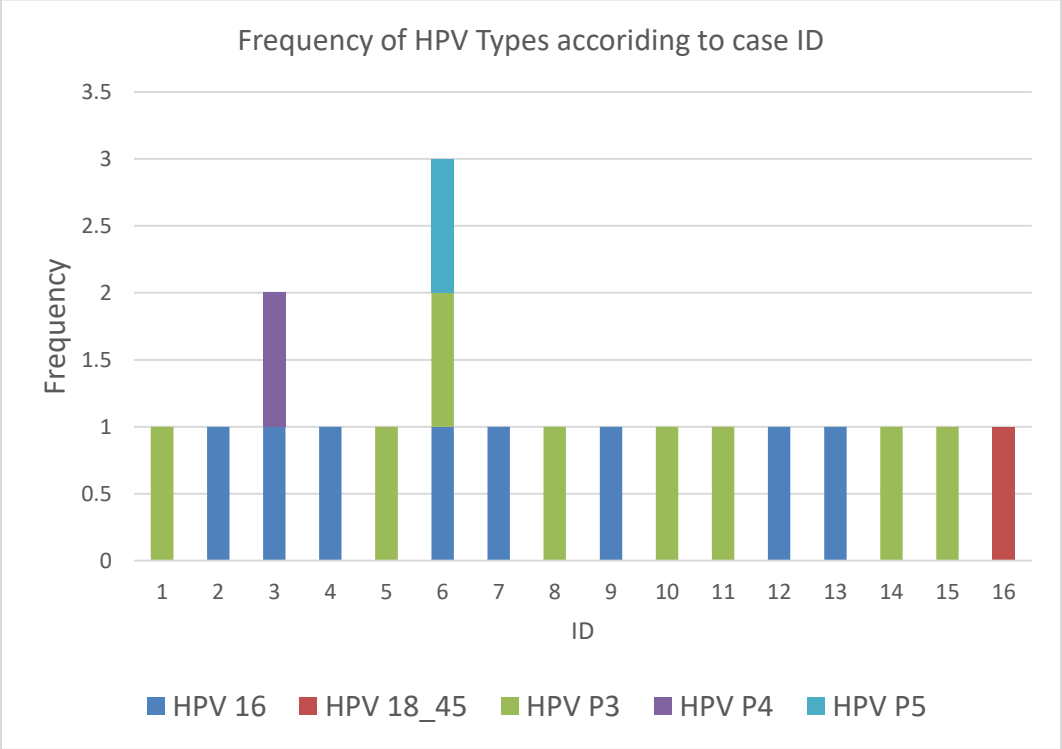

Figure 39: Frequency of HPV types in neoplastic lesions

4.4.7. Prevalence of HR HPV among benign lesions-condyloma acuminatum

a) Prevalence of any HR HPV in benign lesions-condyloma acuminatum

Two (66.7 percent) out of three cases of condyloma acuminatum were positive for HR HPV (*Table 16*).

b) Prevalence of specific HR HPV benign lesions-condyloma acuminatum

HPV 16 and 18_45 were not present in any of the three cases of condyloma acuminatum. P3 was present in two (66.7 percent) while P4 and P5 were each present in one (33.3 percent for each) (Table 17).

4.4.8. Prevalence of HR HPV among vulvar intraepithelial neoplasia (VIN)

a) Prevalence of any HR HPV among vulvar intraepithelial neoplasia (VIN)

Vulvar intraepithelial neoplasia samples tested for HR HPV were in aggregate a total of 17 cases. Out of these, 13 (76.5 percent) were positive for any HR HPV. As can be seen in *Table 16*, among the four cases of LSIL, three (75 percent) were positive for any HR HPV. Among the 13 cases of HSIL, 10 (76.9 percent) were positive for any HR HPV.

b) Prevalence of specific HR HPV among vulvar intraepithelial neoplasia (VIN)

All the three cases of LSIL were positive for HPV 16. None of them was positive for any of the other HR HPVs. HPV 16 was present in eight (61.5 percent) of the

13 cases of HSIL. P3 (31, 33, 35, 52 & 58) and P5 (39, 56, 66 & 68) were present in 5 (38.5 percent) and 2 (15.4 percent) of HSIL respectively. HPV 18_45 and P4 were not present in any of the cases of HSIL (Table 17).

4.4.9. Prevalence of HR HPV among SCC

a) Prevalence of any HR HPV among SCC

Sixteen (88.9 percent) of the 18 cases of SCC were positive for any HR HPV (Table 16).

b) Prevalence of specific HR HPV among SCC

As can be seen in Table 17, HPV 16 and P3 (31, 33, 35, 52 & 58) were each present in 8 cases (50 percent for each) while HPV 18_45, P4 (51 & 59) and P5 (39, 56, 66 & 68) were present in 1 case each (6.3 percent for each).

CHAPTER V

DISCUSSION

This study is the first to provide clinical and pathologic insight about vulvar lesions among Zambian HIV infected women. The study was centred at the largest referral hospital in Zambia located within the same grounds as the only cancer treatment centre. Therefore, it provided the best setting for finding patients with lesions suspicious for malignancy as most of the patients seen at the hospital are referred from other institutions around the country. Of those recruited in this study, at least 13 (24.5 percent) women were residents of towns other than Lusaka.

It took seven months (19Th July 2017 to 26th February 2018) to recruit 53 participants with lesions suspicious for vulvar cancer. Thus, on average, seven participants were recruited each month. Invasive cancer accounted for 21 cases (39.6 percent of suspected cases). This is consistent with a recent report which showed that vulvar cancer was rare in Zambia, being 1.1/ 100,000 population (Kalubula *et al.,*2018); it is also consistent with reports from other parts of the world (Saraiya *et al.*, 2008; Olsen *et al.*, 2012; Alkatout, Schubert, Weigel, *et al.*, 2015; Kehila *et al.*, 2017; Weinberg and Gomez-Martinez, 2019).

Of the women approached for possible enrolment into the study, two did not meet the inclusion criteria-one was younger than 18 years while the other was an older woman (70 years old) who was not HIV infected.

This study specially targeted HIV infected women. The study showed that the most frequent age group among all participants was 30-39 years (37.7 percent) followed by 40-49 years (24.5 percent) while among those with invasive cancer, the most frequent age group was 40-49 years (38.1 percent) followed by the age group 30-39 years (33.3 percent). These findings are closely related to the findings of the ZAMPHIA study which showed that HIV prevalence peaked in the age group 40-49 years (Ministry of Health, 2019) suggesting a high pathogenic role of HIV infection in HPV driven vulvar cancer among HIV infected women. That is, the age distribution of HPV-driven vulvar cancer may be a mirror of HIV infection. This present study also demonstrated that the mean age increased from

nonneoplastic lesions to invasive lesions (figure 11) which has not been described in previous studies such as by Leonard et al (2014). This study also showed that participants with invasive lesions were significantly older than those who did not have invasive cancer (p=0.0049).

While it was reported previously that there was a broad spectrum of vulvar lesions among all women (Arora *et al.*, 2014), this study revealed not so broad a spectrum among the HIV infected study population. This can be explained by the younger overall age distribution of our population. Furthermore, certain lesions such as lichen sclerosus could occur at any age but were reportedly more common in postmenopausal women (Clement and Young, 2014).

5.1 Clinical features of all vulvar lesions

This study revealed that itching was the most common symptom both as a first symptom experienced and also as a symptom ever experienced among all participants. This was similar to what had been described by others previously. The clinical examination findings are discussed under each diagnostic category.

5.1.1. Clinical features of nonneoplastic lesions

The nonneoplastic lesions diagnosed in this study included acute non-specific inflammation, chronic non-specific inflammation and herpes simplex in contrast to Arora *et al*.(2014) who found that the most frequent lesions were lichen sclerosus and squamous cell hyperplasia. The study by Arora and others was not based solely on HIV infected women and had a different age distribution. These factors could account for the differences. This present study had younger participants predominantly. Participants with nonneoplastic lesions had the youngest age than those with other worse lesions in this present study.

a. Symptoms among nonneoplastic lesions

This study demonstrated that on average the symptoms had been of short duration. The most frequent symptom among these lesions was itching as described in previous literature for vulvar lesions in general (Pirog, 2011; Arora *et al.*, 2014).

b. Clinical examination findings of nonneoplastic lesions

The clinical findings particularly as described for herpes simplex were consistent with other literature (Clement and Young, 2014) . These lesions more commonly involved the labia majora than the other regions of the vulva.

5.1.2. Clinical features of benign lesions-condyloma acuminatum

This study found that the affected patients were all adults with the youngest in the second decade of life and the oldest in the sixth decade; this agrees with what has been described in other literature: vulvar condyloma acuminatum could occur at any age but was more common in sexual reproductive years.

a. Symptoms of benign vulvar lesion-Condyloma acuminatum

The findings of this study are similar to reports by others regarding condyloma acuminatum (Léonard *et al.*, 2014). The most common symptom of condyloma acuminatum was itching.

b. Clinical examination findings of vulvar condyloma acuminatum

According to previous literature (Léonard *et al.*, 2014) vulvar condyloma acuminatum could be found on any site on the vulva; however, this study found that these lesions affected the labia majora and minora; furthermore, Leonard et al. (2014) described a wide range of shapes of vulvar lesions; in this study, the condylomas were described as either warty or fungating. This may be due to the fact that there were only three cases of condyloma acuminatum and hence these findings could not be generalized.

5.1.3. Clinical features of vulvar intraepithelial neoplasms (VIN)

Participants with VIN were younger than those with invasive carcinoma in this present study. This study's finding regarding the most frequently affected age group by VIN was almost consistent with that described by Leonard et al. (2014); Leonard reported that the highest frequency was in women of 20–35 years old whereas this study found that it was in the age group 30-39 years.

a. Symptoms in VIN

This study showed that on average VIN had a short duration with 80 percent of cases having duration of fewer than five years, suggesting that the lesions were fast growing. Itching was the most frequent symptom for VIN as reported by others (Clement and Young, 2014).

b. Clinical examination findings of VIN

While Clement and Young (2014) reported that VIN was more common in the labia minora then followed by the labia majora, perianal skin and peri clitoral skin, this present study found that VIN more commonly occurred in the labia majora and the labia minora (70 percent for each) followed by clitoris (4 percent) and perianal skin (2 percent).

5.1.4. Clinical characteristics of invasive cancer

This study showed that the frequency of vulvar cancer peaked in the age group 40-49 years much in agreement with the findings of Kalubula et al. (2018); Kalubula et al (2018) found that the incidence was highest in the same age group at 1.61/ 100,000. This study has contributed to this body of knowledge by demonstrating that the mean age of women with invasive cancer was statistically significantly higher than that of non-invasive lesions (p value 0.049). Also, in terms of immunity, most of the participants had CD4 counts within the range of 200-499 cells/ul compared to those with depressed immunity. The fact that participants with invasive lesions had been on ARV treatment for a long enough time (mean=6.7 years)

suggests that ARV treatment may not offer any protection against developing invasive cancer. Why this is so, has not been established in the literature.

This study found that the frequency of smoking was low among women with invasive cancer suggesting that smoking had a low role in the pathogenesis of vulvar cancer among our study population.

a. Symptoms among participants with invasive cancer

This study showed that a high proportion of participants with invasive cancer had symptoms for less or equal to one year (62 percent). This shows that the tumours were fast growing in most cases. This was confirmed by a high Ki67 proliferation index. As for all the other vulvar lesions, itching was the most common first symptom while pain was the most common symptom ever experienced; on the other hand, in other literature, itching was the most frequent symptom (Alkatout, Schubert, Weigel, *et al.*, 2015). The symptoms of pain, bleeding and lesion discharge were more common among invasive carcinomas than among non-invasive lesions (p= 0.012, 0.001 and 0.014 respectively). These symptoms were reported by Crum et al. (2014), to occur in more advanced cases of vulvar cancer suggesting that the women in this study had to a large extent advanced lesion.

b. Clinical examination findings of invasive cancer

In this study, the most frequent site of invasive cancer was the labia majora, followed by the labia minora, clitoris, vagina, and perianal skin. This is consistent with previous reports/ studies (Hacker, 2004). Compared to other reports that showed that vulvar cancer more commonly presented as a vulvar lump or mass and was only rarely a fungating mass (Hacker, 2004), this study found that fungating masses were more common.

5.2 Histopathologic features of vulvar lesions

5.2.1 Histopathologic features of nonneoplastic vulvar lesions

Herpes simplex ulcer features were characteristic of those described in standard books (Clement and Young, 2014) including the presence of multinucleated giant

keratinocytes with moulding of nuclei and the presence of intranuclear inclusions. The findings of non-specific inflammatory lesions were inconclusive diagnoses. Further studies such as microbiologic studies were necessary to determine the cause of the vulvitis. However, this was beyond the scope of this study.

5.2.2 Histopathologic features of benign lesion-condyloma acuminatum

The lesions of condyloma acuminatum that were examined showed the classical features of condyloma (Léonard *et al.*, 2014) including acanthosis, hyperkeratosis, parakeratosis, and koilocytic atypia among other features.

5.2.3 Histopathologic features of vulvar intraepithelial neoplasms

In this study, all the cases of VIN that were identified were of the classic/ usual type. None of them were of the differentiated type. This is consistent with previous literature (Crum *et al.*, 2014) in that differentiated VIN was reported to occur in older women above the age of 65. This study, in contrast, consisted of women who were of a younger age (mean 40 years).

5.2.4 Histopathologic features of invasive carcinoma

While previous studies have reported that SCC accounted for 90 to 95 percent of vulvar cancer (Pirog, 2011; Alkatout, Schubert, Garbrecht, *et al.*, 2015; Ellenson and Pirog, 2015), this study found that all the cases of invasive cancer were SCC. Furthermore, among the SCC, keratinizing squamous cell carcinoma was not reported; all the cases were of the warty and basaloid types. This finding is consistent with age distribution of the study population as reported by others previously (Crum *et al.*, 2014). Whereas the study population for this study consisted of young women, the keratinizing type is reported to occur in women who are older than 65 years. The high Ki67 proliferation index showed that the tumours were fast growing which could explain the short duration of symptoms in most cases. Immunohistochemistry for P53 was only positive in one case of warty SCC. This was consistent with previous literature (Pirog, 2011). Warty and basaloid SCC only rarely stain positive for P53. Lymph vascular invasion was only

identified in one case; this may be due to one of two possibilities: either there was indeed no vascular invasion, or the invasive component was missed due to sampling error.

5.3 Prevalence of high-risk human papillomavirus among neoplastic lesions

5.3.1 Prevalence of HR HPV in vulvar intraepithelial neoplasm

While HPV prevalence among usual VIN ranged from 66 to 100 percent in various studies which utilized different detection methods (Pino, Rodriguez-carunchio and Ordi, 2013), this study revealed that HR HPV prevalence was 76 percent for all cases of VIN, while among those with LSIL and HSIL it was 75 and 76.9 percent respectively. Only HPV 16, P3 (31, 33, 35, 52 & 58), and P5 (39, 56, 66, & 68) were present in VIN while HPV 18_45 and P4 were not observed. This suggests that the HPV 16, P3 and P5 could have a more important pathogenic role in causing invasive cancer than HPV 18_45 and P4. This would need evaluation by another study as the study population was small in this study. Further, this finding shows a variation from previous reports where HPV 16 and 18 were the most prevalent types.

5.3.2 Prevalence of HR HPV in invasive cancer

According to studies as reported by Pino, Rodriguez-carunchio and Ordi,(2013), the prevalence of HPV among invasive cancers varies widely ranging from 15 to 79 percent. This study found a high prevalence of 88 percent suggesting that almost all the cancers evaluated were potentially driven by HR HPV infection. This can be explained based on the premise that the subtypes of invasive SCC identified are associated with HPV.

Of the cases evaluated for HPV in this study (N= 16), it was determined that the relative contribution of HPV 16, 18_45 and P3 (31.33, 35, 52, 55, 58) was 8 cases (50 percent), one case (6.3 percent) and 8 cases (50 percent) respectively (Table

17). Comparatively, Hartwig *et al.* (2015) reported relative prevalence in terms of HPV 16/18 and HPV 16,18, /31/33/45/52/58 being 73.6 percent (95% CI: 66.4–79.9) and 84.0 percent (95% CI: 77.6–89.0) respectively. The prevalence as found by Hartwig for HPV 16 was much higher than that in this present study suggesting that HPV 31, 33, 35, 52, and 58 play an equally important pathogenic role as does HPV 16.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Vulvar lesions among HIV-infected women affected the young (mean age 40 years), and they most frequently clinically presented as itchy warty lesions. Histologically, the lesions were diagnosed as nonneoplastic (acute and chronic non-specific inflammatory lesions), benign lesions (condyloma acuminatum), Classic VIN (including LSIL and HSIL) and Warty/basaloid type of SCC. The prevalence of any type of HR HPV among all neoplastic lesions (in aggregate) and among invasive lesions was 81.6% and 88.9% respectively, and HPV 16 and P3 (31, 33, 35, 52 & 58) were the most common genotypes among all neoplastic lesions.

6.2. Recommendations

I recommend that a larger multicentre study is done to include both HIV infected and uninfected women, to characterize the vulvar lesions further and to follow up on the survival of those with invasive cancer. This study should include among other things Immunohistochemistry for P16/INK4A or in-situ hybridization to show causation and the documentation of the exact size of the lesions.

As is the case for cancer of the cervix, the only most effective preventive strategy for HIV infection is vaccination against HPV. Vaccination of young girls now will protect them from possible future complications of infection with HR HPV. As shown in the recent report about the positive outcome of vaccination in Scotland, vaccination may potentially provide, not only individual protection but also a group protection against HR HPV. Vaccination against HPV would then provide individual protection and group protection against HPV related malignancies, including vulvar cancer.

As treatment of the vulva is largely dependent on the stage of the disease, efforts to improve early recognition and treatment of vulvar lesions among HIV infected women especially should be accelerated to help reduce morbidity and mortality of HIV infected women. As stated by Canavan et al. (1998), it is not possible to differentiate a malignant lesion from a benign lesion; hence biopsy is the only sure way to diagnose cancer. Clinicians should therefore have a high index of suspicion for vulvar cancer and biopsy all suspicious lesions. More studies need to be done to evaluate factors associated with late presentation of vulvar cancer and appropriate interventions put in place.

Current research is inconclusive about the relationship of HPV and HIV. As also observed in this research, ARV treatment seems not to have any protective effect against HPV infection and its associated diseases. More research needs to be done to solve this puzzle for the HIV infected persons.

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APPENDICES

Appendix 1: Information Sheet for Participation in A Research Study

University of Zambia, School of Medicine

Title of Study: Clinicopathologic Characterisation of Vulvar Cancer Among Human Immunodeficiency Virus (HIV) Positive Women at The University Teaching Hospital in Lusaka, Zambia

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Introduction

I am a doctor who is studying for my Master of Medicine Degree at the University of Zambia. I am also working in the department of Pathology and Microbiology at the University Teaching Hospital. My job is to diagnose diseases in human tissue that is brought to the lab to assist in the care of patients.

What are some general things that you should know about this research study?

You are being asked to take part in a research study. To join the study is voluntary.

You may refuse to join, or you may withdraw your consent to be in the study, for any reason, without penalty.

Research studies are designed to obtain new knowledge. This new information may help people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies. Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of Zambia. You do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study.

You will be given a copy of the consent form. You should ask the researcher (myself) and supervisors named above, or staff members who may assist them, any questions you have about this study at any time.

This is an information sheet. It gives you information about this study. The study staff will talk with you about this information. If you decide to take part in this study, you will be asked to sign or thumbprint a consent form and you will be given a copy of it.

What is the purpose of this study?

Cancer of the vulvar (also known as vulvar cancer) is a cancer that affects the visible parts of a woman's private parts (the vulva) otherwise called the external genitalia. It is an uncommon cancer but recently here at the University Teaching Hospital we have observed an increase in the number of HIV positive women who are coming to the hospital with this cancer. Vulvar cancer is caused by

mainly chronic irritation of the vulvar or infection with some types of a virus called Human papillomavirus (HPV). Most women get the HPV infection at an early stage in their adult lives and it nearly always goes away on its own and is harmless to them. In a few cases the virus causes abnormal changes in the tissues of the vulva (pre-cancer) which later turns into cancer. HPV is the virus that is also responsible for causing cancer of the entry.

The purpose of this research study is to determine the types of cancer of the vulvar that are found in HIV positive women and their possible causes. Also, I would like to find out the possible risk factors of vulva cancer among women who are HIV positive. The results of this study will be used for encouraging the conduct of a larger research that can provide information about vulvar cancer in HIV positive women to doctors and health workers who make decisions about women's health. When the doctors know the causes of vulvar cancer, they can put up appropriate prevention measures including the conducting of vaccination of women and young girls against Human Papillomavirus. Without this vital information, doctors may not formulate good plans on how to care for HIV positive women with vulvar cancer and how to prevent this type of cancer.

Who can participate in this study?

Women 18 years or older who are HIV positive, have an area of concern on the vulvar suspicious for cancer, and freely agree to participate in the research will take part in the study.

Are there any reasons you should not be in this study?

You should not be in this study if you have a bleeding disorder or you are too ill to take part in the research as guided by your attending medical personnel.

How many people will take part in this study?

There will be approximately fifty-three (53) people in this research study.

How long will your part in this study last?

The time you will spend on this study is short. It is anticipated that should you choose to take part in this study, your part in the study will last about one hour to two hours. The only other time you will be contacted is so as to hand over to you the initial laboratory results which will be important for your care. There is no

follow up in this study. Some of the specimen that will be collected will be stored for one to two years or more.

What will happen if you take part in the study?

c) After you sign the consent form, you will be asked some structured questions in the form of a questionnaire. The questionnaire will collect some personal information about you, your past medical history and some risk factors for vulvar cancer. The research study will not report that this particular information came from you much in the same way a census report does not state the names of the people who are counted. You are free to skip questions that you will deem personal or otherwise. A health worker may also check your medical files to look for particular information relating to your health. A doctor will proceed to conduct a thorough examination of your body. The doctor will then conduct a punch biopsy procedure of the area of concern on the vulva. Punch biopsy is a simple procedure after which you can go home once it is done. It will be described in more detail below. In doing a punch biopsy, a small piece of skin from the area of concern will be taken to the laboratory for evaluation to determine if it is cancer or not. If the laboratory results of the punch biopsy reveal that you have cancer or early form of cancer (also known as pre- cancer), you will be immediately referred to the department of obstetrics and gynaecology for routine care.

The punch biopsy procedure involves firstly using some medicine to remove the sense of feeling in the area of concern. A small instrument is then used to remove a tiny piece of skin (less than one third- 1/3 of a centimetre) by using slight pressure in the area. The procedure is quick but you may feel some slight discomfort while the medicine to take away the feeling is being injected but this will pass. Two punch biopsies will be collected. The purpose of the punch biopsy is to know whether the area of concern is a cancer or not. The two biopsies will serve two functions; one will be examined in the laboratory to see if there is cancer and the other to determine the type of human papillomavirus infection present.

You may also have 2mls of your blood taken for CD4 count if the result for the last six months is not present in your medical file.

Information collected from the questionnaire and the results of the test results will be aggregated into a report that will be presented locally as well as in international journals and conferences.

What are the possible benefits from being in this study?

This research study is designed to possibly benefit other women in the future and society by gaining new knowledge. There is little chance you will directly benefit from being in this research study.

What are the possible risks or discomforts involved from being in this study?

Blood collection for CD4 count: Should a CD4 be required for you, please note that the procedure for blood collection will be short and will pose very minimal risk to you. Disposable needles will be used. You will experience some pain at the site where blood is taken from. Bleeding may occur and necessary procedures will be done to stop any bleeding. Drawing blood is part of routine hospital care.

There are some general risks of blood drawing that you should know about. These are highlighted below including what may be done to prevent or mitigate them.

1. Exposure to blood borne viruses through reuse of needles, syringes and lancets, or contaminated work surfaces
    a. Sterile single-use needles will be used
    b. Work surfaces will be cleaned with disinfectant
2. Infection at blood sampling site
    a. hand hygiene performed
    b. Your skin will be cleaned with disinfectant such as 70% isopropyl alcohol and allowed to dry
    c. Sterile needle and syringe will be removed from the packaging just before use
3. Pain at blood sampling site
    a. Only a well-trained person will take the blood sample
    b. A needle of smaller gauge than the selected vein will be used
4. Haematoma or thrombus

a. The selected vessel will be entered at an angle of 30 degrees or less
b. Gauge of needle smaller than the vein will be used
c. pressure to a straight arm for 3–5 minutes will be applied after drawing blood

5. Extensive bleeding
    a. You will be asked whether you are on anticoagulants or have a history of bleeding
    b. A gauge of needle smaller than the vein will be used
6. Nerve damage Use a

a. Antecubital vessels will be used when possible and
b. Probing will be avoided

7. Vasovagal reaction Syncope, fainting
    a. If you are dehydrated, you will be hydrated and a postural blood pressure taken
    b. The attending person will try to reduce your anxiety
    c. Should you express concern, you will be made to lie down or
    d. audio-visual distraction will be provided
8. Allergies
    a. You will be asked whether you have any allergies to latex, iodine and alcohol before starting the procedure.

Punch Biopsy Procedure: For this procedure, risks of complications are very rare but may include: pain or soreness, excess bleeding from the biopsy site or infections at the biopsy site which may require antibiotic treatment.

<u>If you choose not to be in the study, what other care options do you have?</u>

You do not have to be in this research study in order to receive care. You will still have a biopsy and other tests done by your attending doctors even if you do not participate in this research.

<u>How will information about you be protected?</u>

Confidentiality of your personal and medical records in the study will be maintained to the full extent permitted by law. Authorized study assistants and my supervisors from the University will have access to your relevant medical records.

No information will be disclosed to anyone unrelated to the conduct or evaluation of the study.

To protect your identity, you will be assigned a special identification number. Only this identification number will be used in reporting the research findings. The presentation of the results will not state that the information came from you.

Information collected from this study will be entered in a computer and will be password protected and will be accessible only to the principal investigator and supervisors. The computer will be placed in a lockable room with restricted access.

Information that is identifiable to you will be accessed only by members of the study team.

The computerized data will be password protected and will be accessible only to the principal investigator and supervisors.

Should you withdraw from the study, the data collected will also be withdrawn and not further used in the study.

Will you receive results from research involving your specimens?

The results of the biopsy procedure will be directed to you to assist in your care as mentioned above. However, there are no plans to re-contact you or other participants with information about research results.

What will happen if you are injured by this research?

It is unlikely that you will be injured as a result of taking part in this study. UJMT Fogarty global Health Scholars Fund has not set aside funds to pay you for any injury. However, if you are injured as a result of being in this study, I will personally ensure that you receive care by trained medical personnel. This care will be the standard available in the Government Health Institutions in Zambia. However, by signing this form, you do not give up any of your legal rights. If you have any questions about this study or if you experience any side effects, illness or injury that you believe were results from this study you may contact:

What if you want to stop before your part in the study is complete?

You can withdraw from this study at any time without penalty.

You will receive any payment for taking part in this study?

You will not receive any payment for taking part in this research.

Will you have to pay any money to participate in this research?

You will not have to pay any money to take part in this this study.

Are there any potential conflicts of interest?

The principal investigator and the above-mentioned supervisors have no financial interest in the outcome of this study. This research is funded by the U.S. National Institute of Health (NIH) through the consortium of University of North Carolina at Chapel Hill, Johns Hopkins University, Moore house School of Medicine and Tulane University (UJMT) Fogarty Global Health Fellows grant. The researcher and his supervisors do not have a direct financial interest with the sponsor or in the final results of the study.

What if you have questions about this study?

You have the right to ask, and have answered, any questions you may have about this research. If you have questions about the study, complaints, concerns, or if a research-related injury occurs, you should contact the principal investigator and/ or supervisors listed on the first page of this form.

What if you have questions about your rights as a research participant?

All research is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research participant or comments regarding the conduct of this research, you may contact:

The Chairperson
University of Zambia Biomedical Research Ethics Committee Ridgeway Campus, Nationalist Road
P.O. Box 50110
Lusaka, Zambia
Phone: 0211-256067
Email: unzarec@zamtel.zm

Appendix 2: Consent Form for Participation in Research Study

University of Zambia School of Medicine

Title of Study: Clinicopathologic Characterisation of Vulvar Cancer among HIV positive women at the University Teaching Hospital in Lusaka, Zambia

Principal Investigator:
Dr. Fred Maate,
Postgraduate Student, Master of Medicine in Pathology,
Department of Pathology and Microbiology, School of Medicine,
University of Zambia, Lusaka, Zambia; **+ 260- 977-704487**

I, the undersigned, ……voluntarily agree to take part in this study.

I have been given a detailed explanation of the nature and purpose of the study and what I will be expected to do. I understand that the tests and procedures may cause some side-effects and that I am free to withdraw from the study at any time without the need to justify my decision. Also, I will be free to skip questions in the questionnaire I will deem personal or otherwise. This will not affect me or my family availing health care from the Institution and public health services in any way. I understand that my medical records will be seen by representatives of the sponsor or their agents, auditors and regulatory authorities and I agree to the disclosure of this report and any results to regulatory authorities. All data will be treated as confidential and kept for as long as required by law. I hereby give my voluntary, free and informed consent to take part in this study and comply with all the regulations, procedures and interventions stipulated in the study protocol.

……..

……

Signature/thumb impression of Participant Date

……..

Please print name

……...

…….

Signature of Person Conducting Informed Consent Date

……..

Please print name

……..

……

Signature of Impartial Witness Date

……..

Please print name

Appendix 3: Questionnaire on Demographic Data, Medical History and Risk Factors of Vulvar Cancer

Title: Clinicopathologic Characterisation of Vulvar Cancer Among HIV Positive Women at The University Teaching Hospital in Lusaka, Zambia.

Principal Investigator: Dr. Fred Maate

A. Date of interview and participant ID number.

Date	**Participant ID number**
dd/mm/yyyy	

B. Demographic/ Personal Data

1. How old are you? ☐ **Years**
2. Do you live in Lusaka? 1. Yes ☐ 2. No ☐
3. What kind of a residential area do you live in?

(1). Low cost residential area? ☐

(2). Medium cost residential area? ☐

(3) High cost residential area? ☐

(4). Rural area? ☐

4. What is your current marital status?

(1). Single ☐

(2). Married ☐

(3). Divorced/Separated ☐

(4). Widowed ☐

(5). Not married but living with partner ☐

5. Have you ever been employed? 1. Yes ☐ 2. No ☐

6. What is your highest level of school?

(1). No education ☐

(2). Primary ☐

(3). Secondary ☐

(4). More than secondary ☐

C. Sexual behaviour

7. Have you ever had sex? 1. Yes ☐ 2. No ☐

If No, go to question 10.

8. How old were you when you first had sex? ☐ Years old.

9. Throughout your life, what is the number of partners with whom you had sex? (Approximately): ☐

D. Gynaecologic and Obstetric History

10. How long ago (in years) did you notice the area of concern (disease presence) on your vulva?

1. Less than a year ☐
2. More than a year ☐ **specify number of years** ☐

11. What was the first symptom that you experienced on the area of concern on your vulvar?

(1). Itching ☐
(2). Pain ☐
(3). Painful sexual intercourse ☐
(4). Discharge ☐
(5). Other (Specify) _____

12. Since the development of the abnormal condition on your vulva started, have you ever experienced any of the following on the area of concern?

(1). Itching ☐
(2). Pain ☐
(3). Painful sexual intercourse ☐
(4). Bleeding ☐
(5). Discharge ☐

13. Have you ever been examined to see if you had cancer of the cervix? 1. Yes ☐ 2. No ☐

If the answer is No skip to question 16.

14. Which mode of examination of the cervix was used?

(1). Pap smear testing ☐
(2). The clinician/ nurse looked into your cervix after applying some liquid (vinegar) to it? ☐
(3) I do not know ☐

15. Upon examination of the cervix, were you found to have any of the following? ☐

(1). A condition which can lead to cancer (CIN)

(2). Cancer of the cervix ☐

(3). Normal cervix ☐

(4). I do not know ☐

16. Are you pregnant? 1. Yes ☐ 2. No ☐

17. Have you ever experienced sexually transmitted diseases (STDs or infection with herpes, Chlamydia, gonorrhoea, syphilis) in the past? **1.** Yes ☐

2. No **3.** I do not know ☐

If yes, how many times? **1.** Once ☐ 2-4 times ☐ 5 or more times ☐

E. Medical History

18. Are you experiencing one or more of the following health problems?

Yes ☐ No ☐

(**If YES tick against appropriate response?**)

(1). Diabetes (Sugar disease) ☐

(2). Tuberculosis (TB) ☐

(3) Any form of cancer ☐

(4). Had an organ transplant ☐

F. Drug History

19. Are you currently taking antiretroviral medication (ARVs)? **1.**

Yes ☐ **2.** No ☐

If answer is No, skip to question 21

20. How long have you been on ARVs?

1. Less than a year ☐

2. More than a year ☐ **specify number of years** ☐

Skip to question 22

21. Have you ever been on ARVs in the past? 1. Yes ☐ 2. No ☐

If yes, why are you currently **NOT** on ARV medication?

(1). Personal decision to stop without medical advice ☐

☐

(2). According to medical advice

22. Have you ever experienced any of the following? Yes ☐ No ☐

(If YES tick against appropriate response)

(1). Use of steroid medication such as cortisone (injection or pills) in the last 10 years ☐

(2). Used anticancer medication in the last 10 years ☐

G. Life habits

23. Are you a current smoker? 1. Yes ☐ 2. No ☐

If yes, how many cigarettes do you smoke a day? ☐

Also, how long (in years) have you been smoking? ☐

24. **If answer to question 23 is No**, have you ever smoked? 1. Yes ☐

2. No ☐

If yes, how long (in years) has it been since you stopped smoking? ☐

25. Do you currently snuff (inhalation of or sniffing powdered tobacco)? 1. Yes ☐ 2. No ☐

If yes (1) How many times do you snuff in a day? ☐

(2) How long in years have you been snuffing? ☐

26. Do you drink alcohol? 1. Yes ☐ 2. No ☐

If yes (1) how long (in years) have you been drinking? ☐

(2) How often do you drink alcohol?

1. Every day ☐. More than once a week ☐ Once a week ☐

3. Occasionally ☐

27. Have you ever used any form of birth control method (family planning)?

Yes ☐ No ☐

If yes what kind?

(1). Birth control pills ☐

(2). Condoms ☐

(3). Diaphragm ☐

(4). Depo- Provera (injections) ☐

(5). Intrauterine device (IUD) ☐

(6). Rhythm, calendar, natural method ☐

28. Which of the following methods of cooking do you use?

1. Stove ☐ 2. Brazier ☐ 3. Firewood ☐

If you use **firewood**,

(1). how often do you use it?

1. Every day ☐ 2. Once a week ☐ 3. At least three times per week ☐

(2). How long in years have you been using **firewood** for cooking?

1. Less than a year ☐
2. More than a year ☐ **specify number of years** ☐

Appendix 4: Clinical Examination Form

CLINICOPATHOLOGICAL CHARACTERISATION OF VULVAR CANCER AMONG HIV POSITIVE WOMEN AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA ZAMBIA 2017PRINCIPAL

INVESTIGATOR: DR. FRED MAATE

To be completed by clinician

DATE:

PARTICIPANT ID #: VCS

CLINICAL EXAMINATION FINDINGS

1. Site involved on vulva

1. Labia majora ☐
2. Labia minora ☐
3. Clitoris ☐

2. Other sites other than vulva

1. Vagina ☐
2. Cervix ☐

3. Number of lesions of vulva

1. Single lesion ☐
2. Multiple lesions ☐

4. Size (widest diameter): if lesions are multiple take the size of the largest lesion

1. Less than 2cm ☐
2. Greater than 2cm ☐

5. **Type of lesion**:

1. Exophytic lesion
   i. Smooth exophytic lesion ☐
   ii. Warty lesion ☐
   iii. Fungating lesion ☐
2. Flat lesion ☐
3. Ulcerated Lesion ☐

6. Enlarged regional lymph nodes

d) 1. Yes 

e) 2. No 

Completed By:

Signature: _____

Dr _____

Appendix 5: Gross Examination Protocol

Clinicopathologic Characterisation of Vulvar Cancer Among HIV Positive Women at The University Teaching Hospital in Lusaka, Zambia.

Principal investigator: Dr. Fred Maate

Guidelines and Procedure

- Punch biopsy is performed in the evaluation of inflammatory disorders and occasionally as an initial biopsy for neoplasms.
- Read the acquisition form first (very important)! This will tell you how to go about processing the specimen
- Measure the skin surface diameter and the depth of excision. Use nomenclature of dermatology to describe any lesions.
- Occasionally, you may receive a punch biopsy which looks as if a piece has been chopped off. The submitting physician may have sent a portion to microbiology for culture, as a separate specimen for immunofluorescence or to another lab as part of a research protocol or for other special studies. Describe it as a semi-circular punch biopsy.

Sections for Histology

- Submit the entire biopsy
- Request that sections be oriented on edge.

Dictation Template

Received without fixative/ in formalin, labelled _____and "_____" is a _____ (diameter) x _____ (length) mm skin punch biopsy. The skin surface is _____ (colour) with _____ (centrally/ eccentrically located _____ (colour---- darkly pigmented, red) _____ (configuration--- papule, macule, etc.) measuring _____cm in diameter with is_____ (border). The biopsy is longitudinally bisected to reveal a depth of _____mm of the lesion on the cut surface. Specimen entirely submitted in cassette #_____.

1 block: 1. **H&E** 2. **Immunohistochemistry**: p 53, p16, ki67 (MIB1) or any other appropriate immunohistochemical stain.

Reference

1. Skin and appendages: Skin Punch Biopsy. *e-Manual for Specimen Gross Examination in Surgical Pathology (3rd Ed).* [Online] Copyright 2006 - 2013, NuoNuo Medical Informatics, LLC. [Cited: November 21, 2016.] http://www.essentialpathology.info/Gross_manual/index.html.

Appendix 6: Haematoxylin and Eosin Staining Protocol

Clinicopathologic Characterisation of Vulvar Lesions Among HIV Positive Women at The University Teaching Hospital in Lusaka, Zambia.
Principal investigator: Dr Fred Maate

Purpose

H&E is the main diagnostic staining method in histopathology. It is effective in the demonstration of major histological tissue structures particularly nuclei which are the most important structures in the viewing of histological sections for pathological changes. Since H&E is almost always the initial stain, it will invariably provide clues as to which other stains are required.

Principle

Haematoxylin a dark purplish positively charged dye will stain negatively charged tissue components (nuclei particularly chromatin), and the pink to red negatively charged eosin will stain positively charged tissue components (i.e. cytoplasmic elements, connective tissue and muscle) in various shades of orange/pink highlighting the general tissue structure. The affinity of dyes for tissue elements is affected by a number of factors such as the structure and shape of the dye molecule, the charge distribution of the dye and the solvent characteristics.

Haematoxylin itself is not a stain but is oxidized to haematin through a charged mordant). In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant. There are different types of Haematoxylins (alum, iron, tungsten, molybdenum and lead Haematoxylin-the naming according to the mordant used. Alum (for aluminium) Haematoxylins are the most commonly used for routine H&E staining. They include; Mayer's, Enrlich, Harris, Carrazi, Cole, Gills and Delafield's Haematoxylin.

The choice of Haematoxylin is according to personal preference and the staining times with alum haematoxylins may vary according to:

- type of Haematoxylin used
- age and intensity of use of stain
- method of staining

- pre and/or post treatment of sections.

Two methods are used for H&E staining

1. Progressive staining-No differentiation
2. Regressive staining- differentiation is employed

Differentiation is the use of acidic solutions to remove excess background staining by Haematoxylin. It also helps to sharpen the nuclear detail.

There are three commonly used forms of eosin-eosin Yellowish (tetrabromofluorescein, disodium salt Cl 45380), eosin Bluish (the dinitro-dibromo-derivative Cl 45400), and eosin Alcohol Soluble (the ethyl derivative Cl 45386), the former is preferred. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenated fluorosceins) and the staining intensity is highly dependent on PH. 1%Eosin is routinely used.

Equipment, Reagents, Supplies and PPEs (Personal Protective Equipment)

Equipment	Reagents	PPE
Automated tissues stainer or coplin jars for manual staining. Slide holder racks	Haematoxylin, Eosin, Xylene, Alcohol (absolute, 95% and 80%), bluing agent, 1% acid alcohol, water.	Gloves. Lab coat

Specimen

Slide with tissues which are appropriately sectioned, well labelled and adequately dried.

Special safety precautions

Wear appropriate PPE, when handling acids, alcohols, xylene and dyes.

Procedure

- Take sections to water, that is;

- Xylene, three changes to deparaffinize (1 minute each) then hydrate through graded alcohols-absolute 1 minute, 95% 1 minute and 80% 1 minute then water for 2 minutes.
- Stain in Mayer's alum Haematoxylin for 10-15 minutes
- Rinse in running tap water for 2 minutes
- Differentiate in 1% acid alcohol for 30 seconds
- Blue in running tap water or any bluing agent for 2-5 minutely until sections are again blue
- Wash in tap water 2 minutes
- Stain in 1% eosin for 3-5 minutes
- Rinse in tap water for 2-5 minutes
- Dehydrate through alcohols 1 minute each
- Clear in three changes of xylene 1 minute each
- Mount in DPX.

Quality Control

- Ensure that sections are adequately dried as inadequately dried sections will not deparaffinize sufficiently and will not pick up the stain consistently
- Always use fresh absolute alcohol for removal of xylene when before hydrating
- Filter Haematoxylin to remove the metallic sheen that can cause precipitates on the stained slides. Filtering will also help to monitor the condition of the Haematoxylin (refer to reagent test quality protocol for H&E).
- The addition of a little acetic acid (0.5ml to 1000 ml stain) is said to sharpen the staining
- Washing with water after eosin staining must be monitored carefully to prevent the removal of too much eosin.
- Always stain a control slide and check for the quality before staining the test slides.
- Check for staining quality and identify the tissues microscopically before giving the slides to the pathologist.

Reference range/Test Interpretation

- Haematoxylin- bright purple blue (sharp nuclear detail) and not obscured by eosin
- Eosin-at least three colour shades of red, pink and/or orange respectively.
- No or less background staining

Notes and Limitations

Needs a greater knowledge on how to optimize or modify the technique to achieve the

Desired results

- Lack of PH monitoring or stain consistency.
- Nonspecific staining
- Different preferences of stain intensities by individual pathologists.

References

1. McGill (2005), Blood Laboratory, McGill University, Montreal, Canada.
2. Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.
3. John D Bancroft, Marilyn Gamble (sixth edition) Theory and Practice of Histological techniques.

Appendix 7: Manual Staining Protocol Hidef HRP Detection

1. Trilogy/ pressure cooker set for 15 min on high (120 C). 10Trilogy: 190ml DH2O

a. Ensure pressure release button is on “pressure”.

b. When 15 minutes is complete, using face shield ad heat protective gloves turn pressure release button to “pressure release”.

c. Wait for pressure indicator (red button) to recede.

d. Open pressure cooker, place slide sin to the hot rinse (second trilogy container)

2. Trilogy hot rinse→ 2min
3. Wash with DI water→30 seconds (rinse)
4. Peroxide block→10 min (incubate on the slide
5. Wash with TBS BUFFER→60 seconds agitate
6. Apply primary antibody→30 minutes (incubate on the slide)
7. Wash with TBS buffer→60 seconds (Agitate)
8. HRP HiDef Amplifier (HiDef)→10 minutes (incubate on the slide)
9. Wash with DI water→60 seconds (rinse)
10. STOP! Prepare Chromogen FRESH!!!
11. DAB Chromogen→3min (3-5min) (incubate on the slide)

a. CAUTION! DAB is light sensitive; protect slides from light during incubation

12. Wash with DI water→60 seconds (rinse)
13. Counterstain with Haematoxylin incubate 1 minute on the slide
14. Wash with DI water→60 seconds (rinse)
15. Cover slipping
16. Dehydrate slides as follows
17. 95% Alcohol→3 minutes
18. 100% Alcohol→3 minutes
19. Xylene→5 minutes (x3)
20. Place 2-3 drops of permanent mounting medium onto the slide and apply coverslip, spreading the mounting medium by capillary action
21. Allow slides to dry and view under the microscope

Appendix 8: Excerpt from College of American Pathologists (Cap) Protocol for the Examination of Specimens from Patients with Primary Carcinoma of the Vulva

Authors

Saied Movahedi-Lankarani, MD*; Uma Krishnamurti, MD, PhD*; Debra A. Bell, MD; George G. Birdsong, MD; Charles V. Biscotti, MD; Christopher N. Chapman Jr, MD; Blake Gilks, MD; Veronica Klepeis, MD, PhD; Anthony G. Montag, MD

With guidance from the CAP Cancer and CAP Pathology Electronic Reporting Committees.

** Denotes primary authors. All other contributing authors are listed alphabetically.*

Surgical Pathology Cancer Case Summary

Protocol posting date: June 2017

VULVA:

Select a single response unless otherwise indicated.

Procedure (Note A)

___ Local excision

___ Wide excision

___ Partial vulvectomy

___ Total vulvectomy

___ Radical vulvectomy

___ Other (specify): _____

___ Not specified

Tumour Site (select all that apply)

___ Right vulva

+ ___ Labium majus

+ ___ Labium minus

+ ___ Bartholin gland

___ Left vulva

+ ___ Labium majus

+ ___ Labium minus

+ ___ Bartholin gland

___ Clitoris

___ Other (specify): _____

___ Not specified

Tumour Size (Note B)

Greatest dimension (centimetres): ___ cm

+ Additional dimensions (centimetres): ___ x ___ cm

___ Cannot be determined (explain): _____

Tumour Focality

___ Unifocal

___ Multifocal

___ Cannot be determined (explain): _____

___ Not specified

Histologic Type (Notes C and D)

___ Squamous cell carcinoma, NOS

___ Squamous cell carcinoma, keratinizing

___ Squamous cell carcinoma, nonkeratinizing

___ Squamous cell carcinoma, basaloid

___ Squamous cell carcinoma, verrucous

___ Squamous cell carcinoma, warty

___ Squamous cell carcinoma, papillary

___ Adenocarcinoma, NOS

___ Adenocarcinoma, mammary gland type

___ Adenocarcinoma, skene gland type

___ Adenocarcinoma, sweat gland type

___ Adenocarcinoma, intestinal type

___ Adenocarcinoma, with associated Paget disease

___ Adenosquamous carcinoma

___ Transitional cell carcinoma

___ Adenoid cystic carcinoma

___ Adenoid basal carcinoma

___ Small cell neuroendocrine carcinoma

___ Large cell neuroendocrine carcinoma

___ Undifferentiated carcinoma

___ Other histologic type not listed (specify): _____

___ Carcinoma, type cannot be determined

Histologic Grade

___ G1: Well differentiated

___ G2: Moderately differentiated

___ G3: Poorly differentiated

___ G4: Undifferentiated

___ Other (specify): _____

___ GX: Cannot be assessed

___ Not applicable

Depth of Invasion (Note E)

Specify depth of invasion (millimetres): ___ mm

___ Cannot be determined (explain): _____

+ Tumour Border (Note F)

+ ___ Pushing

+ ___ Infiltrating

Other Tissue/ Organ Involvement (select all that apply)

Note: Any organ not selected is either not involved or was not submitted.

___ Not applicable

___ Not identified

___ Vagina, lower one-third

___ Vagina, upper two-thirds

___ Urethra, lower one-third

___ Urethra, upper two-thirds

___ Anus

___ Bladder mucosa

___ Rectal mucosa

___ Pelvic bone

___ Other organs/tissue (specify): _____

___ Cannot be determined (explain): _____

Margins

Peripheral Margin

___ Cannot be assessed (explain): _____

___ Uninvolved by invasive carcinoma

+ Distance of invasive carcinoma from margin (millimetres): ___ mm

+ Specify location: _____

___ Involved by invasive carcinoma

Specify location(s), if possible: _____

___ Uninvolved by intraepithelial neoplasia

___ Involved by high-grade squamous intraepithelial lesion (VIN 2-3)

+ Specify location(s): _____

___ Involved by vulvar intraepithelial neoplasia, differentiated (simplex) type (dVIN)

+ Specify location(s): _____

___ Involved by Paget disease

+ Specify location(s): _____

Deep Margin

___ Cannot be assessed (explain): _____

___ Uninvolved by invasive carcinoma

+ Distance of invasive carcinoma from margin (millimetres): ___ mm

+ Specify location: _____

___ Involved by invasive carcinoma

Specify location(s), if possible: _____

___ Uninvolved by intraepithelial neoplasia

___ Involved by high-grade squamous intraepithelial lesion (VIN 2-3)

+ Specify location(s): _____

___ Involved by vulvar intraepithelial neoplasia, differentiated (simplex) type (dVIN)

+ Specify location(s): _____

___ Involved by Paget disease

+ Specify location(s): _____

Lymph vascular Invasion (Note G)

___ Not identified

___ Present

___ Cannot be determined (explain): _____

Regional Lymph Nodes

Note: Only inguinal and femoral nodes are considered regional lymph nodes. Any other involved nodes should be categorized as metastases (pM1) and be commented on in the distant metastasis section. Presence of isolated tumour cells no greater than 0.2 mm in regional lymph node(s) is considered N0 (i+).

___ No lymph nodes submitted or found

Lymph Node Examination (required only if lymph nodes are present in specimen)

Number of Nodes with Metastasis 5 mm or Greater: ____

___ Number cannot be determined (explain): _____

Number of Nodes with Metastasis Less than 5 mm (excludes ITCs): ____

___ Number cannot be determined (explain): _____

Number of Nodes with Isolated Tumour Cells (ITCs) (0.2 mm or less) (if applicable) [#]: ____

___ Number cannot be determined (explain): _____

[#] *Reporting the number of lymph nodes with isolated tumour cells are required only in the absence of metastasis greater than 0.2 mm in other lymph nodes.*

Specify Lymph Node(s) with Tumour (if applicable) [#]: _____

[#] *Note: Information should include location and laterality of sentinel or non-sentinel regional lymph nodes with tumour.*

Additional Lymph Node Findings (select all that apply) (required only if applicable) (Note I)

___ None identified

___ Extranodal extension

___ Fixed/ulcerated nodes

___ Other (specify): _____

___ Cannot be determined (explain): _____

Total Number of Nodes Examined (sentinel and non-sentinel): ____

___ Number cannot be determined (explain): _____

Specify Site(s)[#]: _____

[#] *Note: Information should include location and laterality of sentinel or non-sentinel regional lymph nodes examined.*

Number of Sentinel Nodes Examined: ____

___ Number cannot be determined (explain): _____

Pathologic Stage Classification (pTNM, AJCC 8th Edition) (Note H)

Note: Reporting of pT, pN, and (when applicable) pM categories is based on information available to the pathologist at the time the report is issued. Only the applicable T, N, or M category is required for reporting; their definitions need not be included in the report. The categories (with modifiers when applicable) can be listed on 1 line or more than 1 line.

TNM Descriptors (required only if applicable) (select all that apply)

___ m (multiple primary tumours)

___ r (recurrent)

___ y (posttreatment)

Primary Tumour (pT)

___ pTX: Primary tumour cannot be assessed

___ pT0: No evidence of primary tumour

___ pT1: Tumour confined to the vulva and / or perineum[#]

___ pT1a: Lesions 2 cm or less, confined to the vulva and/or perineum, and with stromal invasion of 1.0 mm or less [##]

___ pT1b: Lesions more than 2 cm, or any size with stromal invasion more than 1.0 mm, confined to the vulva and/or perineum

___ pT2: Tumour of any size with extension to adjacent perineal structures (lower/distal third of the urethra, lower/distal third of the vagina, anal involvement)

___ pT3: Tumour of any size with extension to any of the following: upper/proximal two-thirds of the urethra, upper/proximal two-thirds of the vagina, bladder mucosa, or rectal mucosa, or fixed to pelvic bone

[#] *Multifocal lesions should be designated as such. The largest lesion or the lesion with the greatest depth of invasion will be the target lesion identified to address the highest pT stage. Depth of invasion is defined as the measurement of the tumour from the epithelial–stromal junction of the adjacent most superficial dermal papilla to the deepest point of invasion.*

[##] *Note: The LAST definition of superficial invasive squamous cell carcinoma (SISCCA) conforms to AJCC pT1a/FIGO IA*

Regional Lymph Nodes (pN) (select all that apply)

+ Modifier

+ ___ (sn)

+ ___ (sn)(i-)

+ ___ (sn)(i+)

Category (pN)

___ pNX: Regional lymph nodes cannot be assessed

___ pN0: No regional lymph node metastasis

___ pN0(i+): Isolated tumour cells in regional lymph node(s) no greater than 0.2 mm

___ pN1: Regional lymph node metastasis with one or two lymph node metastases each less than 5 mm, or one lymph node metastasis ≥ 5 mm

___ pN1a: One or two lymph node metastasis each less than 5 mm[#]

___ pN1b: One lymph node metastasis ≥ 5 mm

___ pN2: Regional lymph node metastasis with three or more lymph node metastases each less than 5 mm, or two or more lymph node metastases ≥ 5 mm, or lymph node(s) with extra nodal extension

___ pN2a: Three or more lymph node metastases each less than 5 mm[#]

___ pN2b: Two or more lymph node metastases ≥ 5 mm

___ pN2c: Lymph node(s) with extranidal extension

___ pN3: Fixed or ulcerated regional lymph node metastasis

[#] *Includes micro metastasis, N1mi and N2mi.The site, size, and laterality of lymph node metastases should be recorded.*

Distant Metastasis (pM) (required only if confirmed pathologically in this case)

___ pM1: Distant metastasis (including pelvic lymph node metastasis) [#]

Specify site(s), if known: _____

[#] *Internal iliac/hypogastric, external iliac, and common iliac lymph nodes are considered distant metastasis.*

+ <u>FIGO Stage (2015 FIGO Cancer Report)</u>

+___ I: Tumour confined to the vulva

+___ IA: Lesions ≤2 cm in size, confined to the vulva or perineum and with stromal invasion ≤1.0 mm, no nodal metastasis[#]

+___ IB: Lesions >2 cm in size or with stromal invasion >1.0 mm, confined to the vulva and/or perineum, with negative nodes

+___ II: Tumour of any size with extension to adjacent perineal structures (lower third of urethra, lower third of vagina, anus) with negative nodes

+ ___ III: Tumour of any size with or without extension to adjacent perineal structures (lower third of urethra, lower third of vagina, anus) with positive inguinofemoral nodes

+___ IIIA: With 1 lymph node metastasis (≥5 mm)

+___ IIIA: With 1 to 2 lymph node metastasis(es) (<5 mm)

+___ IIIB: With 2 or more lymph node metastases (≥5 mm)

+___ IIIB: With 3 or more lymph node metastases (<5 mm)

+___ IIIC: With positive nodes with extracapsular spread

+ ___ IV: Tumour invades other regional (upper two-thirds urethra, upper two-thirds vagina), or distant structures

+___ IVA: Tumour invades any of the following: upper urethral and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to pelvic bone, or fixed or ulcerated inguinofemoral lymph nodes

+___ IVB: Any distant metastasis including pelvic lymph nodes

[#] *Note: The LAST definition of superficial invasive squamous cell carcinoma (SISCCA) conforms to AJCC pT1a/FIGO IA.*

+ Additional Pathologic Findings (select all that apply) (Note I)

+ ___ None identified

+ ___ Condyloma accuminatum

+ ___ High grade squamous intraepithelial lesion (VIN 2-3)

+ ___ Low grade squamous intraepithelial lesion (VIN 1)

+ ___ Vulvar intraepithelial neoplasia, differentiated (simplex) type (dVIN)

+ ___ Lichen sclerosus

+ ___ Other (specify): _____

+ Comment(s)