HPV strain prevalence and HPV-related biomarker expression in vulvar carcinoma at Tygerberg Academic Hospital.

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Declaration

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Abstract

The incidence of vulvar carcinoma in younger women has increased, possibly due to augmented human papillomavirus (HPV) infection. Variable geographic prevalence of HPV that are associated with vulvar carcinoma exists. In South Africa, histological subtyping is not always specified and the HPV prevalence in invasive vulvar squamous cell carcinoma (iVSCC) is largely unknown. High HIV prevalence could also be problematic as one of the risk factors for HPV-dependent vulvar cancer is impaired immunological status (Saidu 2016). The objectives of this study are to identify HPV subtypes in vulvar intraepithelial neoplasia (VIN) and iVSCC FFPE tissue using quantitative real-time polymerase chain reaction (qPCR) and to correlate HPV strain prevalence results with HIV status, clinicopathological features and biomarker expression.

The study samples comprised of FFPE vulvar tissue from patients diagnosed with VIN and iVSCC at Tygerberg Academic Hospital between January 2003 and June 2018. Altogether, 72 VIN and 68 iVSCC FFPE tissue samples were included. DNA and RNA were isolated from FFPE vulvar tissues and the quantity and purity were assessed using fluorometry and spectrophotometry, respectively. The integrity of the derived DNA was determined through amplification of a 205 bp fragment of the human β-globin gene, while that of RNA was analysed with the RIN number and DV_{200} score of the Agilent 2100 Bioanalyzer. The presence of HPV DNA was investigated using qPCR employing primers targeting the E6/E7 gene of HPV 16, 18, 11 and 35. The expression of the HPV 16 and 18 E7 mRNA transcripts was examined with specific primers and probes using qPCR. HPV DNA detection results were correlated with histological diagnosis and subgroup as well as HIV status. Furthermore, tissue microarrays were also constructed of $p16^{INK4a}$ block positive immunohistochemistry samples and the staining pattern between the TMA and full sections were compared.

Results show that patients between the ages of 36 to 59 years diagnosed with VIN and iVSCC at Tygerberg Academic Hospital between January 2003 and June 2018 were the largest proportion of our study population. Additionally, a significant proportion of the patients who were younger than 59 years, irrespective of diagnosis, were found to be HIV positive, with a large portion even being between 18 and 35 years old. Total DNA and RNA were extracted from the included FFPE vulvar tissue blocks. Overall, DNA of sufficient quality and integrity

was obtained as a 205 bp human β -globin gene fragment was amplified in all FFPE vulvar samples, suggesting that the DNA was fit for qPCR analysis. Similarly, RNA was extracted from all applicable samples and was of adequate quality for use in qPCR as indicated by the DV_{200} scores, RIN numbers and the expression of GAPDH in samples converted to cDNA. HPV DNA was detected in 77.8 % of VIN and 66.2 % of iVSCC samples with HPV 16 being the most prevalent strain detected in both diagnoses. The majority of patients for which HPV DNA were detected were found to be co-infected with HIV. When histopathological subtypes were compared with HPV DNA detection, results showed that 82 % of VIN II/III (HSIL) and 37.5 % of VIN I (LSIL) samples tested positive for HPV. Notably, a high HPV prevalence was also found in the keratinizing variants of the samples representative of iVSCC. Regarding mRNA transcript analysis, overall, the proportion of samples in which HPV 16 and HPV 18 mRNA was expressed were slightly less than for HPV DNA detected. However, a high concordance was found between the two HPV detection methods, with high sensitivity and specificity obtained. Two tissue microarrays, one with HIV positive and the other with HIV negative samples, were constructed using samples that were block positive for $p16^{INK4a}$ IHC staining. When comparing the specificity and sensitivity of the $p16^{INK4a}$ IHC staining on full sections slides to that of TMA slides, results showed good agreement between scoring.

In conclusion, these findings showed that VIN and iVSCC were diagnosed in women of much younger age in our study population compared to more developed countries. A high HIV prevalence was noticeable in HPV positive samples, for which HPV 16 was the most common subtype detected. Results also indicated that iVSCC keratinizing variants were largely associated with HPV in our study population which were also in contrast to what is reported. The findings of this study provide some insight into the prevalence of vulvar carcinoma and its precursor lesions, emphasizing the strong association of HPV in disease development in our setting. Lastly, it was also concluded that TMA's may be utilized for ancillary testing of vulvar specimens, with a view of exploiting this technology for its multitude of advantages, especially in the context of pre-invasive HPV associated research. The search for new biomarkers, to predict progression from precursor lesions to invasive disease is still ongoing and inconclusive, therefore, further studies are needed to aid the better understanding of the aetiology and pathophysiology of vulvar cancer in South Africa.

Opsomming

Die toename in die voorkoms van karsinoon van die vulva in jonger vroue kan moontlik aan 'n verhoogde infeksie met menslike papillomavirus (MPV) toegeskyf word. ‘MPV subtipes wat met vulvêre karsinoom geassosieer word, verskil in verskeie liggings. In Suid-Afrika word die histologiese subtipes van die karsinoom nie altyd gespesifiseer nie en die MPV-voorkoms in indringende vulvar plaveiselkarsinoom (iVPK) is grootliks onbekend. Die hoë voorkoms van MIV kan ook problematies wees, aangesien ‘n verswakte immuuunsisteem een van die risikofaktore vir die ontwikkeling van MPV-afhanklike vulvêre kanker is. Die doel van hierdie studie is dus om, met behulp van kwantitatiewe “real-time” polimerase kettingreaksie (kPKR), die MPV genotipes in formalin gefikseerde, paraffienwas-ingebede vulvêre intraepiteel neoplastiese (VIN) en iVPK weefsel te identifiseer. Die voorkoms van MPV subtipes word met MIV-status, sowel as kliniese en patologiese kenmerke asook die uitdrukking van biologiese merkers gekorreleer.

‘n Totaal van 72 VIN en 68 iVPK gevalle wat tussen Januarie 2003 en Junie 2018 by Tygerberg Akademiese Hospitaal gediagnoseer was, was in die studie ingesluit. DNS en RNS was geekstraeër en die kwaliteit en kwantiteit daarvan was met behulp van fluorometrie en spektrofotometrie bepaal. Die integriteit van die DNS was deur die kPKR amplifisering van 'n 205 bp fragment van die menslike ß-globien-geen, terwyl die van RNS met behulp van die RIN-nommer en die DV200-telling van die Agilent 2100 Bioanalyzer geanaliseer was. Die teenwoordigheid van MPV DNS was deur middel van kPKR en die gebruik van “primers” wat die E6 / E7-gene van MPV 16, 18, 11 en 35 teiken, ondersoek. Die uitdrukking van die MPV 16 en 18 E7 mRNA-transkripsies is met spesifieke “primers en probes” deur middel van kPKR ondersoek. Die resultate van MPV tipering is met histologiese diagnose en subgroepe sowel as MIV-status vergelyk. Verder is weefsel “microarrays” (WMA) ook saamgestel uit monsters met $p16^{INK4a}$ blok positiewe immunohistochemie en die kleurpatroon was tussen die WMAs en die volledige weefsel skyfies vergelyk.

Resultate toon dat die grootste deel van ons studiepopulasie uit pasiënte tussen die ouderdomme van 36 tot 59 jaar wat gediagnoseer was met VIN en iVPK in die Tygerberg Akademiese Hospitaal tussen Januarie 2003 en Junie 2018, bestaan het. Boonop is daar gevind

dat 'n beduidende deel van die pasiënte jonger as 59 jaar oud en MIV-positief was, ongeag van die diagnose, met 'n groot gedeelte selfs tussen 18 en 35 jaar oud. Oor die algemeen was DNS van voldoende gehalte en integriteit verkry en die menslike ß-globiengeenfragment is in alle vulvêre monsters geamplifiseer, wat daarop dui dat die DNS geskik was vir kPKR-ontleding. Net so was RNS uit alle geskikte monsters geekstraeër en dit was van voldoende gehalte vir die gebruik in kPKR, soos aangedui deur die DV200-tellings, RIN-getalle en die uitdrukking van GAPDH in monsters wat omgeskakel was na komplimentêre DNS. MPV-DNS is in 77,8 % van die VIN en 66,2 % van die iVPK-monsters opgespoor, met MPV 16 as die mees algemene MPV tipe wat by albei diagnose gevind was. Die meerderheid van die pasiënte waarvoor MPV-DNA gevind was, was ook met MIV geïnfekteer. Toe histopatologiese subtipes met die MPV DNA bevindings vergelyk was, was daar getoon dat 82 % van die VIN II / III (HSIL) en 37,5 % van die VIN I (LSIL) monsters positief vir MPV getoets het. 'n Verhoogde voorkoms van MPV was ook in die keratiniserende variante van die iVPK monsters gevind. Wat die analise van mRNA-transkripsie betref, was die proporsie van monsters waarin MPV 16 en MPV 18 mRNA uitgedruk was, effens minder as vir die waar MPV-DNA gevind was. Daar was egter 'n hoë ooreenkoms tussen die twee MPV-ontledingsmetodes gevind en 'n hoë sensitiwiteit en spesifisiteit was ook getoon. Twee WMA’s, een met MIV-positief en die ander met MIV-negatiewe monsters, was saamgestel uit monsters wat blok-positief vir $p16^{INK4a}$ IHC-kleuring was. Die spesifisiteit en sensitiwiteit van die $p16^{INK4a}$ IHC-kleuring op volledige skyfies en die TMA-skyfies was vergelyk en ‘n goeie ooreenkoms tussen die uitkoms van IHC kleuringspatroon was getoon.

Ten slotte, hierdie bevindings het getoon dat VIN en iVPK by baie jonger vroue in ons studiepopulasie gediagnoseer was, in vergelyking met meer ontwikkelde lande. 'n Hoë voorkoms van MIV was opvallend in MPV-positiewe monsters, met MPV 16 die algemeenste subtipe wat gevind was. Resultate het ook aangedui dat die keratiniserende iVPK variante grootliks met MPV in ons studiepopulasie geassosieer was, wat ook teenstrydig is met wat gerapporteer word. Die bevindings van hierdie studie bied 'n mate van insig in die voorkoms van vulvêre karsinoom en hul voorloper letsels , wat die sterk assosiaise tussen MPV en die ontwikkeling van die kanker in ons omgeweing uitbeeld. Laastens is die gevolgtrekking gemaak dat WMA's gebruik kan word vir aanvullende toetsing van vulvêre monsters, met die doel om die tegnologie se talle voordele te benut, veral in die konteks van pre-infiltrerende MPV-geassosieerde navorsing. Die identifisering van nuwe biomerkes, wat die vordering

vanaf voorloper letsels na infiltrerende siekte is steeds deurlopend en onbeslis, daarom word verdere navorsing egter benodig om die etiologie en patofisiologie van vulvêre kanker in Suid-Afrika beter te verstaan.

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Chapter 1

The role of human papilloma virus in the development of vulvar intraepithelial neoplasia and invasive vulvar squamous cell carcinoma.

1.1 Background

Vulvar cancer incidence has been increasing in the last few decades while the age of patients at diagnosis has decreased (De Vuyst *et al.*, 2009; De Sanjosé *et al.*, 2013; Preti, Scurry and Marchitelli, 2014; Trietsch *et al.*, 2015; Bradbury *et al.*, 2016; Saidu, 2016). This may be linked to an increase in human papillomavirus (HPV) infection (Saidu, 2016), which is one of the risk factors for vulvar pre-cancer and cancer development. A recent study suggests the average age of women with vulvar carcinoma in South Africa to be 52.5 years (Butt and Botha, 2017). A significant difference in age groups thus exists as 64 and 70 years is described for women, diagnosed with vulvar carcinoma, in international literature from high-income countries such as the United States of America, Australia and the Netherlands (Ramanah *et al.*, 2012; Tan *et al.*, 2012 as cited in Butt and Botha, 2017). A recent study shows that the prevalence of HPV in women from Cape Town aged 16 to 22 years is 68.2 % (Mbulawa, Schalkwyk, Hu, Meiring, Barnabas, Dabee, Jaspan, Kriek, Jaumdally, Muller, Bekker, Lewis, Dietrich, Gray, Passmore & Williamson, 2018). According to this study, cervical samples of Capetonian women are more likely to have multiple HPV infections compared to that of women residing in the Gauteng region (Mbulawa *et al.*, 2018). Globally, human papillomavirus (HPV) was the cause of 640 000 out of 2.2 million (approximately 29 %) new cancer cases in 2012, making HPV infection one of the major causes of oncoviral related cancers (Plummer, de Martel, Vignat, Ferlay, Bray & Franceschi, 2016; Serrano, De Sanjosè, Tous, Quiros, Muñoz, Bosch & Alemany, 2015). HPV infection is linked to 30 % to 43 % of invasive vulvar carcinoma cases and approximately 85 % of high-grade vulvar intraepithelial neoplasia (VIN) cases (Serrano *et al.*, 2015). Furthermore, 25,6 million people in Sub-Saharan Africa and 7.7 million people in South Africa were living with HIV in 2018 (Global UNAIDS report, 2019). High HIV prevalence could be problematic as one of the risk factors for HPV dependent vulvar cancer is impaired immunological status (Saidu, 2016). HPV-related cancer statistics in South Africa is lacking,

though the last data published by the National Cancer Registry was in 2014, indicated an incidence of 343 histologically diagnosed vulvar cancer cases. However, this total has remained unchanged for the years 2013 and 2014, suggesting a lack of updated statistics for this cancer type. Nevertheless, a slight increase in vulva diagnosis has been seen in South Africa as 323 cases were diagnosed in 2012 according to the National cancer Registry (Herbst, 2016). Additionally, the latest HPV summary report for South Africa, updated and released in 2019, reports vulvar carcinoma incidence of 22 cases between 2008 to 2012 for the Eastern Cape only and still have no available data on HPV prevalence among vulvar cancer cases elsewhere in South Africa (Bruni *et al*., 2019). There is clearly a void in knowledge regarding the prevalence of vulvar cancer in South Africa. Therefore, an increase in studies pertaining to vulvar cancer and other low incidence anogenital malignancies are much needed in Sub-Saharan Africa.

In the text that follows, a literature review is presented. An overview of the epidemiology of HPV and vulvar carcinoma is provided followed by the clinical and histological features of vulvar carcinoma. Next, a brief insight is given into the classification system used for vulvar neoplasia and carcinoma diagnosis followed by a summary of the treatment, prevention and management of HPV and vulvar malignancies. A detailed explanation of the molecular mechanism of HPV-related vulvar carcinoma is then provided, followed by the influence of HIV, a known risk factor for HPV infection, on this disease. As the source material used in this study is formalin-fixed paraffin embedded (FFPE) vulvar tissue, insight into the utility of FFPE-derived nucleic acids in sensitive molecular applications such as qPCR is provided. Lastly, a short information piece on the use of biomarkers for HPV-related cancers such as vulvar carcinomas as well as the use of tissue microarray is given.

1.2 Epidemiology of HPV and vulvar carcinoma

According to a worldwide meta-analysis study, HPV prevalence has increased in less developed countries (15.5 %) compared to developed countries (10.0 %) with the highest HPV incidence occurring in women younger than 25 years (De Sanjosé *et al.*, 2007). HPV prevalence was found to be the highest (22.1 %) in African women with normal cytology while a single study included in this meta-analysis estimated the HPV occurrence in South African women to be 15.5 % (De Sanjosé *et al.*, 2007). A significant geographical difference in HPV

prevalence rates was observed by Reade, Eiriksson and Mackay, 2014, making the identification of prevalent HPV strains in individual settings a relevant and important research aspect. HPV prevalence therefore differs from country to country with another study referring to Africa having the highest HPV prevalence of 32 % while that of Asia was a mere 6 % (Hinten, Meeuwis, van Rossum & de Hullu, 2012).

Squamous cell carcinoma (SCC) of the vulva is preceded by the precursor lesion vulvar intraepithelial neoplasia (VIN). VIN can either develop via an HPV associated pathway resulting in usual VIN (uVIN) or an HPV independent pathway leading to differentiated VIN (dVIN) (Alkatout, Schubert, Garbrecht, Weigel, Jonat, Mundhenke & Günther, 2015; Hoang, Park, Soslow & Murali, 2016; Léonard, Kridelka, Delbecque, Goffin, Demoulin, Doyen & Delvenne, 2014; Saidu, 2016). Differentiated VIN mainly occurs in older, post-menopausal women (55 to 85 years) and shows a low rate of HPV infection (Alkatout *et al.*, 2015; Bradbury *et al.*, 2016). Chronic vulvar dermatosis such as Lichen sclerosis has been described as a predisposing risk factor for the development of dVIN as it causes severe pruritus which may lead to squamous cell hyperplasia, progression to atypia, development of VIN and eventually invasive vulvar carcinoma (Alkatout *et al.*, 2015; Bradbury *et al.*, 2016; De Sanjosé *et al.*, 2013).

Unlike dVIN, uVIN usually affects younger women (35 to 65 years old) and is associated with smoking and impaired immunological status (Saidu, 2016). Other predisposing risk factors include previous sexual transmitted diseases and low economic status (Alkatout *et al.*, 2015). Although spontaneous regression of disease does occur in some cases, women with untreated u-VIN have an increased risk for disease progression, as approximately 80 % of women with untreated VIN III (high grade lesion) develop invasive carcinoma (Canavan and Cohen, 2002; Alkatout *et al.*, 2015; Hoang *et al.*, 2016). Approximately 18 % to 52 % of women diagnosed with u-VIN also presents with squamous dysplasia in other anogenital sites (Hoang *et al.*, 2016).

Several types of vulvar cancer such as basal cell carcinoma, adenocarcinoma, sarcoma and melanoma exist but squamous cell carcinoma (SCC) is the most common type of vulvar cancer

and accounts for 90 % to 95 % of vulvar malignancies (Alkatout *et al.*, 2015; Léonard *et al.*, 2014; De Sanjosé *et al.*, 2013). Histological subtypes of vulvar squamous cell carcinoma (VSSC) include basaloid and warty SCC which are normally associated with HPV infection (Knopp *et al.*, 2009; Preti, Sccurry and Marchitelli, 2014). Other histological subtypes include verrucoid, keratinizing and non-keratinizing SCC and are often unrelated to HPV infection (Knopp *et al.*,2009; Preti, Sccurry and Marchitelli, 2014).

1.3 Clinical and histological features of vulvar carcinoma

Symptoms of vulvar carcinoma include a visible lump in or around the vulva area as well as a long history of pruritus (itchy skin), pain, discharge and bleeding (Alkatout *et al.*, 2015; Bradbury *et al.*, 2016). As no current screening method for vulvar cancer exists, VIN is usually diagnosed after symptoms have already occurred (Preti, Sccurry and Marchitelli, 2014). During clinical evaluation the site, size, shape, amount and colour of the lesion is recorded and will indicate if further testing is necessary (Preti, Sccurry and Marchitelli, 2014).

1.3.1. uVIN

Usual VIN (uVIN) is often defined as unifocal or multifocal with elevated lesions on the opening of the vaginal canal and labia minora and normal surrounding mucosa (Preti, Sccurry and Marchitelli, 2014). Positive diagnosis of VIN in association with HPV infection does not always lead to invasive vulvar carcinoma and may persist, progress or resolve spontaneously without treatment (Saidu, 2016). However, although data is limited, it was found that HIV-positive women with a HPV co-infection plus multifocal and multicentric VIN lesions have an increased risk of recurrent disease and development of invasive vulvar carcinoma (Bradbury *et al.*, 2016). Studies done on HPV strain prevalence in cervical cancer cases also showed the tendency of HIV positive patients to be infected with multiple high-risk HPV types, which could be linked to the rapid disease progression (Williamson, 2015). As HPV is strongly associated with the development of uVIN, identifying the prevalent HPV strains present in HIV positive and negative patients could be extremely valuable for prognosis, preventative treatment and for the evaluation of HPV vaccine efficiency.

During the development of uVIN, atypical basaloid cells start to proliferate in the basal layer and continue to do so throughout the full thickness of the squamous epithelium until maturation into squamous cells near the surface of the epithelium (Preti, Sccurry and Marchitelli, 2014). Atypical mitoses and apoptotic bodies are common features in atypical basaloid cell proliferation while koilocytosis (HPV-induced cellular changes leading to structurally altered squamous epithelial cells) only becomes evident after the development of mature squamous cells (Preti, Sccurry and Marchitelli, 2014). As previously mentioned, HPV associated SCC arises from uVIN lesions and can be histologically described as basaloid or warty, however an intermediate form, warty-basaloid VIN is also common (De Sanjosé *et al.*, 2013; Preti, Sccurry and Marchitelli, 2014). Warty VIN is characterized by the presence of HPV-associated cytopathogenic changes such as koilocytes while basaloid VIN contains atypical basaloid cells (Preti, Sccurry and Marchitelli, 2014).

1.3.2. dVIN

Clinical symptoms of dVIN include poorly defined white or pink plaque lesions in combination with a long history of itching, soreness, pain, burning, bleeding, dryness and other common symptoms of lichen sclerosis or lichen planus (Preti, Sccurry and Marchitelli, 2014). These lesions are often treatment-resistant which is another indication of dVIN and do not commonly affect surrounding areas such as the vagina, cervix and anus (Preti, Sccurry and Marchitelli, 2014). A diagnosis of dVIN is challenging to make as it has subtle histological criteria to distinguish it from lichen sclerosis (Preti, Sccurry and Marchitelli, 2014). dVIN is histologically identified through basal nuclear atypia and premature maturation of cells above the basal layer (Preti, Sccurry and Marchitelli, 2014).

1.4. Vulvar pre-cancer lesion and carcinoma classification

A variety of pathological classification systems for vulvar pre-cancer lesions and vulvar carcinoma exists such as the American Joint Committee on Cancer TNM as well as the International Federation of Gynecology and Obstetrics (FIGO) staging systems (Alkatout *et al.*, 2015). These two systems classify vulvar carcinoma based on the size of the tumour, whether lymph nodes are affected as well as metastasis of the tumour to distant sites (Alkatout *et al.*, 2015). The International Society for the Study of Vulvovaginal Disease (ISSVD) began

to use the term VIN in 1986 which was divided into three grades according to the extent of the dysplasia throughout the epithelial thickness (Hoang *et al.*, 2016). VIN I referred to cases where dysplasia occurred throughout the lower one-third of the epithelial thickness, VIN II was diagnosed where two-thirds of the epithelial thickness was affected and VIN III was diagnosed when more than two-thirds of the epithelial thickness was affected (Hoang *et al.*, 2016). The ISSVD also introduced another category termed VIN III differentiated type (Hoang *et al.*, 2016). However, it later became evident that this classification system was not sufficient as the majority of VIN I cases were associated with low-risk HPV types and very seldom progressed to vulvar cancer while VIN II and III was linked to high-risk HPV and had an increased risk for progression to iVSCC (Hoang *et al.*, 2016). The classification system was therefore changed in 2004 referring to all high-grade lesions as VIN only while the VIN I classification was removed and these lesions now referred to as a wart or HPV infection (Preti, Sccurry and Marchitelli, 2014; Hoang *et al.*, 2016). The ISSVD then further changed the classification to usual type VIN (uVIN) which includes lesions previously diagnosed as VIN II and VIN III, differentiated type VIN (dVIN) and unclassified VIN (VIN, not otherwise specified (NOS)) (Preti, Sccurry and Marchitelli, 2014; Hoang *et al.*, 2016). Due to the small amount of VIN I cases that is linked to high-risk HPV types, the World Health Organization (WHO) continues to classify uVIN into three grades (VIN I – III), where VIN I would be equivalent to low grade squamous intraepithelial lesion (LSIL) while VIN II and III would be regarded as high-grade squamous intraepithelial lesions (HSIL) according to the classification used by the Lower Anogenital Squamous Terminology (LAST) guidelines published in 2012 (Preti, Sccurry and Marchitelli, 2014). The classification of vulvar lesions has since been standardized as the "squamous intraepithelial lesion (SIL)" terminology proposed in this document by the College of American Pathologist (CAP) and American Society for Colposcopy and Cervical Pathology (ASCCP) was widely accepted by a variety of committees (Hoang *et al.*, 2016). The WHO and ISSVD also later accepted the SIL terminology but also includes dVIN as a separate category (Hoang *et al.*, 2016).

1.5. Treatment, prevention and management of HPV, vulvar intraepithelial neoplasia and invasive vulvar carcinoma

No specific HPV test is available at Tygerberg Academic Hospital and an HPV positive diagnosis is mainly reliant on the observation of histological HPV changes such as koilocytosis (Butt and Botha, 2017). Recently, the option to request $p16^{INK4a}$ immunohistochemistry (IHC) staining for some cases, a known surrogate marker for HPV-related morphological changes, has been implemented at Tygerberg Academic Hospital (Personal communication with Dr R. Razack, pathologist).

A variety of PCR-based HPV DNA testing methods have been developed such as the HPV linear array kit (Roche), Cobas® HPV test (Roche) and the HPV Direct Flow Chip kit (Master Diagnostica). Other approaches available for the testing of the presence of HPV are IHC as well as In situ hybridization (ISH) (Molijn *et al*., 2005). However, HPV testing for vulvar carcinoma patients may be redundant, as symptoms have often already occurred when VIN is diagnosed and a biopsy or surgery is the next step of action. Treatment methods available for vulvar carcinoma are invasive as radical surgery, sometimes in combination with pre- or postsurgical radiotherapy or chemotherapy is often the mainstay of treatment (Butt and Botha, 2017; Knopp, *et al.*, 2009; Wills and Obermair, 2013). At Tygerberg Academic Hospital, surgery was the primary treatment for 62.7 % of vulvar carcinoma patients between January 2001 and December 2014, with 40.7 % of these patients undergoing adjuvant therapy, such as radiotherapy, post-surgery (Butt & Botha, 2017). The type of surgery performed is dependent on the size and extent of the invasion of the lesion (Herbst, 2016). A wide local excision is commonly performed in early-stage disease cases where the lesion, as well as a margin of surrounding healthy tissue, is excised (Alkatout *et al.*, 2015; Herbst, 2016). Other types of surgery include partial vulvectomy, radical vulvectomy with or without the removal of adjacent infected organs, reconstructive surgery and removal of nearby lymph nodes (Alkatout *et al.*, 2015; Herbst, 2016). These harsh treatment methods can lead to severe physical, sexual and psychological dysfunction (Wills & Obermair, 2013). Post-surgical complications such as lymphedema, hematoma, wound infection, breakdown, cellulitis and hernia formation may also occur (Wills & Obermair, 2013). Several techniques have been developed to decrease these complications. For example, sentinel lymph node biopsy which may allow fewer lymph nodes to be removed by identifying the lymph node where the cancer is most likely to spread to first

(Herbst, 2016). At some institutions wound healing disorders are minimized by replacing the traditional *en bloc* resection with the triple incision (Alkatout *et al.*, 2015).

Despite differences in aetiopathogenesis and cellular mechanism of HPV dependent and independent iVSCC, treatment options for both iVSCC types remain the same. However, current research is evolving around developing targeted agents and differing treatment strategies for iVSCC (Reade, Eiriksson & Mackay, 2014). Targeted treatment works similarly to chemotherapy but is specially produced to target cancer cells with a specific type of alteration. For example, as an increase in epidermal growth factor receptor (EGFR) was observed in vulvar squamous cell carcinoma, the use of anti-EGFR tyrosine kinase inhibitor drugs such as erlotinib and gefitinib have been investigated as a form of targeted treatment (Horowitz, Olawaiye, Borger, Growdon, Krasner, Matulonis, Liu, Lee, Brard & Dizon, 2012; Inrhaoun, Elghissassi, Gutierrez, Brain & Errihani, 2012; Olawaiye, Lee, Krasner & Horowitz, 2007). Increase in EGFR gene copy numbers was however associated with the absence of high-risk HPV types indicating that this form of therapy may not be beneficial for vulvar cancer caused by high-risk HPV (Horowitz *et al.*, 2012).

A variety of HPV vaccines such as Gardasil®, Cervarix® and Gardasil 9® have been developed to protect against numerous HPV types including HPV 16, 18, 6, 11, 31, 33, 45, 52 and 58 (Buchanan, Graybill and Pierce, 2016). These HPV vaccines have been widely used in developed countries and are reported to prevent up to 90 % of HPV-related anogenital cancers (Buchanan, Graybill and Pierce, 2016). Two HPV vaccines are currently registered in South Africa, Cervarix^{TM}, which prevents infection with HR-HPV types 16 and 18; as well as Gardasil^{TM} containing antigens for HPV 16, 18, 11 and 6 (Botha & Dochez, 2012). However, the determination of prevalent HPV types in different settings is needed to confirm whether current HPV vaccines protect against those specific HPV types. A recent case report study also suggested the use of HPV vaccines as a therapeutic agent as the administration of Gardasil® in a recurrent vulva carcinoma patient prevented disease relapse (Gustafson, Gade & Blaakær, 2014). The Vaccine and Cervical Cancer Screen project (VACCS) were recently completed in South Africa targeting primary school girls in grade 4 to 7 (>9 years old) in the Gauteng and Western Cape provinces (Snyman *et al.*, 2015). Findings of the project was that vaccination programmes can be successfully implemented in primary schools in South Africa and that

appropriate age groups for HPV screening was reached (Botha and Dochez, 2012; Snyman *et al.*, 2015).

1.6. Molecular mechanism of HPV related vulvar carcinoma

HPV have a specific affinity for epithelium while some HPV types may also have a cutaneous or a mucosal specificity making the anatomical site susceptible to infection by a specific virus type (Thomas, Pim & Banks, 1999). The HPV genome is a small circular double-stranded DNA molecule consisting of 6 early genes (namely E1, E2, E4, E5 E6 and E7) and two late genes, called L1 and L2 (Narisawa-Saito & Kiyono, 2007). The HPV subtype is determined by the E6, E7 and L1 gene regions as more than 10 % variation in nucleotide sequence in these regions are required for the HPV strain to be defined as a different HPV type (Burk, Harari & Chen, 2013; Ishiji, 2000; Pande, Jain, Prusty, Bhambhani, Gupta, Sharma, Batra & Das, 2008). More than 100 different HPV types have been identified of which approximately 40 of these genotypes are known to infect the genital mucosa (Narisawa-Saito & Kiyono, 2007; Shikova, Todorova, Ganchev & Kouseva-Dragneva, 2009; Zhang, Zhang & Zhang, 2018; Zhu, Wang, Mao, Zhang & Ma, 2019). Infection with the majority of the HPV subtypes, such as HPV 6 and HPV 11, will lead to non-cancerous proliferative lesions or warts and is therefore named low-risk HPV types. On the other hand, HPV subtypes such as HPV 16, HPV 18 and HPV 35 are categorised as high-risk types, as they are known to be oncogenic and may result in pre-malignant or malignant lacerations (Knopp *et al.*, 2009; Zhu *et al.*, 2019). A recent third HPV subtype classification group namely "possibly high-risk" is used to classify HPV subtypes which may have the ability to be cancer-causing such as HPV 26 and HPV 30 (Zhu *et al.*, 2019).

1.6.1 Mechanism of HPV associated malignancy

The initial HPV infection is regulated by receptors of HPV, known as Heparan sulfate proteoglycans (HSPG) as well as α6-integrin and laminin-5 facilitates ensure sufficient initial HPV infection (Senapati, Senapati & Dwibedi, 2016). HPV then gains entry into the exposed basal cells of the epithelial mucosa after tissue trauma such as micro-tearing has occurred, a process which may take up to 12 hours (Senapati *et al.*, 2016; Tong, Zheng, Zhao, Xing, Li, Lin, Zhang & Jin, 2016). HPV DNA integration begins at integration "hotspots", which are sites throughout the genome which are not repaired after DNA damage induced by factors such

as oxidative stress as well as the E1, E6 and E7 HPV proteins (Senapati *et al.*, 2016). As the cell attempt to repair itself using the DNA damage response pathway, viral particles known as episomal HPV, takes advantage of this pathway to replicate (reviewed in Senapati, Senapati and Dwibedi, 2016). This leads to overexpression of viral E6 and E7 early genes causing the production of E6 and E7 oncoproteins (Tong *et al.*, 2016). HPV E6 gene products degrade p53, a human tumour suppressor protein, via the ubiquitin pathway (Figure 1.1) (Bernard, Robinson, Nominé, Masson, Charbonnier, Ramirez-Ramos, Deryckere, Travé & Orfanoudakis, 2011; Narisawa-Saito & Kiyono, 2007; Thomas *et al.*, 1999). E6 oncoproteins interact with E6-AP, an E3 ubiquitin-ligase, which modifies the affinity of E6-AP for p53 (Figure 1.1) (Bernard *et al.*, 2011; Narisawa-Saito & Kiyono, 2007; Thomas *et al.*, 1999). Subsequent binding of E6-AP to p53 then leads to ubiquitination of p53 which is then degraded by the 26 S proteasome complex (Figure 1.1) (Bernard *et al.*, 2011; Narisawa-Saito & Kiyono, 2007; Thomas *et al.*, 1999). p53 is responsible for the regulation of the cell cycle as this protein is expressed in reponse to DNA damage or initiation of unscheduled DNA replication (Gaëlle *et al.*, 2007). Degradation of p53 thus results in loss of cell cycle arrest, survival of damaged cells and suppression of apoptosis which leads to uncontrolled cellular replication (Gaëlle *et al.*, 2007; Tong *et al.*, 2016).

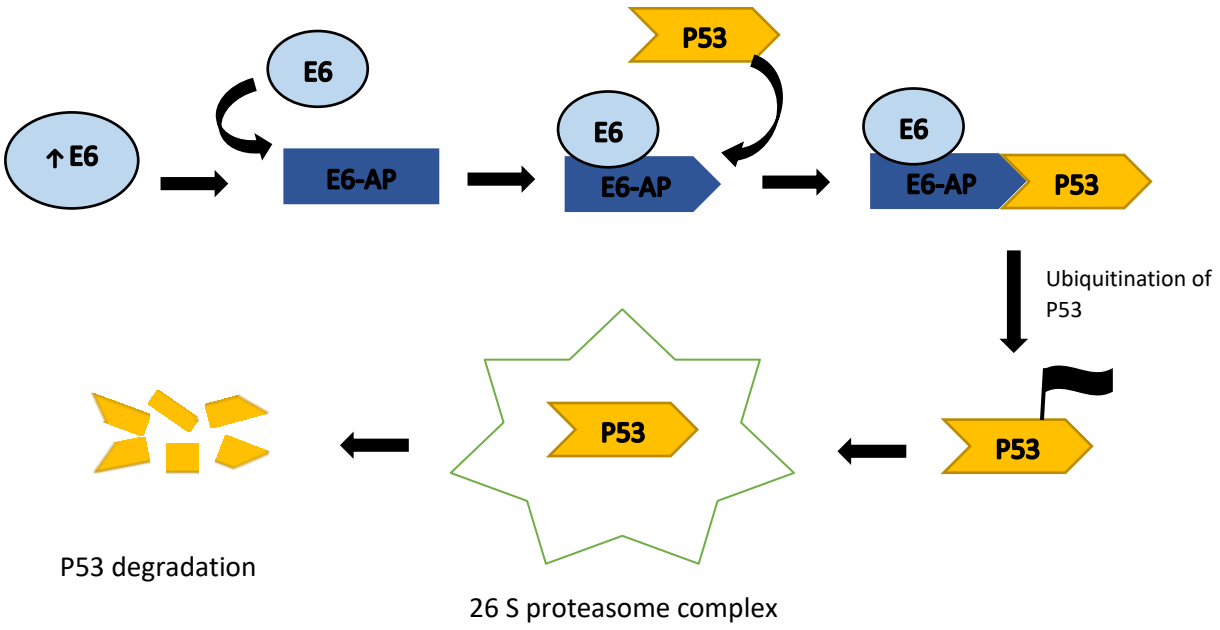


Figure 1.1: E6 oncoprotein induced degradation of P53 (Adapted from Thomas, Pim and Banks, 1999 and Narisawa-Saito and Kiyono, 2007).

Association of HPV E7 oncoproteins with the tumour suppressor gene product, retinoblastoma (pRb) protein, leads to the dissociation of the pRb-E2F complex (Figure 1.2) (Giarrè, Caldeira, Malanchi, Ciccolini, Jo, Le & Tommasino, 2001; McLaughlin-Drubin & Münger, 2009; Narisawa-Saito & Kiyono, 2007). Unphosphorylated pRb binds to transcription factors such as E2F to inhibit cell cycle progression and therefore acts as a transcriptional repressor (Giarrè *et al.*, 2001; McLaughlin-Drubin & Münger, 2009; Narisawa-Saito & Kiyono, 2007). G1 cyclin-dependent kinases phosphorylate pRb, causing E2F to dissociate from pRb. E2F is thus no longer inhibited and can therefore promote cell progression to the S phase and activate transcription (Narisawa-Saito & Kiyono, 2007). Binding of HPV E7 oncoproteins inactivates pRb resulting in the dissociation of bound E2F as well as the inability of pRb to bind to free E2F transcription factors causing cells to enter the S phase of the cell cycle prematurely (Giarrè *et al.*, 2001; McLaughlin-Drubin & Münger, 2009; Narisawa-Saito & Kiyono, 2007). This leads to hyperproliferation and immortalization of infected cells (Giarrè *et al.*, 2001; McLaughlin-Drubin and Münger, 2009; del Pino, Rodriguez-Carunchio and Ordi, 2013).

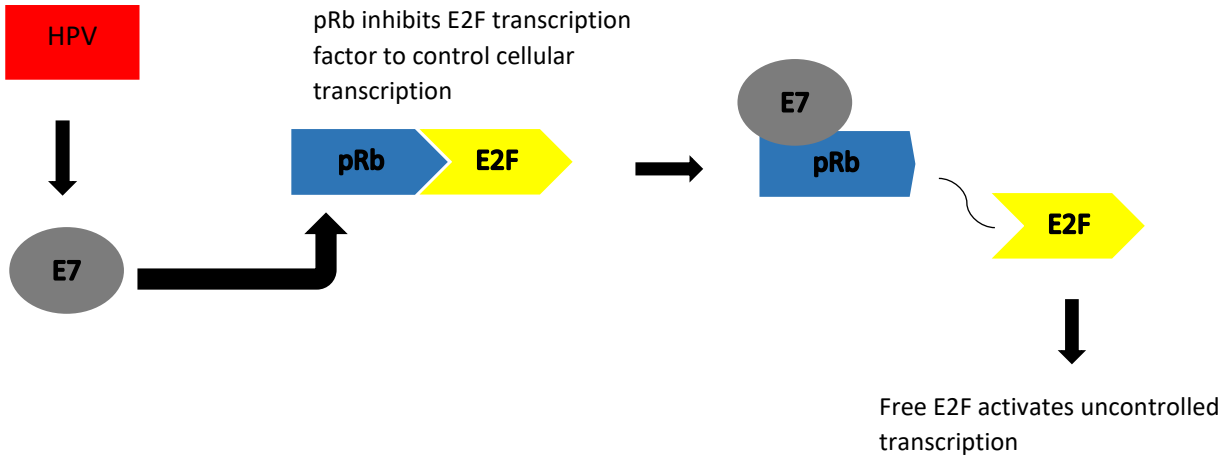


Figure 1.2: Interaction of E7 with pRb, activating E2F transcription factor (Adapted from Narisawa-Saito and Kiyono, 2007).

1.6.2 Influence of HIV on HPV related vulvar carcinoma

Immunosuppression is recognized as one of the risk factors for developing HPV associated diseases such as vulvar cancer. This may be due to the inability of the compromised immune system to fight the HPV infection (Marchetti, Comi, Bini, Rovati, Bai, Cassani, Ravizza,

Tarozzi, Pandolfo, Dalzero, Opocher, Romagnoli, Carrassi, Bosari & d'Arminio Monforte, 2013; Saidu, 2016). However, HIV infected cells also secrete a transactivator protein (Tat) and gp120 which disrupts the epithelial tight junctions, resulting in easier penetration of HPV into the target cells (Saidu, 2016). Patients infected with HIV are prone to infection with multiple HPV types, reactivation of latent HPV infection as well as HPV infection persistence (Saidu, 2016). This may be due to continued immune suppression caused by HIV infection. HIV may also play a role in disease progression from HPV infection to squamous intraepithelial neoplasia and subsequent invasive carcinoma as HIV positive patients have increased uVIN prevalence, post-treatment disease recurrence, uVIN persistence and disease progression to invasive vulvar carcinoma (del Pino, Rodriguez-Carunchio and Ordi, 2013; Preti, Sccurry and Marchitelli, 2014; Bradbury *et al.*, 2016; Saidu, 2016). A recent study found that uVIN recurrence was significantly higher and also occurred earlier in HIV positive women (Bradbury *et al.*, 2016). The same study also concluded that progression to invasive vulvar carcinoma occurred more frequently in younger HIV positive women (Bradbury *et al.*, 2016).

1.7 The use of FFPE derived nucleic acids for sensitive molecular application

FFPE tissue is a rich resource of macromolecules and has therefore become more appealing for use in clinical research and molecular analysis. Nucleic acids extracted from FFPE tissue samples may be utilized for molecular biology studies in cancer research and genetic control of cell growth and differentiation as well as screening for genetic variations associated with diseases (Srinivasan *et al.*, 2002). However, formalin fixation is known to have a deleterious effect on macromolecules such as DNA and RNA which may reduce the efficiency of downstream molecular applications.

One of the major negative effects formalin fixation has on nucleic acids are fragmentation and/or degradation, which leads to decreased quantity and quality of nucleic acids extracted from FFPE specimens (Bass *et al.*, 2014, Von Ahlfen *et al.*, 2007). The average fragment length obtained from FFPE tissue is reported to be 300-400bp (Bonin *et al.*, 2003; Huijsmans *et al.*, 2010). An additional negative effect is formalin-induced macromolecule modification through crosslink formation (Bass *et al.*, 2014, Thavarajah *et al.*, 2012), which often leads to a decrease in the utility of DNA and RNA in molecular assays such as genotyping (Baak-Pablo, *et al.*, 2010). Formalin interacts with macromolecules through the formation of covalent bonds

between formaldehyde and nucleophilic groups on amino acids or nucleic acid bases (Hoffman *et al*., 2015). This leads to the formation of a methylol adduct that is subsequently converted to an unstable Schiff base (Hoffman *et al*., 2015). Methylols or Schiff bases can chemically react with another functional group on another molecule leading to the formation of methylene bridges (Hoffman *et al*., 2015). Nucleic acid-macromolecules crosslinks caused by formalin fixation in tissue may lead to impurities being co-extracted with nucleic acids and may result in decreased in efficiency of subsequent molecular applications such as qPCR (Huijsmans *et al*., 2010; Kapp *et al*., 2015, Ludyga *et al*., 2012). Nevertheless, recent advancements in extraction methods have led to improved nucleic acid quality and yield from FFPE tissue blocks which may therefore be suitable for sensitive downstream molecular assays such as PCR (Kokkat *et al*., 2013).

1.8. Biomarker expression for vulvar carcinoma

Currently, no screening method or test, such as the pap smear for cervical cancer, is available for vulvar cancer. This often leads to late diagnosis of vulvar cancer and subsequent severe treatment methods as the disease has already progressed. On the other hand, these treatment methods may also cause overtreatment of patients with early diagnosis and where lymph node metastases have not yet occurred. For this reason, an increase in research on biomarkers for vulvar cancer diagnosis and prognosis is needed to indicate the severity and presence of vulvar cancer as well as to predict clinical behaviour and metastatic potential of the disease (Cao *et al*. 2016).

A variety of studies have investigated $p16^{ink4a}$ as a prognostic biomarker in different cancer types with a recent meta-analysis suggesting that overexpression of $p16^{ink4a}$ may be associated with higher survival and better prognosis of vulvar carcinoma (Cao *et al*. 2016). According to Tringler *et al.*, 2007 groin lymph node status was the strongest prognostic factor, while a recent study reported the most important prognostic factors in vulvar carcinoma is suggested to be the FIGO classification stage, lymph node metastasis and histological grade (Cao *et al.*, 2016). Overexpression of $p16^{ink4a}$ was significantly associated with a FIGO stage of I-II, no lymph node metastasis, age of patient (<55) and HPV positive status (Cao *et al.*, 2016). However, no correlation could be made between the overexpression of this biomarker and histological grade such as VIN I and VIN II (Cao *et al.*, 2016). Literature on the use of $p16^{ink4a}$ as surrogate

marker for HPV-induced vulvar carcinoma is limited as well as conflicted. Cao *et al*. (2016) concluded that $p16^{ink4a}$ correlates strongly with HPV positivity in vulvar carcinoma, while another study suggests that $p16^{ink4a}$ overexpression is not a surrogate marker for high-risk HPV types (Sznurkowski, Żawrocki and Biernat, 2016).

The study of microRNAs as potential markers is well documented for a variety of cancers including cervical cancer but limited research is available on vulvar carcinomas (De Melo Maia, Lavorato-Rocha, Rodrigues, Coutinho-Camillo, Baiocchi, Stiepcich, Puga, Lima, Soares & Rocha, 2013; Yang & Wu, 2016). In a single study characterizing microRNA in vulvar carcinoma, 25 microRNAs were differentially expressed between HPV-positive and HPV-negative carcinomas, while 79 altered microRNAs were observed in tumour tissue only, which was all down-regulated compared to adjacent normal tissue (De Melo Maia *et al.*, 2013). miRNA-590-5p, a novel oncogenic miRNA in iVSCC, was found to be upregulated in association with lymphatic metastasis and research suggests that it may be a valuable therapeutic target in iVSCC (Yang & Wu, 2016). Different types of biomarkers such as proliferation markers, tumour suppressor genes, viral oncogenes and apoptosis markers have been investigated for diagnostic and prognostic value. However, clarification of the clinical value of these markers is lacking (Fons *et al.*, 2007; Cattani *et al.*, 2009; Knopp *et al.*, 2009). This is due to the small number of studies being conducted on biomarkers for vulvar carcinoma, low sample size and absence of multivariate analysis in most of the studies (Knopp *et al.*, 2009).

Validation of biomarkers for diagnostic use requires a large sample size and is often time-consuming and expensive (Knopp *et al.*, 2009). The use of tissue microarray technique (TMA) may effectively reduce costs and turnaround time as it allows for various molecular applications, including immunohistochemical (IHC) and in situ hybridization (ISH) analysis, of hundreds of different tissue samples simultaneously (Cardano, Diaferia, Falavigna, Spinelli, Sessa, DeBlasio & Biunno, 2013; Singh & Sau, 2010). The TMA technique allows for the construction of one FFPE tissue block containing a variety of FFPE tissue cores from different FFPE tissue blocks. This means that areas of interest from a big case with many FFPE blocks, for example, as is common in the case of vulvar cancer diagnoses or target regions from different patients, can be analyzed at the same time using only one slide. The technique may be useful in the validation and verification of biomarkers, for example, tumour progression

studies where biomarker reaction is analyzed over the different stages of tumour progression such as VINI, VINII, VINIII and finally invasive vulvar cancer (Jain, 2018). Research regarding the sensitivity and specificity of the TMA technique could therefore be beneficial to the investigation on the prognostic and diagnostic value of new biomarkers for vulvar carcinoma and for the validation of these biomarkers for use in the clinical or diagnostic setting.

1.9 Outline of this study

The aim of this study was to identify the prevalent HPV strains in VIN and invasive iVSCC patients at Tygerberg Academic Hospital and to determine the expression of biomarkers in these malignancies.

The objectives of this study are to:

(1) Identify the prevalent HPV strains through HPV subtype determination in patients diagnosed with LSIL (VIN I), HSIL (VIN II and VIN III) as well as iVSCC,

(2) to correlate HPV strain prevalence results with HIV status, patient age, histological subtype and biomarker expression,

(3) assess the expression of the high risk HPV E6/E7 oncoviral mRNA transcripts using quantitative real time PCR to detect transcriptionally active HPV infection, and

(4) to assess the expression of well-known diagnostic marker $p16^{INK4a}$ in iVSCC FFPE tissue using IHC on full section and TMA slides to compare the results and assess the sensitivity and specificity of antibodies using the TMA technique,

Chapter 2 Materials and Methods

2.1 Materials

All materials used in this project are detailed in the methods section accompanied by the manufacturer and catalogues numbers.

2.2 Methods

2.2.1 Ethics approval

Ethics approval for this study was obtained from the Undergraduate Health Research Ethics Committee at the Faculty of Medicine and Health Sciences at Stellenbosch (U17/08/038). A waiver of consent was granted. Ethics approval was renewed annually throughout the duration of this study.

2.2.2 Case selection and FFPE tissue block retrieval

Data received from the Central Data Warehouse of the National Health Laboratory Service (NHLS) as well as clinical lists of cases diagnosed at Tygerberg Academic hospital from January 1997 to June 2018 were used to identify suitable cases for inclusion in this study. Criteria for inclusion in this study were: (i) FFPE tissue samples from patients with a diagnosis of vulvar intraepithelial neoplasia (VIN) or invasive vulvar squamous cell carcinoma (iVSCC), which were archived in the Division of Anatomical Pathology at Stellenbosch University/ NHLS, (ii) known age at the time of diagnosis, and (iii) sufficient representative tumour tissue available for DNA and RNA extractions. All cases for which the FFPE tissue blocks could not be retrieved, cases with discrepant diagnosis after the secondary validation as well as FFPE blocks with insufficient representative tumour tissue were excluded from the study. The sample size required to achieve 95 % power, as determined with the help of a biostatistician and OpenEpi version 3.01 (Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version. www.OpenEpi.com, updated 2013/04/06), were 72 FFPE tissue blocks representative of VIN and 68 tissue blocks representative of iVSCC diagnoses. The HIV status of patients at the time of diagnosis, where available, was also recorded.

The original diagnosis was validated by a dedicated pathologist using representative hematoxylin and eosin (H&E) slides and a light microscope. Discrepant diagnoses were resolved in a secondary validation by a second pathologist. Additionally, H&E slides were also appraised for the presence of adequate representative tissue and visible HPV-induced changes such as the presence of koilocytes. The histological subtypes were recorded for each case. For tissue microarray construction, suitable tumour and normal tissue (where present) regions were demarcated on H&E slides using a permanent marker. All specimens were de-identified and a unique study number consisting of the diagnosis group and consecutive specimen number such as VIN-001 and VSCC-001 were allocated.

2.2.3 Preparation of FFPE tissue sections using microtomy

A rotary microtome (Leica Biosystems #RM2245, Wetzlar, Germany) was used to section the FFPE tissue blocks. Prior to sectioning, a sterile disposable scalpel was used to trim off excess paraffin around the embedded tissue, using a new scalpel for each block to minimize cross-contamination. As the tissue blocks were exposed to air during storage, the first two tissue sections cut were discarded. The microtome and tools, such as forceps and brushes, were cleaned using 10 % (v/v) bleach and 70 % (v/v) ethanol after sectioning of each FFPE block. A new microtome blade was used to cut each FFPE tissue block. Two to four 10 µm sections were cut for respective DNA and RNA extractions. Sections of 3 µm thickness were cut for immunohistochemistry (IHC) analysis. The sections used for IHC were placed on a labelled positively charged Histobond+ adhesion microscope slide (Leica Biosystems catalogue# 0810401) (Wetzlar, Germany) and left at room temperature (RT) to dry before staining procedures were done. Appropriate personal protective equipment (PPE) was used.

2.2.4 Nucleic acid extraction

FFPE tissue sections were dewaxed using deparaffinization solution (catalogue #19093) (Qiagen, Hilden, Germany). Total DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. Briefly, tissue were lysed using ATL buffer and proteinase K. Samples were incubated overnight (at least 16 hours) at 56 ^{o}C and immediately thereafter placed at 90 ^{o}C for 1 hour to aid the reversal of DNA crosslinks. Samples were spun at 11 000 x g in a benchtop centrifuge and the lower clear

phase were transferred to a clean 2 ml microcentrifuge tube. In order to digest residual RNA, 2 µl of RNase 1 (10U/µl) (catalogue# EN0602) (Thermo Fisher Scientific, Life Technologies, USA) were added to each tube and incubated at 37 ^{o}C for 30 minutes. Buffer AL and molecular grade ethanol (catalogue #E7023) (Sigma-Aldrich St. Louis, MO, USA) were added to each sample and the lysates were transferred to a QIAamp minelute column. The columns were washed with buffer AW1 and buffer AW2, respectively and spun down at 11 000 x g for each step. Finally, DNA was eluted using a total volume of 75 µl ATE buffer. DNA was stored at -20 ^{o}C until subsequent use.

Total RNA was extracted using the miRNeasy FFPE tissue kit (catalogue #217504) (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, PKD buffer and proteinase K were added to each sample for tissue lysis. Samples were incubated at 56 ^{o}C for 15 minutes followed by another 15 minute incubation step at 90 ^{o}C. The lower clear phase of the samples was transferred to a clean 2 ml microcentrifuge tube, incubated on ice for 3 minutes and then spun for at 20 000 x g 15 minutes. The supernatant were transferred to a clean 5 ml tube followed by the addition of DNase booster and DNase 1 to remove residual DNA. Samples were then incubated at room temperature for 15 minutes, after which buffer RBC and molecular grade ethanol (catalogue #E7023) (Sigma-Aldrich St. Louis, MO, USA) were added. The lysates were then transferred to minelute spin columns and spun at 8000 x g for 30 seconds until all of the fluid passed through the column. Columns were washed twice with Buffer RPE. RNA was eluted using 30 µl of nuclease free water and subsequently stored at -20^{o}C until further utilization.

2.2.5 Nucleic acid purity and quantity assessment

Nucleic acid purity was assessed by measuring the $A260_{nm}/A280_{nm}$ and $A260_{nm}/A230_{nm}$ absorbance ratios using the Biodrop µLite spectrophotometer (Biodrop LTD, Cambridge, UK). The following categories specified in Table 2.1 were used to score the nucleic acid purity.

Table 2.1 Categories used for the assessment of nucleic acid purity.

Purity Category	DNA A260nm/A280nm	RNA A260nm/A280nm	DNA and RNA A260nm/A230nm
I	<1.8	<1.9	<2.0
II	1.8 to 1.9 (inclusive of values)	Category II (1.9 to 2.0) (inclusive of values)	2.0 to 2.2 (inclusive of values)
III	>1.9	>2.0	>2.2

Nucleic acid concentrations were determined using the Qubit 3.0 fluorometer (Life Technologies, Thermo Fisher Scientific, California, USA) according to manufacturer's recommendations. The double-stranded DNA concentrations were measured using the Qubit dsDNA Broad Range Assay kit. RNA concentrations were measured using the Qubit RNA high sensitivity (HS) assay kit.

2.2.6 Nucleic acid integrity assessment

DNA integrity was assessed through amplification of the 205 bp human β-globin gene fragment using a SYBR green based quantitative Real-Time Polymerase Chain Reaction (qPCR) (Table 2.2). The 10 µl qPCR reaction contained 100 ng DNA, 0.4 µM of the respective forward and reverse primers (Greer, Peterson, Kiviat, Manos & Ph, 1990), 1X PowerUp™ SYBR™ Green Master mix (Thermo Fisher Scientific, Life Technologies, USA) and nuclease free water. The PCR cycling conditions were 50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The dissociation curve conditions were 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds according to manufacturer's recommendations. Human genomic DNA (Cat# PRG3041) (Promega, Madison, WI, USA) and a non-template control (NTC) was used as positive and negative controls, respectively. All samples and controls were done in triplicate.

Table 2.2 Human β-globin (Greer *et al.*, 1990) ***and HPV subtype-specific*** (Karlsen, Kalantari, Jenkins, Pettersen, Kristensen, Holm, Johansson & Hagmar, 1996) ***primer sequences and expected fragment sizes.***

Primer	Primer sequence	Expected fragment size
β-globin: KM29	5'-GGT TGG CCA ATC TAC TCC CAG G-3'	205 bp
β-globin: PC04	5'-CAA CTT CAT CCA CGT TCA CC-3'	
HPV 18 F	5'-CCG AGC ACG ACA GGA GAG GCT-3'	172 bp
HPV 18 R	5'-TCG TTT TCT TCC TCT GAG TCG CTT-3'	
HPV 16 F	5'-ATA TAT GTT AGA TTT GCA ACC AGA GAC AAC-3'	196 bp
HPV 16 R	5'-GTC TAC GTG TGT GCT TTG TAC GCA C-3'	
HPV 35 F	5'-CCCGAG GCA ACT GAC CTA TA-3'	230 bp
HPV 35 R	5'-GGG GCACAC TAT TCC AAA TG-3'	
HPV 11 F	5'-ATG GAA AGT AAA GAT GCC TCC ACG T-3'	200 bp
HPV 11 R	5'-CAA CAG GCA CAC GCT GCA AG-3'	

RNA integrity and fragment size distributions were assessed using the Agilent HS RNA kit on the Agilent® 2100 Bioanalyser (Agilent Technologies, California, United States of America). The RNA integrity number (RIN) and DV200 were used as quality indicators. RNA was deemed suitable for qPCR analysis a RIN score ≥ 2 and a DV200 score ≥ 30 %. In order to assess the adequacy of the RNA samples for use in qPCR as well as the presence of likely inhibitors of the qPCR reactions, RNA samples were reverse transcribed into cDNA, and the expression of the human GAPDH mRNA transcript was investigated as described in section 2.2.8.2.

2.2.7 Detection of HPV DNA using qPCR

Detection of HPV 16, 18, 35 and 11 DNA were done by qPCR using HPV strain-specific primers (Karlsen *et al.*, 1996) (Integrated DNA Technologies (IDT)) (Iwoa, USA) targeting the E7 genes (Table 2.2). The 10 µl qPCR reactions contained 100 ng DNA, 0.4 µM of the respective forward and reverse primers, 1X PowerUp™ SYBR™ Green Master mix (ThermoFisher Scientific, Life Technologies, USA) and nuclease free water. The PCR cycling conditions for amplification of HPV 18, 11 and 35 were 50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. Amplification conditions for HPV 16 were similar with the exception of an annealing temperature of 55 °C instead of 60 °C and an additional extension step for 1 min at 72 °C as recommended by the manufacturer.

The dissociation curve conditions were 95 ºC for 15 seconds, 60 ºC for 1 minute and 95 ºC for 15 seconds according to manufacturer's recommendations. HPV plasmid DNA (HPV 16 catalogue #45113D, HPV 18 catalogue #45152D, HPV 35 catalogue #40330, HPV 11 catalogue #45151D) (ATCC, Manassas, United States of America) was used as respective positive controls while a NTC represented the negative control. qPCR reactions for all samples and controls were done in triplicate.

2.2.8 Detection of HR HPV 16 and 18 E6/E7 mRNA transcripts

2.2.8.1 Reverse transcription of total RNA samples into cDNA

The High-Capacity cDNA Reverse Transcription kit (catalogue #4368814) (Life Technologies, Thermo Fisher Scientific, California, USA) was used to convert total RNA to cDNA, according to the manufacturer's instructions. All reactions were done in duplicate. To detect possible carryover of residual genomic DNA in the qPCR reaction, a no reverse transcriptase (no-RT) reaction was included for each RNA sample. The 20 µl reverse transcription reaction contained 300 ng RNA, 1X RT buffer, 4 mM dNTPs, 1X random primers, 1 µl of MultiScribe reverse transcriptase (50 U/µl), 1 µl of RNase inhibitor (20 U/µl) (catalogue# N8080119) (LTC Tech, Johannesburg, South Africa) and nuclease-free water. The cycling conditions for the reverse transcription reactions were 25 ºC for 10 minutes, 37 ºC for 120 minutes and 85 ºC for 5 minutes. The respective duplicate cDNA samples were pooled and used in subsequent qPCR analysis.

2.2.8.2 Detection of human and viral HPV 16 and 18 E6/E7 mRNA transcripts

The Quant Studio 5 real time PCR machine (Thermo Fisher Scientific, California, USA) was used for the analysis of mRNA transcript expression. As previously stated, the human GAPDH was used as a housekeeping gene as well as to give an indication of the adequacy of the RNA samples. The 20 µl GAPDH TaqMan gene expression assay reaction consisted of 2X TaqMan fast advance master mix (catalogue #4444557) (Life Technologies, Thermo Fisher Scientific, California, USA), 20 X TaqMan assay (catalogue #4331182) (Life Technologies, Thermo Fisher Scientific, California, USA) and nuclease free water. The HPV 16 and HPV 18 subtype-specific primers and probe sequences were designed according to Lamarcq *et al.*, 2002. The qPCR assays each contained 2X TaqMan fast advance master mix (catalogue #4444557) (Life

Technologies, Thermo Fisher Scientific, California, USA), 0.25 µM for each of the forward and reverse primers, 0.125 µM probe and nuclease-free water. Each qPCR reaction contained 2 µl of pooled cDNA. cDNA reverse transcribed using RNA from HeLa and SiHa cells were used as HPV 18 and HPV 16 positive controls, respectively. The no template controls were used as negative controls. No-RT reactions of each RNA sample were included in the GAPDH assay reactions to detect possible residual genomic DNA. The qPCR reactions were done in triplicate for all samples and cycling conditions were 2 minutes at 50 ^{o}C and 2 minutes for 95 ^{o}C for the hold stage, followed by the PCR stage of 40 PCR cycles at 95 ^{o}C for 1 second and 20 seconds at 60 ^{o}C .

2.2.9 Immunohistochemical staining using p16^{INK4a} antibodies

The BOND-III Fully Automated IHC and ISH Stainer (serial# 3211341) (Leica Biosystems, Wetzlar, Germany) was used for p16^{INK4a} (catalogue# 805-4713) (Roche, Basel, Switzerland) IHC staining. All steps were carried out at room temperature unless otherwise stated. Briefly, FFPE tissue slides were deparaffinised using Bond Dewax Solution (catalogue# AR9222) (Leica Biosystems, Wetzlar, Germany) for 30 seconds (twice at 72 °C and once at room temperature), washed with alcohol for 30 seconds (three times) and then washed three times with Bond Wash solution (catalogue# AR9590) (Leica Biosystems, Wetzlar, Germany) for 30 seconds (X3). Slides were then subjected to Bond Epitope Retrieval solution 2 (catalogue# AR9640) (Leica Biosystems, Wetzlar, Germany) for 30 seconds (twice) followed by a 20 minute step at 100 °C and another 30 second step at room temperature. Samples were then washed with Bond wash solution (catalogue# AR9590) (Leica Biosystems, Wetzlar, Germany) for 30 seconds (four times) followed by a 3 minute wash step. The p16^{INK4a} antibody) was then applied for 15 minutes followed by Bond Wash solution (catalogue# AR9590) (Leica Biosystems, Wetzlar, Germany) for 2 minutes, 1 minute and 1 minute, respectively. A post primary step was then applied for 8 minutes followed by three wash steps of 30 seconds each. The slides were then covered with Bond^{TM} polymer refine detection (catalogue# DS9800) reagent for 8 minutes followed by two wash steps of 2 minutes each using Bond wash solution (catalogue# AR9590) (Leica Biosystems, Wetzlar, Germany). Slides were then washed with deionized water for 30 seconds followed by incubation with peroxide block reagent for 5 minutes. Slides were once again washed with Bond wash solution for 1 minute followed by two 30 second wash steps. Deionized water was then applied for 30 seconds followed by mixed DAB Refine once for 30 seconds and once for 10 minutes. The samples were washed three

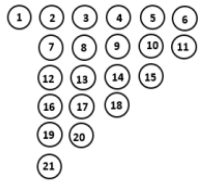
times with deionized water for 30 seconds each and stained with Hematoxylin for 5 minutes. This is followed by deionized water for 30 seconds, Bond wash solution (catalogue# AR9590) (Leica Biosystems, Wetzlar, Germany) for 30 seconds and another deionized water step for 30 seconds. IHC slides were then analysed blindly by a dedicated pathologist for $p16^{INK4a}$ positivity.

2.2.10 Tissue Microarray (TMA) construction

After sections were cut for DNA and RNA extractions, the leftover FFPE blocks were inspected for adequate tissue thickness for inclusion in the TMA construction. The H&E stained slides of full tissue sections were used to identify the representative regions of interest (tumour) for TMA production. The tape method was used tor TMA construction according to Glinsmann-Gibson *et al.* (2019) (Figure 2.1 and Figure 2.2). Briefly, Formula R tissue embedding infiltration medium (catalogue #3801450) (Leica Microsystems, Wetzlar, Germany) was melted completely at 60 °C. Grid paper was attached to the base of a stainless steel mold (catalogue #4123) (Sakura Finetek Europe, AJ Alphen aan den Rijn, The Netherlands) using clear, double sided tape to aid in the alignment of the tissue cores. The marked H&E slides were then overlaid with the corresponding FFPE tissue blocks to identify where the tissue core should be taken. A 1 mm core was then taken from the respective FFPE tissue blocks using the UNITMA microarayer (catalogue #IW-UT06) (IHC World, Ellicott City, United States of America). For each sample included in the TMA, the FFPE core was then slowly released from the microarrayer and a needle stick was used to pick up the core and place it in the pre-identified location on the grid paper in the base mold. After each FFPE tissue core were extracted and placed in the desired position, the base mold is covered with a uni-cassete (catalogue #4157) (Sakura Finetek Europe, AJ Alphen aan den Rijn, The Netherlands). Small weights were placed on two corners of the cassette and the mold was gently filled with liquid paraffin. The TMA block was left at room temperature for 30 minutes to cool and another 30 minutes at 4 °C to ensure the paraffin block was properly set. The base mold was then removed and the FFPE TMA block was cut as described in section 2.2.3.

TMA name

FROST



Spot	ACSR ID or other Identifiers	H&E core present ? (Y,N or NA)	H&E tumor present / control satisfactory? (Y,N or NA)	CD 20 forTumour	
				Pos/ Neg (control satisfactory?)	F/ M/ A
1	p16/ki67 control				
2	VSCC-008				
3	VSCC-014				
4	VSCC-019				
5	VSCC-025				
6	VSCC-033				

Figure 2.1 Illustration of an example of a TMA map.

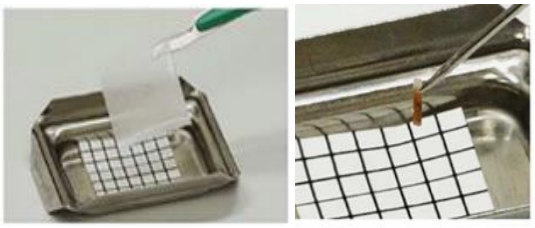


Figure 2.2 Example of the paper grid used to aid in alignment of tissue cores (Glinsmann-Gibson et al., 2019).

2.2.11 Statistical analysis

Data was analysed using IBM® SPSS® statistics version 25 (IBM Corp. Released 2017, IBM SPSS Statistics for Windows, Version 25.0; Armonk, NY: IBM Corp) and Stata version 15.1. HPV genotyping data and other categorical variables were summarised using frequencies and proportions. Association between HPV strains (i.e. presence/absence) and categorical variables such as HIV status and $p16^{INK4a}$ biomarker expression (coded as either positive or negative) were assessed using Chi-Square test. The strength of the relationships was quantified using a correlation coefficient. All statistical tests were performed at 5 % level of significance and 95 % confidence intervals will be reported. Sensitivity and specificity of the TMA slide was evaluated using full section slides as the gold standard using EpiCalc 2000 (Version 1.02, Gilman and Matt, 1998).

Chapter 3 Results and Discussion

3.1 Specimen selection and validation

Approximately 2 500 possible VIN and iVSCC cases diagnosed at Tygerberg Academic Hospital between January 1997 to June 2018 were obtained from clinical lists and data from the NHLS central data warehouses. Of these plausible cases, merely 312 had suitable diagnosis and were available for retrieval as the NHLS at Tygerberg Academic Hospital only stored H&E slides from 2003. The H&E slides of cases prior to 2003 were no longer available as these were discarded by the NHLS according to their standard operating procedures and policies regarding long term specimen storage. Therefore, the cases included in this study were from the time period of January 2003 to June 2018. A total of 82 cases had discrepant diagnoses upon validation and were re-validated by a second pathologist. Of these, 47 cases were excluded from the study as no consensus could be reached on the diagnosis after the secondary validation.

The population size (N) for this study was defined as the number of suitable cases diagnosed with VIN and iVSCC, respectively and for which the FFPE tissue blocks were retrieved from the NHLS archive, that is, the number of tissue blocks in-hand. The population proportion required for a 95 % confidence level was calculated as 72 FFPE tissue blocks representative of VIN and 68 tissue blocks representative of iVSCC. Therefore, enough FFPE tissue blocks for both diagnosis groups were obtained for the study to have 95 % power. Clinical information such as age of the patient at the time of diagnosis, HIV status and histopathological subtype were also recorded where possible. All specimens included in this study had sufficient tumour and/or pre-cancerous tissue required for RNA and DNA extractions and thus met the selection criteria. Specimens were de-identified by allocating a unique study number for example VIN-001 or VSCC-001.

3.2 Analysis of age and HIV status of women diagnosed with VIN and iVSCC

As previously mentioned, VIN and invasive vulvar carcinoma incidence have increased over the last few years with the affected patients being of a much younger age (Bradbury *et al.*,

2016; De Vuyst *et al.*, 2009). Increased diagnosis in younger populations may be due to high HIV prevalence as immunosuppression is one of the major risk factors for developing HPV related diseases (Bradbury *et al.*, 2016). HIV infection also accelerates the progression of VIN to invasive vulvar carcinoma which may lead to increase iVSCC diagnosis in younger women (Auvert, Marais, Lissouba, Zarca, Ramjee & Williamson, 2011; Bradbury *et al.*, 2016). In order to ascertain the age distribution in our population, the age of women at diagnosis was assessed and correlated to HIV status. Other features such as histopathological subgroups were also compared to HIV status to investigate the role of HIV in the development of VIN and iVSCC.

3.2.1 Younger women were more frequently diagnosed with VIN and iVSCC

Data analysis using the Pearson Chi-square test showed no significant difference ($p>0.05$) in the age at which patients were diagnosed with pre-cancerous lesions and invasive squamous cell carcinoma of the vulva (Figure 3.1 and Table 3.1). A similar trend for both diagnosis groups was observed, with the mean ages being 49.4 ±17.5 years ranging from 23 to 88 years for VIN, and 49.3±14.9 within a range of 24 to 83 years for iVSCC (Figure 3.1).

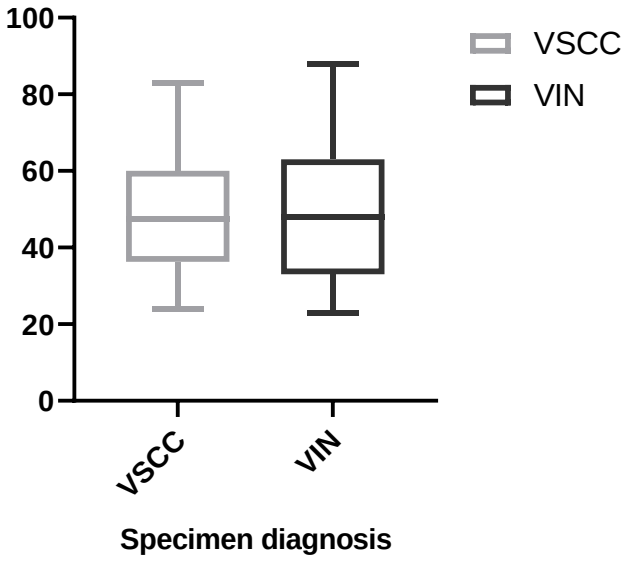


Figure 3.1: Age distribution of women diagnosed with VIN and VSCC. This box and whisker plot illustrates the distribution of the ages centred on the respective population means of patients diagnosed with VIN and iVSCC.

Results show that 41.7 % (30/72) of patients diagnosed with VIN and 54.4 % (37/68) of those diagnosed with iVSCC were between the ages of 36 to 59 years (Table 3.1). Moreover, a proportion of women were 35 years and younger, that is, 28 % (20/72) of these particular

women were diagnosed with VIN and 19 % (13/68) with iVSCC (Table 3.1). Only 30.6 % (22/72) VIN and 26.4 % (18/68) iVSCC patients were older than 60 years at the time of diagnosis. However, of these, only 7 % (5/72) and 3 % (2/68) were older than 75 for the respective VIN and iVSCC diagnoses. Our data thus show that women diagnosed with vulvar carcinoma and its precursors in our study population at Tygerberg Academic Hospital were generally younger than 59 years, with 69 % (50/72) receiving a diagnosis of VIN and 73 % (50/68) with iVSCC. Our results are therefore in accordance with the findings of the clinical study also done at Tygerberg Academic Hospital but between 1 January 2001 and 31 December 2014, by Butt and Botha (2017). Their results showed that the mean age of patients diagnosed with HPV-related vulvar cancer and its precursors during the indicated time period were 50,4 years. Overall, our findings are contrary to reported international studies where the mean age of women diagnosed with VIN and iVSCC is 64 and 70 years. Our results indicate that the majority of women whose specimens were included in this study received a diagnosis of VIN and iVSCC at a much younger age with a large proportion being even younger than 35.

Table 3.1: The average age and number of HIV positive and HIV negative patients as well as patients with unknown HIVstatus for each age group per diagnosis.

Diagnosis	Age group		HIV status		
		Total (mean age±SD)	Negative (mean age±SD)	Positive (mean age±SD)	Unknown (mean±SD)
VIN	18-35	20 (29.5±3.3)	2 (29.5±0.7)	16 (30.1±3.3)	2 (25±0.0)
	36-59	30 (46.7±6.4)	6 (49.3±7.5)	17 (43.9±5.6)	7(51±4.4)
	60-75	17 (67.2±4.9)	9 (66.3±5.5)	2 (71.5±0.7)	6 (67.2±4.5)
	>75	5 (85±3.9)	0	0	5 (85±3.9)
	Total	**72 (49.4±17.5)**	**17 (56.0±14.0)**	**35 (39.2±11.5)**	**20 (61.8±18.7)**
iVSCC	18-35	13 (31.1±3.7)	0	13 (31.1±3.7)	0
	36-59	37 (46.1±8.1)	18 (50.3±7.7)	19 (42.2±6.5)	0
	60-75	16 (67.3±5.4)	12 (69.0±5.1)	4 (62.3±2.1)	0
	>75	2 (81.0±2.8)	2 (81.0±2.8)	0	0
	Total	**68 (49.3±14.9)**	**32 (59.2±12.5)**	**36 (40.4±10.7)**	**0**

3.2.2 Age of VIN and iVSCC patients according to HIV status

As stated previously, HIV is known to exacerbate HPV related diseases as HIV positive women are more likely to contract HPV and also have the propensity for rapid disease progression

(Auvert *et al*., 2011; Williamson, 2015). In our study, the iVSCC group comprised of 52.9 % (36/68) HIV positive and 47.1 % (32/68) HIV negative patients (Table 3.1). On the other hand, the VIN group included 48.6 % (35/72) HIV positive, 23.6 % (17/72) HIV negative and 27.8 % (20/72) of patients with unknown HIV status (Table 3.1). The proportion of samples of patients with unknown HIV status was thus excluded from the analysis pertaining to HIV status. Results showed that 88.9 % (32/36) of HIV positive patients with iVSCC and 94.3 % (33/35) of HIV positive patients with VIN were younger than 59 years old (Table 3.1). HIV positivity was thus the highest in younger age groups with 45.7 % and 48.6 % of HIV positive VIN patients being between the ages of 18 to 35 and 36 to 59 years respectively, while 36.1 % and 52.8 % of iVSCC patients for the respective age groups were HIV positive (Table 3.1). Using the Pearson Chi-square test, a significant relationship between HIV status and age of patients diagnosed with VIN and iVSCC in our sample population was established ($p<0.05$). The majority of patients for both VIN and iVSCC categorized in the older age groups were HIV negative.

3.3 Nucleic acid purity and quality assessment

Formalin fixation of tissues is known to result in macromolecule modification through cross-link formation, fragmentation and degradation of extracted nucleic acids that leads to decreased quantity and quality, which in turn may affect downstream molecular applications (von Ahlfen, Missel, Bendrat & Schlumpberger, 2007; Baak-Pablo, Dezentje, Guchelaar & van der Straaten, 2010; Bass, Engel, Greytak & Moore, 2014; Thavarajah, Mudimbaimannar, Rao, Ranganathan & Elizabeth, 2012). Human and viral nucleic acids templates of subpar quantity and quality may interfere with sensitive PCR-based applications such as genotyping. Therefore, to obtain successful and meaningful results, the quantification and quality assessment of FFPE-derived nucleic acids are essential to gauge suitability for qPCR and genotyping.

3.3.1 Nucleic acid purity assessment

DNA and RNA isolated from FFPE vulvar tissue were evaluated using a Biodrop µLite and a Qubit 3.0 fluorometer. Indicators used for purity assessment were the $A260_{nm}/A280_{nm}$ and $A260_{nm}/A230_{nm}$ spectrophotometric absorbance ratios which were each classified into categories as indicated in Table 2.1. The generally accepted $A260_{nm}/A280_{nm}$ ratios for pure

DNA and RNA are 1.8 and 2.0, respectively. An $A260_{nm}/A280_{nm}$ ratio less than 1.7 indicates protein contamination. The $A260_{nm}/A230_{nm}$ ratio is an ancillary measurement of pure nucleic acids where values between 2.0 and 2.2 indicate pure DNA or RNA, whilst values less than 2.0 suggest the presence of contaminants such as residual nucleic acid extraction reagents (Ludyga, et al., 2011).

All samples yielded pure DNA fit for utilization in PCR reactions. The average $A260_{nm}/A280_{nm}$ ratios (Figure 3.2 A and Table 3.2) for VIN DNA samples were 1.91±0.08 and data ranging from 1.68 to 2.15. The DNA extracted from iVSCC samples also had an average of 1.91 but with a standard deviation 0.10 and data ranged from 1.81 to 2.02. VIN and iVSCC DNA samples had an average $A260_{nm}/A230_{nm}$ reading (Figure 3.3 A and Table 3.3) of 2.38±0.18 and 2.23±0.79, respectively. Hence, the majority of the extracted DNA, irrespective of diagnosis group, could be classified as either category II and III of the $A260_{nm}/A280_{nm}$ ratios, with only 4 VIN and 1 iVSCC samples falling into category I. A similar trend was observed for the $A260_{nm}/A230_{nm}$ ratios as most samples fell into categories I and II, with 8 of the VIN and 7 of the iVSCC samples being assigned to category III. These results, with the exception of a few outliers, indicated that pure DNA, free of protein contamination and with minimal reagent carryover, was isolated. The average DNA yields for VIN and iVSCC samples were 5 µg and 9 µg, respectively. All samples thus had sufficient DNA yield and were included in this study as the minimum amount of DNA needed was 1.5 µg.

VIN and iVSCC RNA samples had an average $A260_{nm}/A280_{nm}$ ratio (Figure 3.2 B and Table 3.2) of 1.89±0.06 and 1.92±0.04 as well as an $A260_{nm}/A230_{nm}$ ratio (Figure 3.3 B and Table 3.3) of 1.84±0.33 and 1.94±0.24, respectively. Contrary to the DNA $A260_{nm}/A280_{nm}$ ratio categorisation, most RNA samples were assigned to categories I and II (Table 3.2), with only one sample in each diagnosis group being in category III. Similarly for the $A260_{nm}/A230_{nm}$, most RNA samples were categorised in categories I and II, with 5 VIN and 6 iVSCC being assigned to category III (Table 3.3). As in the case with the extracted DNA, these results, with the exception of a few outliers, suggested that pure RNA was extracted from FFPE vulvar tissue. The average RNA yield were substantially less than that of DNA with VIN samples having an average yield of 1.5 µg and iVSCC samples an average of 2.3 µg.

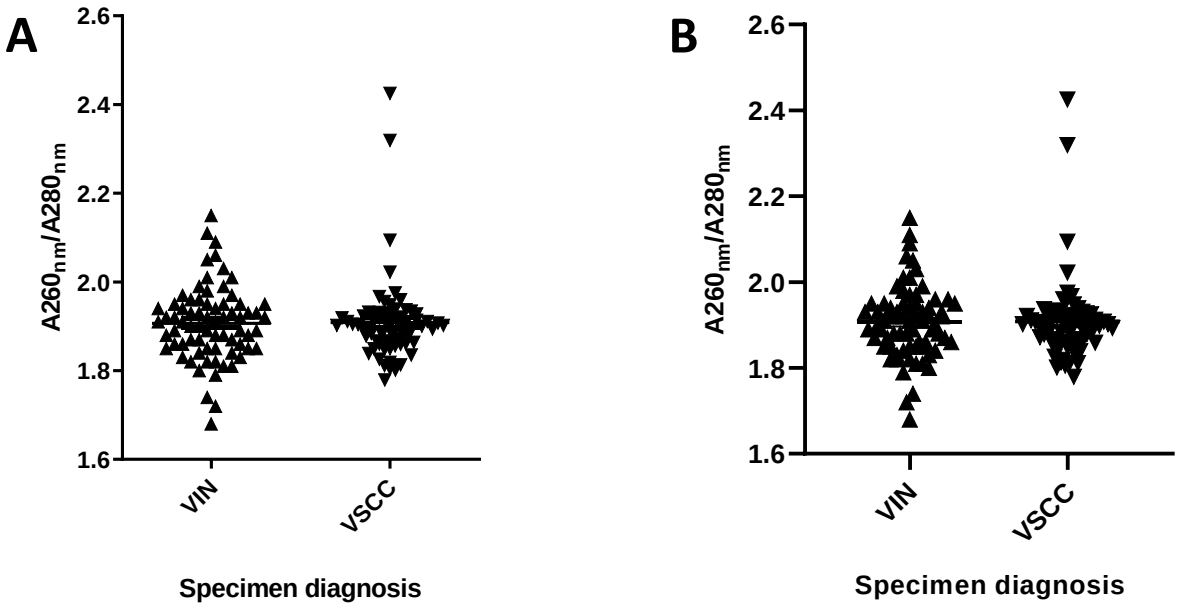


Figure 3.2: DNA (A) and RNA (B) A260$_{nm}$/A280$_{nm}$ purity results. This figure shows the distribution of A260$_{nm}$/A280$_{nm}$ ratios for nucleic acids extracted from VIN and iVSCC samples.

Table 3.2: Proportion of DNA and RNA samples divided into purity categories according to the A260$_{nm}$/A280$_{nm}$ ratio.

	Sample type			Sample type	
	DNA A260nm/A280nm			RNA A260$_{nm}$/A280$_{nm}$	
Purity categories	**VIN (n = 72)**	**iVSCC (n = 68)**	**Purity categories**	**VIN (n = 72)**	**iVSCC (n = 68)**
Category I (<1.8)	4	1	**Category I (<1.9)**	30	16
Category II (1.8 to 1.9)	31	32	**Category II (1.9 to 2.0)**	41	51
Category III (>1.9)	37	35	**Category III (>2.0)**	1	1
Average ratio	1,91 (±0,08)	1,91 (±0,10)	**Average ratio**	1.89 (±0.06)	1.92 (±0.04)

A minimum of 0.9 µg RNA per sample (300 ng x 3 reactions) was needed for efficient cDNA conversion. Therefore, 11 VIN samples and 2 iVSCC samples were excluded from qPCR analysis due to insufficient RNA yield. Furthermore, all samples included for HPV genotyping analysis thus had sufficient nucleic acid concentrations.

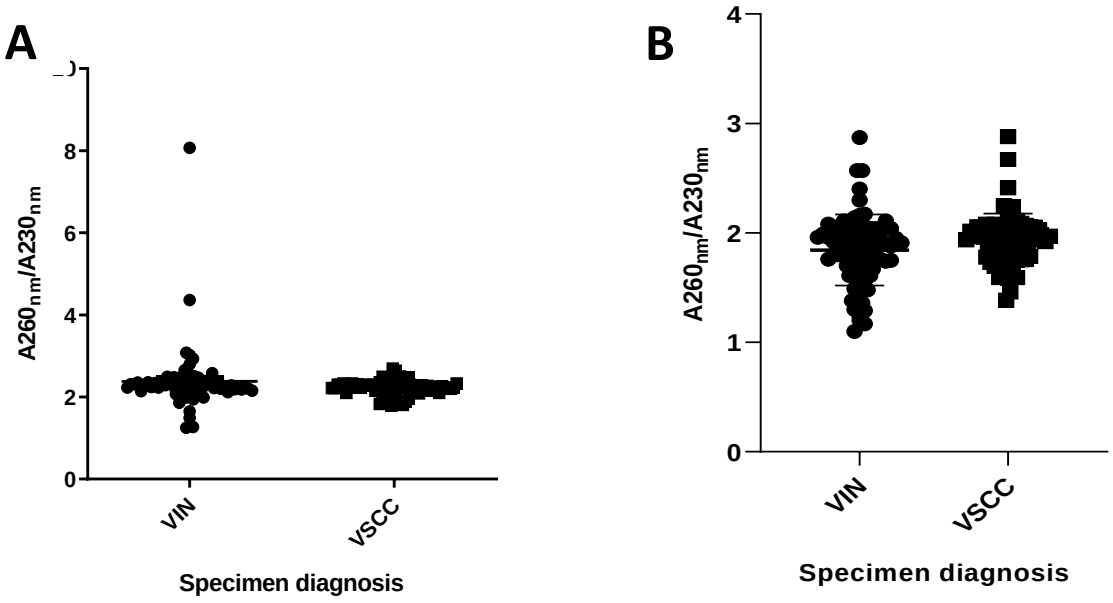


Figure 3.3: DNA (A) and RNA (B) $A260_{nm}/A230_{nm}$ purity results. This figure shows the distribution of nucleic acid purity results according to the $A260_{nm}/A230_{nm}$ ratios for VIN and iVSCC.

Table 3.3: Proportion of DNA and RNA samples divided into purity categories according to RNA RNA $A260_{nm}/A230_{nm}$ ratio.

	Sample type			
	DNA A260nm/A230nm		RNA A260nm/A230nm	
	VIN (n = 72)	iVSCC (n = 68)	VIN (n = 72)	iVSCC (n = 68)
Category I (<2.0)	8	7	51	42
Category II (2.0 to 2.2) inc	14	13	16	20
Category III (>2.2)	50	48	5	6
Average ratio	2,38 (±0,79)	2,23 (±0,18)	1.84 (±0.33)	1.94 (±0.24)

3.3.2 DNA integrity

DNA integrity was determined by the amplification of a 205 bp fragment of the highly abundant human β-globin gene using qPCR. As FFPE-derived DNA is often highly fragmented, amplification of the β-globin fragment was used as verification of the ability to obtain amplicons of around 205 bp as well as to verify the sufficiency of human DNA. In addition, the successful amplification of the β-globin fragment also indicated that there was no interference of any possible PCR inhibitors. This would thus demonstrate that the FFPE-

derived DNA was suitable for use in qPCR analysis, especially for amplification of other gene fragments of similar sizes. This particular β-globin fragment was chosen as it was approximately of similar size as all of the HPV amplicons to be analysed (Table 2.1).

For the analysis of the qPCR reactions, the same threshold was used to assess amplification of all samples. The dissociation curve and melting temperature of the human DNA positive control as well as a Ct value of ≤36 were used to confirm the successful amplification of the unknown samples. No amplification was observed in NTCs. The 205 bp human β-globin gene fragment could be amplified in 100 % (72/72) of VIN as well as 100 % (68/68) of iVSCC samples. All extracted DNA samples were therefore included for analysis as the β-globin fragment was amplified successfully in all samples, indicating that the samples were of adequate DNA integrity for use in PCR-based HPV genotyping.

3.3.3 RNA integrity

RNA integrity was measured using the Agilent 2100 Bioanalyzer and the Agilent Nano 6000 total RNA kit. The RNA integrity number (RIN) was generated as an indication of intact and degraded RNA. Generally, a RIN value of 1 indicates severely degraded RNA while a value of 10 represents highly intact RNA. Another measurement of RNA quality used is the DV200 score which measures the percentage of fragments ranging between 200 and 8000 bp. RNA was deemed of good quality if it had a minimum RIN score of 2 and a DV_{200} score of more than 30 % (Yakovleva *et al.*, 2017, Technical note: RNA sequencing, Illumina, 2016). Although the RIN value of an RNA sample is a reliable metric to evaluate RNA integrity for standard RNA sequencing, the DV_{200} score adds more value to evaluating RNA integrity of samples derived from FFPE tissue as these samples can be much more challenging due to sample degradation (Graf, 2017). The DV_{200} score thus gives an indication of the percentage of fragments in the RNA sample which are larger than 200 nucleotides.

As is tabulated in Table 3.4, the majority of VIN and iVSCC samples had a DV_{200} score of 30 % to 50 % and 50% to 70 % suggesting that a good proportion of RNA samples contained fragments larger than 200 nucleotides and thus were not severely degraded. However, 1 out of 72 VIN and 4 out of 68 iVSCC samples had a DV_{200} score of less than 30 %. A greater proportion of VIN and iVSCC samples had a RIN value of >2 (Table 3.5), suggesting that RNA

was fragmented. The proportion of VIN and iVSCC samples with a RIN value less than 2 were 12 in 72 and 3 in 68, respectively.

Table 3.4 The proportion of VIN and iVSCC RNA samples categorised according to DV_{200} scores.

DV200 Category	VIN (n = 72)	iVSCC (n=68)
<30 %	1	4
30 %-50 %	32	26
50 %-70 %	38	37
>70 %	1	1

Table 3.5 Proportion of VIN and iVSCC RNA samples categorised according to RIN score.

RIN score	VIN (n = 72)	iVSCC (n=68)
<2	12	3
2-3	59	65
>3	1	0
Average RIN score (SD)	2.2 (±0.4)	2.31 (±2.5)

Samples deemed fit for use in qPCR analysis were subjected to cDNA conversion and the expression of the human GAPDH mRNA transcript was evaluated as an indication of specimen sufficiency and for the presence of possible qPCR reaction inhibitors. The presence of residual genomic DNA was assessed through the amplification of RNA samples subjected to cDNA conversion with no reverse transcriptase added to the reaction. Expression of GAPDH mRNA was evaluated using the same threshold for all samples tested as well as a Ct value of ≤36. GAPDH was amplified in 100 % (61/61) VIN and 100 % (66/66) iVSCC samples included in the analysis, confirming that the FFPE-derived RNA was suitable to use in qPCR. Residual genomic DNA was not detected in any of the no-RT controls; hence all samples identified for use in RNA analysis were included.

3.4 Detection of HPV DNA in FFPE vulvar tissue

3.4.1 HPV prevalence in VIN and iVSCC

The prevalence of HPV 16, HPV 18, HPV 35 and HPV 11 in FFPE vulvar tissue from patients diagnosed with VIN and iVSCC were tested with qPCR using DNA samples and HPV type-specific primers targeting the respective E7 gene regions (Karlsen *et al.*, 1996). Reported HPV prevalence results varied from study to study and are largely dependent on the geographical site. Overall, HPV DNA was detected in 77.8 % (95 % confidence intervals (CI): 66.4 % – 86.7 %) of VIN and 66.2 % (95 % CI: 53.7 % – 77.2%) of iVSCC samples (Table 3.6). HPV 16 DNA was more frequently detected in both diagnosis groups compared to the other HPV strains and could be amplified in 69.4 % (95 % CI:57.4 % – 79.8 %) of VIN and 57.3 % (95 % CI: 44.7 % – 69.3 %) of iVSCC samples (Table 3.6). Therefore, results indicate that HPV 16 is the most prevalent HPV subtype detected in our sample population, being detected in slightly more VIN samples than iVSCC. This result correlates with that of De Sanjosé *et al.*, 2013 which found that HPV 16 had a higher prevalence in VIN samples than in iVSCC samples. According to De Sanjosé *et al.*, 2013, this finding is contrary to what is observed in cervical HPV-related lesions where a higher HPV 16 prevalence is seen in more advanced disease.

On the other hand, HPV 18 DNA were present in a higher number of iVSCC samples compared to VIN with amplification in 10.3 % (95 % CI: 4.2 % – 20.1 %) of iVSCC and 4.2 % (95% CI: 0.9 % – 11.7%) of VIN samples (Table 3.6), which is also in concordance to that described in De Sanjosé *et al.*, 2013 for HPV 18 detection on iVSCC and VIN cases. Furthermore, HPV 11 were detected in 11.1% (95 % CI: 4.9 % – 20.7 %) of VIN samples and 5.9 % (95 % CI: 1.6 % – 14.3 %) of iVSCC samples (Table 3.6). The least abundant HPV strain detected in our sample population was HPV 35 as it was detected in 4.2 % (95 % CI: 0.9 % – 11.7 %) of VIN samples and in none of the iVSCC samples (Table 3.6). Although the majority of VIN and iVSCC samples had single HPV infection, more than one HPV type could be amplified in 7 VIN and 5 iVSCC samples. For VIN samples, co-infection with HPV 16 and HPV 11 were detected in 4 samples, whereas co-infections with HPV 18 and HPV 11 as well as HPV 16 and HPV 35 could be detected in 1 sample each. Additionally, a co-infection with HPV 16, HPV 35 and HPV 11 was identified in one of the VIN samples. The detection of both HPV 16 and HPV 18 DNA was observed in three iVSCC samples, while HPV 18 and HPV 11 as well as HPV 16 and HPV 11 could be detected in one iVSCC sample each. Our HPV DNA detection results is

also in agreement with Mbulawa *et al.*, 2016 who concluded HPV 16 and HPV 18 to be the most prevalent high risk HPV strains among South African women. However, a study done among Nigerian women concluded that HPV 18 and 16 prevalence were low in this population (Akarolo-Anthony, Famooto, Dareng, Olaniyan, Offiong, Wheeler & Adebamowo, 2014). This study done by Akarolo-Anthony *et al*., 2014 also found HPV 35 to be the most prevalent strain among Nigerian women using cervical samples, while HPV 35 was the least abundant DNA to be detected in our study population. Our findings show that the HPV prevalence in our study population differ to that in other African countries such as Nigeria and thus could support the idea of variable HPV strain prevalence in different geographical areas if a larger South African population is studied in future.

Table 3.6: HPV strain prevalence in VIN and iVSCC samples. This table indicates the overall HPV DNA detection as well as HPV strain specific prevalence in VIN and iVSCC samples.

Sample type	HPV 16	HPV 18	HPV 35	HPV 11	*HPV infection
VIN (n=72)	50 (69.4 %)	3 (4.2 %)	3 (4.2 %)	8 (11.1 %)	56 (77,8 %)
iVSCC (n=68)	39 (57.3 %)	7 (10,3 %)	0 (0 %)	4 (5.9 %)	45 (66,2 %)

*All samples with one or more HPV strain detection.

The inability to detect HPV DNA in some samples may not be an indication that HPV infection is absent in these samples, but could possibly be attributed to several explanations. One of the major factors which may lead to unsuccessful amplification could be the use of degraded DNA templates, such is characteristic of FFPE DNA. However, as extensive quality assessment of the extracted FFPE DNA was done and all DNA samples were found fit for use in qPCR, this might thus be an unlikely reason. The human β-globin gene fragment was successfully amplified for all FFPE DNA samples, which also confirms that the integrity of the samples as well as the ability to amplify fragments of around 205 bp. Another reason for undetectable HPV DNA may be the low abundance of HPV DNA, which could be ascribed to low HPV DNA co-extraction with human DNA, especially considering the formation of formalin-induced crosslinks between nucleic acids and proteins in fixed tissue. If HPV DNA was too low, it may also have been below the detection range of the qPCR assay, which could then have led to a "negative" HPV DNA result. Another plausible reason could be that tissue samples for which HPV DNA were not detected could have been infected with different HPV

subtypes than those tested for. Therefore, additional investigations using a broader range of HPV subtypes are warranted.

3.4.2 Correlation of HPV DNA detected and histological subtypes

As part of the histological analysis, VIN and iVSCC diagnoses are also further classified into histopathological subgroups. In our study population the VIN group consisted of 8 LSIL (low-grade lesion) and 64 HSIL (high-grade lesion) samples. The VSCC group were further categorized into 5 warty, 7 basaloid, 14 non-keratinizing and 42 keratinizing samples (Table 3.7). According to literature, HPV is more readily associated with warty and basaloid iVSCC subtypes while the keratinizing variants are often linked to absence of HPV infection (Knopp *et al.*, 2009; Preti, Sccurry and Marchitelli, 2014). VIN and iVSCC histological subtype classifications were therefore compared to HPV DNA detection (Table 3.7).

For the VIN subgroups, HPV could be detected in 37.5 % of LSIL (VIN I) and 82.8 % of HSIL (VIN II and VIN III). A reason for the lower HPV detection rate in LSIL samples compared to that of HSIL could be due to possible clearing of the HPV infection in these samples. The observation of increased HPV 16 infection frequency in lower grade lesions compared to more advance disease, is in contradiction to the findings of De Sanjosé *et al*. (2013), where it is found that HPV 16 prevalence were higher in advanced cervical disease.

Results showed that HPV was detected in 60 % (3/5) of warty iVSCC and 57.1 % (4/7) of basaloid iVSCC samples (Table 3.7). However, although the sample numbers are low, this high HPV detection rate in warty and basaloid VSCC samples are consistent with literature findings (Faber, Sand, Albieri, Norrild, Kjaer & Verdoodt, 2017). HPV DNA was detected in 66.7 % (28/42) of keratinizing iVSCC samples (Table 3.7). This is in contrast to the literature as prevalence rates of only 4 % to 21 % of keratinizing iVSCC samples have been previously reported to be associated with HPV (Madeleine, Daling, Schwartz, Carter, Wipf, Beckmann, McKnight, Kurman, Hagensee & Galloway, 1997). Though, more recent reviews also reported HPV DNA to be associated with keratinizing iVSCC. Faber *et al.* (2017) reported that a mere 13.2 % of keratinizing cancerous vulvar lesions were HPV positive, whereas, Pils *et al*., 2017 indicated that 10 % of their keratinizing vulvar samples compared to 77 % of samples with a warty or basaloid morphology were infected with HPV. Additionally, 71.4 % (10/14) of non-

keratinizing iVSCC samples in our study population tested positive for HPV DNA (Table 3.7). However, of the women diagnosed with iVSCC, 18 of them were older than 60 years. Notably, 15 of these 18 cases were histologically classified as keratinizing and/or non-keratinizing iVSCC and of these, HPV DNA was not detected in 10 samples. Considering the advanced age of these women and the lack of HPV DNA detection, these few cases most likely represent HPV independent iVSCC. Background dermatitis with or without dVIN was not correlated for in these cases. As previously stated, diagnosis of dVIN is challenging. The small number of cases again highlights the difference in epidemiology of the two aetiopathogenic pathways in first world versus developing countries. Nonetheless, a diagnosis of dVIN is rare at Tygerberg Academic Hospital and further investigation is needed (personal communication, Dr R. Razack).

Correlation of the histological subtype with the specific HPV genotypes showed that HPV 16 was detected in a significant proportion of VIN samples (69.4 %; 50/7; P <0.05) compared to the iVSCC samples (Table 3.6). A similar observation was made for HPV 11 that was detected in significantly more VIN samples (11.1 %; 8/72; P<0.05) than in samples with the iVSCC diagnosis (Table 3.6).

Our findings indicated that, in contrast to available literature, a higher HPV prevalence was observed in our keratinizing and non-keratinizing iVSCC samples (Table 3.7). Additionally, results also suggested that the relationship between HPV infection and vulvar iVSCC histopathological subtypes might differ to that in other geographical regions.

Table 3.7: Comparison of VIN and iVSCC diagnosis subgroups with HPV DNA detection.

	iVSCC subtype				VIN subtype	
	Keratinizing	**non keratinizing**	**warty**	**basaloid**	**LSIL**	**HSIL**
HPV detected	28/42 (66.7 %)	10/14 (71.4 %)	3/5 (60 %)	4/7 (57.1 %)	3/8 (37.5 %)	53/64 (82.8 %)
HPV Undetected	14/42 (33.3 %)	4/14 (28.6 %)	2/5 (40 %)	3/7 (42.9 %)	5/8 (62.5 %)	11/64 (17.2 %)

3.4.3 Comparison of the HPV subtype DNA detected with HIV status

A compromised immune system is one of the major risk factors for HPV infection. According to literature, HIV infection promotes the progression of an HPV infection to an intraepithelial lesion and subsequently into invasive squamous cell carcinoma (Saidu, 2016). The presence of HPV DNA was correlated with HIV status (Table 3.8). Only samples from patients with known HIV status, albeit positive or negative, were included in this analysis. A total of 41 VIN and 45 iVSCC patients had a general HPV infection; of these 70.7 % (95 % CI: 54.4 – 83.8 %) of VIN and 57.8 % (95 % CI: 42.3 – 72.3 %) of iVSCC samples had a HIV co-infection (Table 3.8). For VIN cases with HPV 16 infection, the percentage of HIV positive patients were higher (46 %) than for HIV negative (24 %) (Table 3.8). No significant difference was seen in the proportion of iVSCC cases with HPV 16 DNA detected and HIV status (Table 3.8). Although the detection rate for HPV 18 was low, it was found that all 3 of the VIN samples and all 7 of iVSCC samples were from HIV positive patients. Both VIN samples in which HPV 35 was detected were also HIV positive. Likewise, 87.5 % (7/8) of VIN and 100 % (4/4) iVSCC samples positive for HPV 11 had a HIV co-infection. However, a Pearson Chi-square test revealed no significant difference between HIV positive and HIV negative VIN and iVSCC samples for which HPV was detected.

Of the cases for which multiple HPV strains were detected, 5 of the 7 VIN samples and all 5 iVSCC samples were from HIV positive patients (refer to 3.5.1). This result is in concordance with available literature which states that HIV infection increases the risk of multiple HPV infection (Lissouba, Van de Perre & Auvert, 2013).

Table 3.8: HPV subtype detection compared to HIV status.

	VIN		iVSCC	
HPV type	**HIV positive**	**HIV negative**	**HIV positive**	**HIV negative**
16	23/35 (66 %)	12/35 (34 %)	20/39 (51 %)	19/39 (49 %)
18	3/3 (100 %)	0/3 (0 %)	7/7 (100 %)	0/7 (0 %)
35	2/2 (100 %)	0/2 (0 %)	0/0 (0 %)	0/0 (0 %)
11	7/8 (87.5 %)	1/8 (12.5 %)	4/4 (100 %)	0/4 (0 %)
HPV prevalence	29/41 (70.7 %)	12/41 (29.3 %)	26/45 (57.8 %)	19/45 (42.2 %)

3.5 Evaluation of HPV 16 and HPV 18 mRNA transcript expression in VIN and iVSCC samples

3.5.1 Expression of HPV 16 and HPV 18 mRNA transcripts

The expression of HPV mRNA transcripts was analyzed by amplification of the E7 genes for HPV 16 and HPV 18 using qPCR. Results showed that mRNA expression of HPV 16 was obtained in 67.2 % of VIN and 50.0 % of iVSCC samples while positive HPV 18 mRNA expression was seen for 1.6 % of VIN and 6.1 % of iVSCC samples (Table 3.9). Results for DNA detection and mRNA transcript expression were compared (Table 3.9). Overall, HPV infection could be detected in 72.1 % and 68.9 % of VIN DNA and RNA samples, respectively while 65.2 % of DNA and 56.1 % of RNA extracted from iVSCC samples had HPV infection (Table 3.9). When comparing HPV 16 DNA detection and mRNA expression in VIN samples, it was found that DNA was identified in 68.9 % of samples compared to 67.2 % of samples where mRNA transcripts were expressed (Table 3.9). A slightly greater difference was observed between the detection of HPV 16 DNA and mRNA transcripts in iVSCC samples. HPV 16 DNA was amplified in 59.1 % of DNA and 50 % of RNA samples. For HPV 18, amplification was possible in 3.3 % of DNA and 1.6 % of RNA VIN samples while 10.6 % of iVSCC DNA and 6.1 % of iVSCC RNA samples were positive for HPV 18 (Table 3.9). Discordance between HPV DNA detection and mRNA HPV expression occurred in some samples such as VIN-040 and VIN-097 for which HPV 16 DNA was amplified but no mRNA transcripts were found. HPV 16 DNA was detected in 6 iVSCC samples for which HPV 16 mRNA transcripts were not detected (Table 3.9). HPV 18 DNA could also be detected in one VIN and three iVSCC samples for which no mRNA transcripts could be amplified. Contrastingly, HPV 16 mRNA transcripts were found in VIN-044, a LSIL sample, but the DNA was undetectable. With regards to samples where mRNA transcripts could not be detected, plausible reasons might be similar to those states in Section 3.4.1. Additional to these reasons could be the HPV infections were not transcriptionally active.

Table 3.9: Comparison of HPV amplification in DNA and RNA VIN and iVSCC samples.

	DNA			RNA		
	HPV 16	**HPV 18**	**HPV infection**	**HPV 16**	**HPV 18**	**HPV infection**
VIN (n=61)	42 (68,9 %)	2 (3,3 %)	44 (72,1 %)	41 (67,2 %)	1 (1,6 %)	42 (68,9 %)
iVSCC (n=66)	39 (59,1 %)	7 (10,6 %)	43 (65,2 %)	33 (50 %)	4 (6,1 %)	37 (56,1 %)

3.5.2 Sensitivity and specificity of HPV detection in DNA and RNA

The sensitivity and specificity for the detection of HPV DNA and mRNA transcripts were compared for HPV 16 and 18 using EpiCalc 2000 (Version 1.02, Gilman and Matt, 1998). DNA amplification were used as the gold standard as the majority of HPV genotyping kits commercially available, are directed at DNA detection. The detection of HPV 16 mRNA in VIN samples (Table 3.10) had a sensitivity of 98 % (95 % confidence intervals (CI): 86 % – 100 %) and a specificity of 90 % (95 % CI: 67 % – 98 %) while that of iVSCC samples (Table 3.11) were 100 % (95 % CI: 87 % – 100 %) and 82 % (95 % CI: 64 % – 92 %), respectively.

Table 3.10 Concordance and discordance of HPV 16 DNA and mRNA transcripts in VIN samples.

		HPV DNA detection (GS)		
		Positive	**Negative**	**Total**
mRNA HPV expression	**Positive**	40	1	41
	Negative	2	18	20
	Total	42	19	61

Table 3.11 Concordance and discordance of HPV 16 DNA and mRNA transcripts in iVSCC samples.

		HPV DNA detection (GS)		
		Positive	**Negative**	**Total**
mRNA HPV expression	**Positive**	33	0	33
	Negative	6	27	33
	Total	39	27	66

The sensitivity and specificity for the detection of HPV 18 mRNA transcripts in VIN samples (Table3.12) were 100 % (95 % CI: 50 – 89 %) and 98 % (95 % CI: 90 – 100 %), respectively, while the sensitivity and specificity for iVSCC samples (Table 3.13) were 100 % (95% CI: 40

– 98 %) and 95 % (95 % CI: 86 – 99 %). These findings suggest that using qPCR to test for the expression of HPV 16 and HPV 18 mRNA transcripts is precise and may be used to distinguish between the proportions of true positive as well as negative results with high accuracy. Furthermore, both the positive and negative predictive values for all comparisons were >80 %, suggesting a high probability for the positive and negative HPV 16 an HPV 18 mRNA expression results to be true.

Table 3.12 Concordance and discordance of HPV 18 DNA and mRNA transcript detection in VIN samples.

		HPV DNA detection (GS)		
		Positive	**Negative**	**Total**
mRNA HPV expression	**Positive**	1	0	1
	Negative	1	59	60
	Total	2	59	61

Table 3.13 Concordance and discordance of HPV 18 DNA and mRNA transcripts in iVSCC samples.

		HPV DNA detection (GS)		
		Positive	**Negative**	**Total**
mRNA HPV expression	**Positive**	4	0	4
	Negative	3	59	62
	Total	7	59	66

3.6 Correlation of HPV DNA detection, koilocytosis and p16^{INK4a} IHC staining of full section slides

The use of p16^{INK4a} IHC as a surrogate marker for HR-HPV infection is widely researched in anogenital malignancies and is commonly used in diagnostic settings as an ancillary test. However, the correlation of the presence of HPV with the overexpression of this biomarker in vulvar cancer is contentious (Sznurkowski *et al.*, 2016). Some studies have reported high concordance of HPV and p16^{INK4a} overexpression in vulvar carcinoma tissue while the opposite is also observed in other studies (Santos *et al.*, 2006 as cited in Sznurkowski, Zawrocki and Biernat, 2016; De Sanjosé *et al.*, 2013). Currently, there are no routine HPV test available at Tygerberg Academic Hospital and a positive HPV diagnosis is mainly reliant on the

observation of HPV-induced cellular changes using cytology and histology (Figure 3.4 and 3.5). Therefore, the results based on the presence of these HPV cellular changes were used as gold standard in the following comparative analysis.

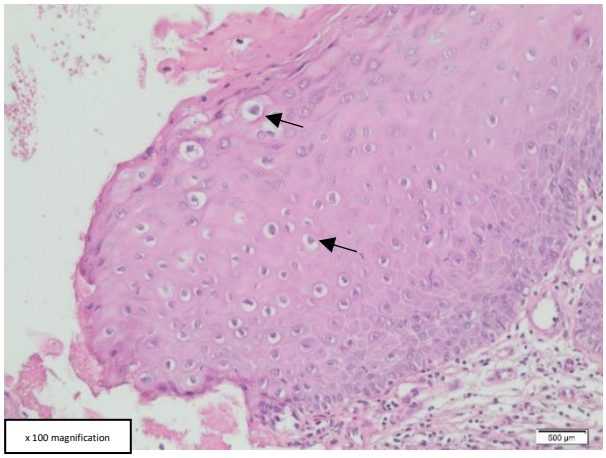


Figure 3.4 This figure illustrates HPV- induced cellular changes known as koilocytes (indicated by arrows) in a LSIL sample (VIN-002).

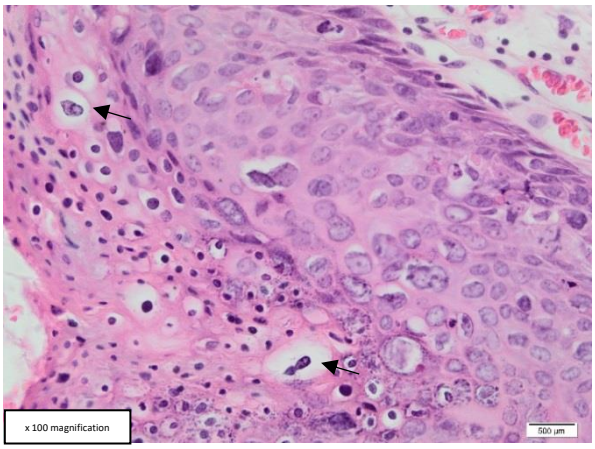


Figure 3.5 This figure illustrates HPV-induced cellular changes known as koilocytes (indicated by arrows) in an HSIL sample (VIN-001).

The expression of $p16^{INK4a}$ in iVSCC samples with adequate FFPE tissue and the presence or absence of HPV-related cellular and structural changes in the tissue, together with the detection of HPV DNA were evaluated and compared. A total of 54 iVSCC were found to have adequate remaining tissue for IHC analysis (and subsequent TMA construction). The scoring of the $p16^{INK4a}$ IHC staining was blinded and done by a pathologist. Tissue were scored positive for $p16^{INK4a}$ IHC staining if block positivity (Figure 3.6) were observed and all other staining patterns were scored as negative (Figure 3.7), in line with the standard operating procedure for the scoring of routine diagnostic specimens. During the histological validation process, cellular changes caused by HPV known as koilocytosis were reported for 37.0 % (20/54) of the selected iVSCC samples. Overall, $p16^{INK4a}$ were positive in 77.8 % (42/54) of samples while HPV DNA could be detected in 63.0 % (34/54) of iVSCC samples.

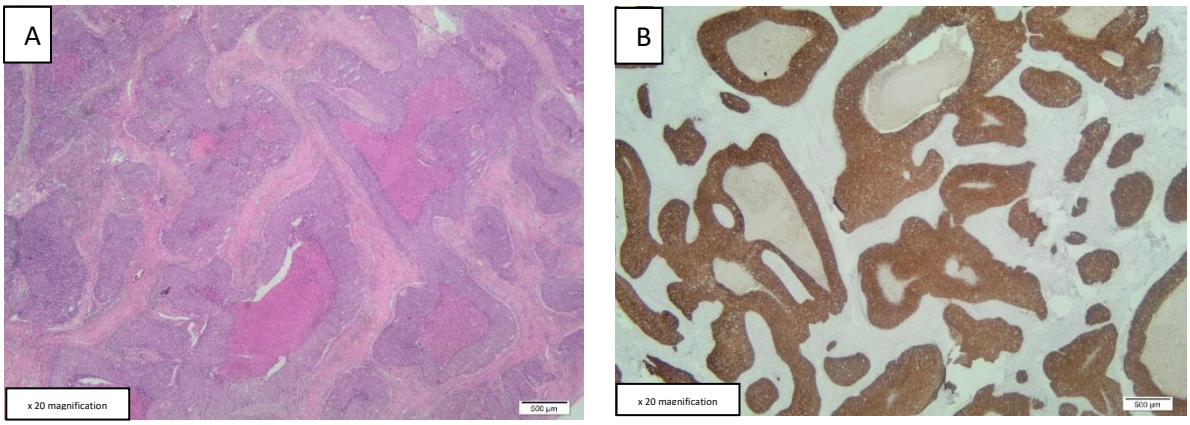


Figure 3.6 An example of iVSCC tissue sample (VSCC-033) stained with H&E (A) and p16^{INK4a} (B) to illustrate the block positive expression of p16^{INK4a}.

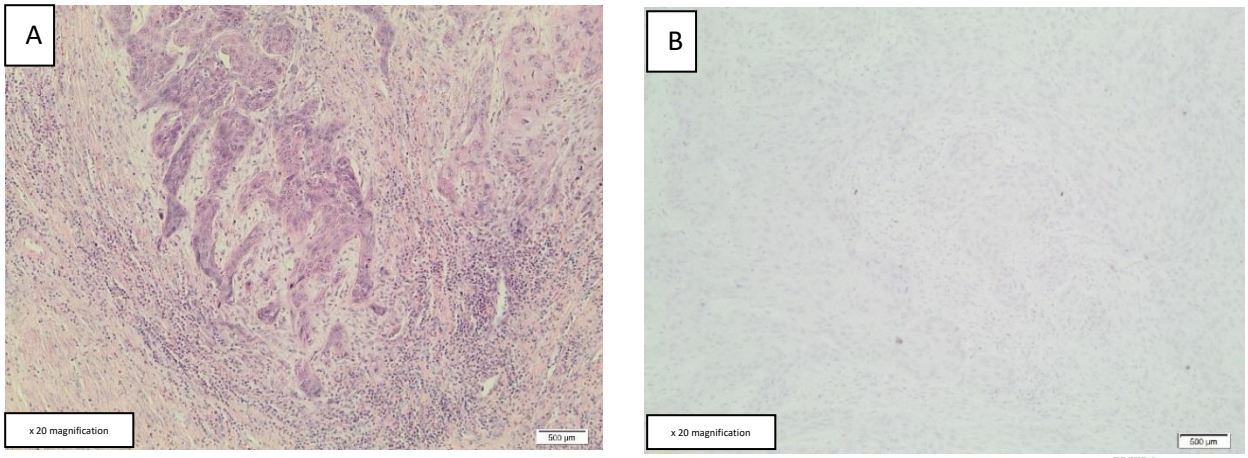


Figure 3.7 An example of iVSCC tissue sample (VSCC-048) stained with H&E (A) and p16^{INK4a} (B) to illustrate a p16^{INK4a} IHC negative result.

When HPV DNA presence was compared with the observation of koilocytes, 24 % (13/54) of samples had both HPV DNA and koilocytic changes, whereas 39 % (21/54) were positive of the HPV DNA only. Koilocytes were observed in 13% (7/54) of cases in which HPV DNA could not be detected (Table 3.14). The presence of HPV DNA in the absence of cellular HPV-related koilocytes changes may be due to the integration of HPV into the human genome, and may thus not be visible in iVSCC tissue. Koilocytic atypia is usually an indicator for low grade lesions and LR HPV, and as demonstrated in this study its utility as a HPV indicator in invasive disease is poor.

Table 3.14 Comparison of HPV presence in iVSCC samples based on koilocytosis and HPV DNA detection.

		Koilocytic changes		
		Positive	**Negative**	**Total**
HPV DNA detection	**Positive**	13	21	34
	Negative	7	13	20
	Total	20	34	54

3.7 TMA construction of iVSCC tissue

3.7.1 The evaluation of TMA quality using H&E staining

A total of 54 iVSCC cases with adequate block thickness were identified for inclusion in the construction of the tissue microarray. IHC staining using antibodies against $p16^{INK4a}$ was performed on the full sections of these 54 tissue blocks in order to identify $p16^{INK4a}$ block positive cases, defined as strong nuclear and cytoplasmic expression in a continuous segment of cells. Of these, only 17 HIV negative and 25 HIV positive cases were diagnosed as $p16^{INK4a}$ block positive and were thus included in the TMA. Two separate TMA's were made for HIV positive and HIV negative samples.

Sections from each TMA were evaluated for tumour and core presence using H&E staining. A total of 22/25 full cores were present on the HIV positive TMA, while there were partial cores for 3 of the 25 cores. All tissue cores except for one partially present core were visible on the HIV negative TMA. Capturing only a proportion of a tissue core on a slide may be due to lack of depth during sectioning of the TMA block or part of the tissue being lost during the staining procedure. The presence of tumour in the TMA FFPE core was an indication of the accurate marking and sampling of the tumour region on the full section slide and FFPE tissue block. For the HIV positive TMA, tumour were present for 22/25 cores while an absence of tumour tissue were reported for 3/25 cores. Only 1/17 cores present on the HIV negative TMA lacked tissue representative of iVSCC. These results may be due to human error as incorrect sampling or marking of the tumour area may occur. As the tissue sections expand during the capturing of

the tissue on the glass slide, it is sometimes difficult to effectively compare the marked H&E slide to the FFPE tissue block which may also lead to incorrect sampling of tissue cores.

3.7.2 p16^{INK4a} IHC staining on full section vs TMA slides

As deeper sections into the TMA block were cut for IHC staining, tissue core presence was re-evaluated for the IHC slides. A similar tissue core presence was observed in the H&E and IHC TMA slides with a total of 22/25 full cores, 2/25 partial cores and 1/25 absent core reported on the HIV positive IHC TMA while 16/17 full cores and 1/17 partial cores were observed in the HIV negative TMA. As the tissue cores are derived from different FFPE blocks, the amount of tissue in each core differs from one another. Therefore, it is possible for some tissue cores to become depleted before others, hence the missing TMA core. A mentioned previously, the absence or partial absence of tissue cores may also be attributed to the H&E or IHC staining procedure as the tissue cores are small an may be easily removed during the numerous wash steps. As the partial cores were sufficient for the pathologist to interpret the IHC staining of the TMA, these samples were included in the comparative analysis while the absent core on the TMA of the tissue from HIV negative patients was excluded. Comparison of the p16^{INK4a} staining pattern for full sections versus TMA cores were done collectively, for both HIV positive and HIV negative samples. As previously stated, tissue cores were only sampled from tissue with a block positive p16^{INK4a} result on full section slides. An example of a TMA core sampled from VSCC-036 and stained with H&E and p16^{INK4a} is illustrated in figure 3.8 A and B.

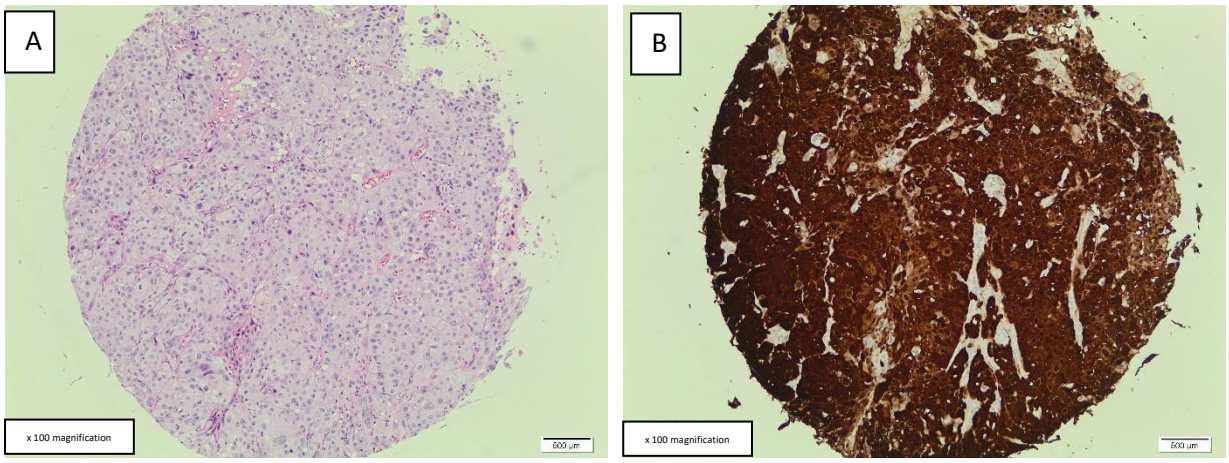


Figure 3.8 An example of a TMA core from sample VSCC-036 stained with H&E (A) and p16^{INK4a} (B).

Results showed 39/41 TMA cores were $p16^{INK4a}$ block positive while 2/41 TMA cores had a negative $p16^{INK4a}$ IHC stain result. A high sensitivity of 95 % (95 % CI:82 – 99 %) were achieved for the use of TMA cores for $p16^{INK4a}$ IHC staining of iVSCC tissue, indicating that there is good agreement between the positive scoring on full tissue sections and TMA sections. However, this finding indicates that TMA's may be sufficient for biomarker studies that do not depend on the interpretation of the staining pattern such as $p16^{INK4a}$ but rather is reliant on scoring of absence or presence or intensity. A drawback of the TMA technique is that cores representative of tumour (and normal) tissue are identified using H&E stained slides and for $p16^{INK4a}$ stain assessment, the staining pattern of the entire excised lesion is normally evaluated. Therefore, if only a representative tissue core is taken, then the $p16^{INK4a}$ stain cannot be accurately analysed. A possible solution for this issue could be to take several cores representative of different histological regions. TMA's could thus be used in ancillary testing of vulvar specimens (even additional to $p16^{INK4a}$) or in the testing of new biomarkers for iVSCC, but care should be taken to sample representative tumour tissue.

Chapter 4 Conclusion

The aim of the present study was to identify the prevalent HPV strains in vulvar pre-cancer and invasive iVSCC samples that were diagnosed at Tygerberg Academic Hospital between January1997 and June 2018. Only samples stored from January 2003 to June 2018 could be included in the study as, of the approximate 2500 listed samples obtained from clinical lists as well as data from the NHLS central data warehouse, only 312 were deemed suitable. The age at which the majority of the patients were diagnosed were analysed and compared to the detection of HPV DNA plus known risk factors such as HIV infection. The distribution of the detected HPV subtypes among the different histological subgroups were also investigated and compared. Furthermore, the sensitivity and specificity of the different methods used to investigate the presence of HPV such as qPCR to amplify DNA and mRNA transcript expression as well as $p^{16INK4a}$ IHC were evaluated. Lastly, we investigated whether TMAs were suitable for the purpose of biomarker expression analysis in vulvar cancer tissue.

It was found that HPV 16 was the most prevalent strain detected among VIN and iVSCC samples included in this study. However, further studies are needed to confirm these results and to be able to extend the findings to the broader community. Contrary to what is reported in other African countries, HPV 35 was the least frequent strain detected in our study population, suggesting that the prevalence of HPV subtypes may vary in different geographical regions (Akarolo-Anthony *et al.*, 2014). However, a comparative study with larger sample numbers from a variety of different African regions is needed to confirm such observations. A positive correlation between HPV infection, diagnoses of VIN and iVSCC and HIV seropositivity was noted. However, statistical tests revealed no significant difference between HIV positive and HIV negative in either VIN or iVSCC samples for which HPV DNA was detected. As the majority of samples, regardless of diagnosis, for which multiple HPV infections were detected had an HIV co-infection, it could be concluded that, in our sample study, HIV infection may increase the risk of being infected with multiple HPV strains or vice versa that HPV infection may increase the risk of contracting HIV, which is in concordance with available literature (Giuliano, Botha, Zeier, Abrahamsen, Glashoff, van der Laan, Papenfuss, Engelbrecht, Schim van der Loeff, Sudenga, Torres, Kipping & Taylor, 2015; Williamson, 2015).

VIN and iVSCC was found to be diagnosed in a substantially younger population, with the majority of diagnoses made for patients younger than 59 years old. This study thus supports the findings from a previous study done at Tygerberg Academic Hospital (Butt and Botha, 2017), which found that vulva carcinoma was not a disease of the elderly in our setting but rather occurred at a much younger age. A significant relationship was also observed between HIV status and patient age, as the majority of patients in the younger age groups (younger than 59) were HIV positive while women in the older age groups were predominantly HIV negative. However, due to the small sample size and the rarity of the disease, more studies are needed to confirm this finding. It is therefore concluded that HIV status may play a role in younger patients developing vulva cancer as immunosuppression is known to increase susceptibility to HPV infection and may accelerate the progression of this disease.

Upon assessing the presence of HPV in different VIN and iVSCC histological subgroups, it was found that the majority of samples in which HPV was detected were classified as HSIL (VIN II/III), keratinizing and non-keratinizing histological groups. According to the WHO Classification of Tumours of the Female Reproductive Organs, keratinizing carcinomas are often HPV independent and $p16^{INK4a}$ negative with the presence of dVIN in adjacent epithelium (Kurman *et al.*, 2014). Current literature frequently concludes higher HPV detection in warty and basaloid iVSCC subgroups, with only a small percentage of keratinizing variants which are not normally associated with HPV (Faber *et al.*, 2017; Madeleine *et al.*, 1997; Pils, Gensthaler, Alemany, Horvat, de Sanjosé & Joura, 2017). However, the majority of iVSCC samples included in this study were classified as keratinizing and non-keratinizing compared to substantially lower amounts of warty and basaloid iVSCC subgroups observed.

When HPV 16 and HPV18 mRNA expression were compared to DNA detection, a high concordance was established between the two techniques. Therefore, detection of mRNA transcripts using qPCR is highly sensitive and may produce high accuracy of true positive and negative HPV results. Likewise, high concordance was found for $p16^{INK4a}$ expression analysed with IHC when the staining of full section slides was compared to that of the TMA slides, bearing in mind that only $p16^{INK4a}$ block positive sample were used. However, in the case of the vulvar, TMA's may be more useful for additional testing of samples to $p16^{INK4a}$. TMA's

may also be suitable for testing of novel biomarkers for iVSCC for which the scoring is more reliant on the presence or absence of the biomarker rather than staining pattern.

A major limitation of this study was the inaccessibility of FFPE tissue blocks either for the samples from January 1997 to December 2002 as well as those which could not be retrieved. Another drawback experienced in this study is the large number of VIN cases for which HIV status was unknown and which could therefore not be included in the HIV-related analyses. As previously stated, due to the low sample size, results cannot be extrapolated to the wider population. Similar ancillary studies in different regions of the Western Cape and South Africa is recommended to determine if HPV 16 is the most prevalent strain in HPV dependent vulvar neoplastic disease. This knowledge will confirm whether the current bivalent HPV vaccine (against HPV subtypes 16 and 18) used in government-operated healthcare facilities will suffice to cover HPV driven vulvar disease, in addition to cervical pathology. The effect of national HPV vaccination will only be evident in the following decades. In the meantime, many vaccination naïve women will only present late with a palpable vulvar lesion, as screening tests for vulvar neoplasms are lacking. Future perspective studies allude to the development of a vaccine that could also have a therapeutic role. Awareness of the prevalent HPV strains for vulvar neoplasia is therefore needed in our setting. More so, once pre-neoplastic disease is already established, the clinical task of constant surveillance, discerning invasive lesions from precursor lesions and deciding on the extent of surgery is challenging and cumbersome. Removing precursor lesions do not always guarantee against invasive disease development. The disfiguring surgery also has far reaching effects on patients' psychosocial being. Until the benefits of HPV vaccination are seen, a large population of unvaccinated woman is still prone to vulvar disease. The search for new biomarkers, anti-tumour agents and predictors for disease outcome, especially to predict progression from precursor lesions to invasive disease is still much needed.

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