Human immunodeficiency virus (HIV) and Human papillomavirus (HPV) infection and cell cycle regulators in preinvasive lesions and invasive carcinomas of the anus

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DECLARATION

I, Dr Louis Johann de Jager, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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ABSTRACT

Introduction: Anal cancer is a rare disease which accounts for 1.5% of gastrointestinal tract malignancies. The majority of these carcinomas are squamous cell carcinomas and are associated with high risk-HPV infection. HIV infection appears to interact synergistically with high risk-HPV in the development of squamous cell carcinoma at this site.

Aims and objectives: To review the pathology of anal carcinomas and anal intraepithelial neoplasia (AIN) diagnosed between 2003 and 2012. To investigate the frequency of high risk-HPV infection and HIV infection in premalignant and malignant epithelial anal lesions using immunohistochemistry and to investigate the effect of these infections on Langerhans cell density. To investigate the role of cell cycle and WNT signalling pathway markers in the pathogenesis of these lesions.

Materials and methods: This was a retrospective study and 51 cases of anal carcinoma and precursor lesions were identified during the study period. Where possible, blocks which contained normal and dysplastic tissue and invasive carcinoma were selected. Ten immunohistochemical stains (p24, p16, pRb, E-cadherin, CD1a, Langerin, Bcl-2, Ki-67, HPV L1 capsid protein and β-catenin) were performed and scored in normal, dysplastic and carcinomatous tissue. Data were analysed to determine if there were statistically significant differences in the expression of markers in different subtypes of carcinomas, grades of differentiation of carcinomas and in the range from normal to carcinoma.

Results: The patients' ages ranged from 24 to 81 years. There were 26 females and 24 males; one patient did not have age or sex information available. Twenty-one cases did not have information available on HIV status. Eleven cases demonstrated squamous cell dysplasia only and 40 cases demonstrated invasive carcinoma, 36 of these being squamous cell carcinomas. p24 was positive in only two known HIV-positive cases. p16 demonstrated block positive staining in 35 out of 36 squamous cell

carcinomas and 14 out of 18 high grade squamous intraepithelial lesions. There was a significant decrease in the proportion of pRb-positive cells from well to poorly differentiated squamous cell carcinomas (p=0.03). HIV status did not influence the expression of markers. The subtype of carcinoma did not have a significant effect on the proportion of pRb-positive cells. Differentiation of squamous cell carcinoma had a significant effect on the E-cadherin expression score (the more well differentiated a carcinoma, the higher the E-cadherin score; p=0.04). There was a significant difference in E-cadherin expression between normal tissue and squamous cell carcinoma, and dysplastic tissue and squamous cell carcinoma (p=0.002 and p=0.004, respectively). Differentiation, subtype of squamous cell carcinoma and HIV status did not influence the density of CD1a/Langerin-positive Langerhans cells. No significant difference in the density of CD1a/Langerin-positive cells was demonstrated amongst normal, dysplastic and squamous cell carcinoma tissue, regardless of HIV status. The differentiation, subtype of squamous cell carcinoma and HIV status, did not have a significant effect on the Bcl-2 expression. There was a significant difference in Bcl-2 expression among normal, dysplastic and cancerous tissue (p=0.02). There was no significant difference in the Ki-67 proliferation index amongst the different subtypes of squamous cell carcinoma and the degrees of differentiation. HPV L1 capsid IHC only stained two squamous cell carcinomas and nine cases with dysplastic squamous epithelium (AIN I and AIN II). There was no case which showed abnormal localisation of β-catenin.

Conclusion: Less than 20% of HIV-positive cases showed positive p24 staining. p24 does not appear to be a useful stain to determine HIV status in non-lymphoid tissues. p16 is known to be a surrogate marker for high risk-HPV infection, and the fact that 35 out of 36 squamous cell carcinomas showed block positive staining suggests that the majority of squamous cell carcinomas in this study were associated with high risk-HPV infection. The mean density of CD1a- and Langerin-positive cells was increased in HIV-positive patients. HPV L1 capsid IHC showed a low sensitivity of detecting AIN and invasive SCC of the anus. Including vaccinations against high risk-HPV in the South African Expanded Programme on Immunisation may reduce the burden of anal dysplastic lesions and invasive squamous cell carcinoma in future.

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LIST OF ABBREVIATIONS

AIN	Anal intraepithelial neoplasia
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATZ	Anal transitional zone
CAP	College of American Pathologists
CDC	Centers for Disease Control and Prevention
CIF	Cellular interference factor
CIN	Cervical intraepithelial neoplasia
CK	Cytokeratin
CK1	Casein kinase 1
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOH	South African National Department of Health
DSS	Disease-specific survival
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMPD	Extramammary Paget disease
EPI	Expanded programme on immunisation
GCDFP-15	Gross cystic disease fluid protein 15
GSK3β	Glycogen synthase kinase 3β
GWAS	Genome-wide association studies
H&E	Haematoxylin & eosin
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HRA	High-resolution anoscopy
HREC	Human Research Ethics Committee
hr-HPV	High risk human papillomavirus
HSIL	High grade squamous intraepithelial lesion
hTERT	Human telomerase reverse transcriptase
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
LAST	Lower anogenital squamous terminology
LAT	Lower anogenital tract
LC	Langerhans cell
LSD	Fisher's least significant difference test
LSIL	Low grade squamous intraepithelial lesion
LS means	Least square means
MCM2	Minichromosome maintenance 2
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men

MSW	Men who have sex with women
NEC	Neuroendocrine carcinoma
NET	Neuroendocrine tumour
NHLS	National Health Laboratory Service
NK	Natural killer
ON	Overnight
OS	Overall survival
PATRICIA	PApilloma TRIal Cervical cancer in young Adults
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
RT	Room temperature
SCC	Squamous cell carcinoma
Siah1	Seven in absentia homologue 1
SIL	Squamous intraepithelial lesion
TCF/LEF	T-cell factor/lymphoid enhancer factor
TEDTA	Tris ethylenediaminetetraacetic acid
TNF-α	Tumour necrosis factor-α
TOP2A	DNA topoisomerase IIA
UCT	University of Cape Town
USA	United States of America
VEPAC	Variance components analysis
WHO	World Health Organisation
WNT	Wingless signalling pathway

CHAPTER 1

INTRODUCTION

Anal cancer principally refers to anal canal squamous cell carcinoma and anal canal adenocarcinoma [1]. These are rare carcinomas which account for 1.5% of gastrointestinal tract malignancies [2]. There is a slightly higher incidence of anal canal squamous cell carcinoma among women than men [3-5]. Most of these carcinomas are associated with high risk human papillomavirus (hr-HPV) infection [3, 5]. Human immunodeficiency virus (HIV) infection appears to interact synergistically with hr-HPV in the development of these carcinomas and precursor lesions [6].

South Africa has a very high burden of HIV infection compared to 1st world countries [7-9]. This was thought to possibly lead to different characteristics in anal carcinomas from our country compared to that from other parts of the world.

Information regarding expression and localisation of certain cellular proteins such as Bcl-2 and β-catenin in anal carcinomas may be useful for oncologists to determine prognosis and to decide on treatment strategies [10-12].

Bivalent and quadrivalent HPV vaccines have been proven to offer very good protection against development of cervical low-grade (LSIL) and high-grade squamous intraepithelial lesions (HSIL), squamous cell carcinoma, adenocarcinoma in situ and adenocarcinoma [13]. Hr-HPV infection is a common aetiological factor in both cervical and anal neoplastic epithelial lesions, and HPV vaccinations have also been found to be effective in preventing anal SIL [14]. Highlighting a high incidence of hr-HPV infection in anal carcinomas in South Africa, coupled with the high burden of HIV in our country may hopefully lead to inclusion of these vaccines into the expanded programme on immunisation (EPI).

To my knowledge, there has not been a South African study of the interaction of HPV and HIV infection in anal canal carcinomas and precursor lesions.

CHAPTER 2

LITERATURE REVIEW

2.1 Normal anatomy and histology of the anal canal

The anal canal represents the distal-most aspect of the gastrointestinal tract (**Figure 2.1**). It starts at the anorectal junction and continues to the keratinising skin of the perineum, the anal verge. It generally measures between 2,5cm and 5cm in an adult [15].

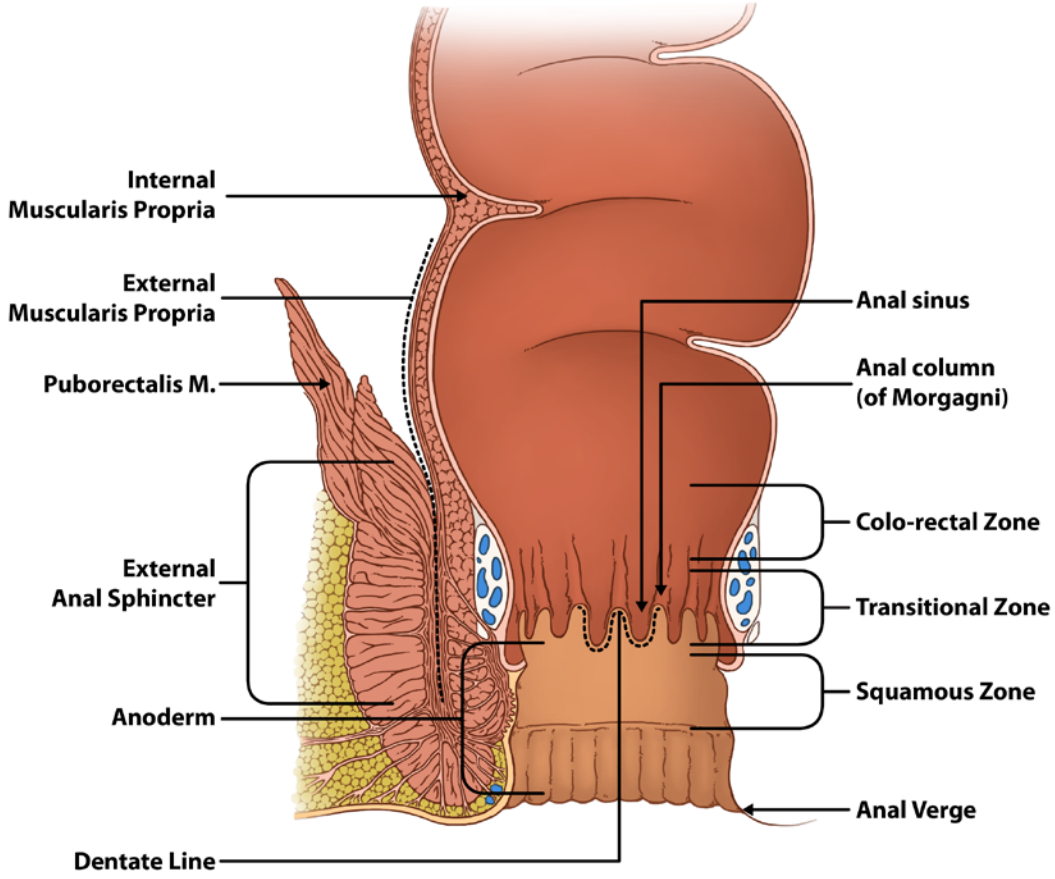

Figure 2.1: Anatomy of the anal canal.

Within the proximal part of the anal canal, columnar epithelium, identical to that of normal rectum is present (colorectal zone) [4]. Immediately distal to this, the anal

transitional zone (ATZ) is found. Historically, a variety of terms has been used for the ATZ, including intermediate and cloacogenic regions [4]. It has an average length of 10mm and is macroscopically recognisable by the anal columns (columns of Morgagni), anal valves and anal sinuses. The anal columns are vertical mucosal folds which join at distal ends to form the anal valves. Anal columns may be more conspicuous in children than adults [15]. Anal glands within the submucosa, with their respective anal ducts, drain into small recesses above the anal valves, the anal sinuses. The number of anal glands may vary between individuals, but the median number is six (may range between three and ten) [4, 16]. The anal glands have a similar lining to that of the ATZ surface epithelium (see below) [4].

Microscopically, the ATZ is defined as the mucosa between uninterrupted colonic epithelium proximally, and uninterrupted squamous epithelium distally [16]. The lining epithelium of the ATZ is a specialised mucosa whose origins are explained by the embryologic fusion of two different germ layers: the endodermal hindgut (superior) and the ectodermal protoderm (inferior). In contrast to the cervical transformation zone, the ATZ surface lining consists of multiple layers of epithelium (usually four to nine cell layers) and may contain basal, columnar, cuboidal, urothelial (transitional) and squamous epithelium [4, 16]. Metaplasia of the ATZ cells reduces the risk of HPV infection and HPV-directed carcinogenesis [17]. Mucin may be found within the ATZ, produced by columnar cells. Melanocytes and endocrine cells may also be present in the basal layer of the ATZ [2].

The dentate line is formed by the anal valves and sinuses, and marks the divide between the ATZ and distal stratified squamous epithelium (anoderm). The anoderm extends for 15mm and appears pale and shiny macroscopically. Adnexal skin structures are absent within this segment, but melanin is always demonstrable [16]. The end of anal canal, the anal verge, is marked by the appearance of skin adnexal structures such as sweat glands, sebaceous glands and hair follicles.

Immunohistochemically, the anal glands, ducts and ATZ are positive for CK7 and negative for CK20 [5]. This is helpful in the identification of carcinomas originating from these sites. Traditionally, different definitions of the anal canal have been used. The *histologic* anal canal stretches from the proximal ATZ to the distal end of the anoderm. The *surgical* anal canal includes proximal rectal-type mucosa and starts from the anorectal ring. The *anatomical* anal canal only refers to the anoderm [2]. The term "anus" classically refers to the anal canal and the peri-anal skin [4].

2.2 Classification of pre-invasive lesions and invasive tumours of the anal canal

Tumours of the anal canal can be broadly categorised as epithelial, mesenchymal and neurogenic. Primary lymphomas and melanomas may also occur in the anal canal [18].

The WHO (World Health Organisation) classification of tumours of the anal canal was updated in 2010 (**Figure 2.2**) [18].

Epithelial tumours
Premalignant lesions
- Anal intraepithelial neoplasia (dysplasia), low grade
- Anal intraepithelial neoplasia (dysplasia), high grade
- Bowen disease
- Perianal squamous intraepithelial neoplasia
- Paget disease

Carcinoma
- Squamous cell carcinoma
- Verrucous carcinoma
- Undifferentiated carcinoma
- Adenocarcinoma
- Mucinous adenocarcinoma

Neuroendocrine neoplasms
- Neuroendocrine tumour (NET)
  - NET G1 (carcinoid)
  - NET G2
- Neuroendocrine carcinoma (NEC)
  - Large cell NEC
  - Small cell NEC
- Mixed adenoneuroendocrine carcinoma

Mesenchymal tumours

Secondary tumours

Figure 2.2: The 2010 WHO classification of tumours of the anal canal.

2.2.1 Epithelial tumours

Primary epithelial tumours can show differentiation towards any of the numerous types of epithelium in the anal canal (the ATZ, stratified squamous epithelium, anal glands and their ducts) [2].

2.2.1.1 Preinvasive / preneoplastic lesions

The anal canal may harbour a condyloma acuminatum (anal wart). This lesion is thought to potentially lead to dysplastic changes and ultimately invasive squamous cell carcinoma [19]. Pathologically, the lesion is recognised as a cauliflower-type (papillary) growth, lined by hyperkeratotic squamous epithelium. Koilocytic atypia of superficial squamous epithelial cells is usually seen [4]. Condylomata of the anal canal are frequently associated with hr-HPV infection. Low-grade and high-grade dysplasia may be present within condylomata [5].

Anal intraepithelial neoplasia (AIN) or flat dysplastic lesions are thought to be the precursors of invasive squamous cell carcinoma and are frequently associated with hr-HPV infection. The edges of a squamous cell carcinoma may harbour AIN [2]. Traditionally, AIN refers to dysplastic changes within the non-keratinised stratified squamous epithelium or ATZ of the anal canal, whereas Bowen disease is the historical term for full-thickness dysplasia of the perianal skin – perianal squamous intraepithelial neoplasia [5, 18]. Dysplastic changes of squamous epithelium consist of decreased (or the absence of) maturation of the epithelium. Cells lose nuclear polarity, nuclear hyperchromasia is present and mitotic figures are conspicuous. Koilocytes may also be present [2].

Extramammary Paget disease (EMPD) may occur in areas rich in apocrine glands, such as the anogenital region [5, 18]. The vulva is most often involved, but it has been described in the perineum, anal margin skin and anal canal [18]. Most often elderly patients are affected [5, 20]. EMPD may be primary or secondary in nature. Primary

disease originates primarily from the epidermis or surface epithelium and is not associated with 'migrating' tumour cells from an underlying malignancy. Invasion of the underlying stroma may occur. Secondary disease is thought to arise from either an underlying synchronous or metachronous visceral malignancy. This is most often due to colorectal adenocarcinoma [5, 18]. Histologically, the squamous epithelium is infiltrated by large cells with pale cytoplasm and large, vesicular nuclei. Immunohistochemically, primary EMPD classically shows positivity for CK7 and GCDFP-15 and is CK20 negative. The Paget cells in secondary EMPD will reflect the immunohistochemical profile of the malignancy of origin. In the case of colorectal adenocarcinoma, this will often show positivity for CK20 and be negative for CK7 and GCDFP-15 [5, 18].

2.2.1.2 Invasive lesions

Squamous cell carcinoma (SCC) is the commonest malignancy of the anal canal. It is usually a malignancy of the elderly (women more frequently afflicted than men) and occurs typically in the 6^{th} or 7^{th} decade [4, 5, 18, 20]. Younger patients, particularly those suffering from HIV infection or other chronic immunosuppression, may also be at risk [4, 18, 20]. Hr-HPV infection plays a major role in development of SCC [2, 5, 18, 20]. Various other risk factors for development of SCC and histologic subtypes of SCC exist, and will be discussed later in the text.

Verrucous carcinoma (or giant condyloma of Buschke and Löwenstein) is considered to be a low-grade form of SCC. Macroscopically it may resemble a typical condyloma, but histologic examination reveals an endophytic growth pattern. Cells may show minimal cytologic atypia, and the invasive front demonstrates a pushing margin with destruction of underlying tissue. Increased cytologic atypia and an infiltrative margin should prompt a diagnosis of well-differentiated SCC. Verrucous carcinoma is usually associated with low-risk HPV infection (HPV-6 and HPV-11). This tumour does not metastasise [5, 18].

Undifferentiated carcinoma is a malignant epithelial neoplasm without any glands or features indicating a line of differentiation. Immunohistochemistry or ultrastructural studies must be used to exclude neuroendocrine differentiation and establish the epithelial nature of the neoplasm [21].

Adenocarcinoma of the anal canal is a rare neoplasm (± 10% of all anal canal carcinomas) which arises from the anal canal epithelium [5, 18, 20]. It can be broadly divided into mucosal and extramucosal (perianal) adenocarcinoma.
Mucosal adenocarcinoma arises from either the ATZ or colorectal-type mucosa above the dentate line. The immunophenotype is identical to ordinary colorectal adenocarcinoma (CK7 negative, CK20 positive) [2].
Extramucosal adenocarcinoma may either arise from anal glands (or their ducts) or fistula tracts [2, 4, 5, 18]. Anal gland adenocarcinoma does not show accompanying overlying mucosal dysplasia and the immunophenotype is usually CK7 positive and CK20 negative [2, 5, 18, 20]. Adenocarcinomas arising from anorectal fistulae may be associated with Crohn's disease [5, 18]. Histologically, it is most often a well-differentiated mucinous adenocarcinoma and usually have the same immunophenotype of an anal gland adenocarcinoma, making the distinction between the two entities difficult [2, 4, 5, 18].

Neuroendocrine tumours may also occur in the anal canal. Most of these tumours are thought to arise from the rectum, although the ATZ is known to harbour neuroendocrine cells. Well-differentiated neuroendocrine tumours (low-grade NETs, carcinoids) usually present in elderly patients and usually follow an indolent course. Morphologically, patterns associated with neuroendocrine tumours elsewhere, may be seen histologically. Ribbons, nests and trabeculae may be present, but a tubular pattern, may be confused with an adenocarcinoma [5, 20]. Poorly differentiated neuroendocrine carcinomas (high-grade NECs) are rare primary tumours of the anal canal [21]. Small cell NEC is an aggressive tumour and has been seen in HIV positive patients and those who have undergone radiation therapy. It may consist of sheets, rosettes and trabeculae of hyperchromatic cells. Smearing artefact and moulding of

nuclei are frequently present. Necrosis and a high mitotic count are commonplace [5, 20, 21]. The differential diagnosis could include poorly differentiated adenocarcinoma, lymphoma and possibly melanoma [5, 20]. Large cell NEC is extremely rare in the anal canal (there are fewer than twenty cases in the English literature). It lacks the classic cytological features of small cell NEC. There are no glandular structures present and squamous differentiation is absent. Cells have abundant cytoplasm and nuclei demonstrate a coarse chromatin pattern and nucleoli [21]. Immunohistochemistry is invaluable to demonstrate the neuroendocrine differentiation of these tumours. Pan-cytokeratins prove the epithelial nature of these tumours. Neuroendocrine markers such as synaptophysin, chromogranin A and CD56 (neural cell adhesion molecule) are usually positive. TTF-1 (thyroid transcription factor-1) may show immunoreactivity in small cell carcinoma [5, 20, 21].

Basaloid carcinoma was historically individualised in publications, sometimes being referred to cloacogenic carcinoma. The current WHO classification recommends that SCC be used for these tumours, with an additional comment on the basaloid differentiation [4]. These tumours are comprised of cells with a high nuclear-cytoplasmic ratio, inconspicuous intercellular bridges and usually demonstrate peripheral palisading [5].

2.3 Epidemiology and risk factors of preinvasive and invasive anal carcinoma

Anal carcinoma is an uncommon disease and accounts for only 1.5% of cancers of the gastrointestinal tract, and 4% of lower gastrointestinal tract cancers [2, 3]. Women have a higher incidence of anal carcinoma than men, but the rate of increase in incidence is higher in men than women [3, 4]. However, the risk for detecting anal canal HPV infection declines with age in women, but does not in men; older, homosexual men may have a greater number of sexual partners than women, attributing to this phenomenon [6].

Anal carcinoma has also increased in incidence over several decades, with the North American incidence reported as being 0.35 per 100 000 individuals in 1973 and rising to 0.98 per 100 000 in 1998. This increase may also be due to an increase in surveillance and better methods of diagnosis.

The incidence of anal carcinoma is 0.9 per 100 000 in the general population of the United States (USA), but this number increases to 35 per 100 000 in HIV-negative homosexual men (men who have sex with men – MSM) and 60 – 70 per 100 000 in HIV-positive MSM [2, 22, 23]. Men who have sex with women (MSW) have a lower incidence of anal HPV infection than MSM in one study with subjects from São Paulo, Brazil, Cuernavaca, Mexico and Tampa, Florida. The rate of acquiring HPV-16 infection was 6.5 times higher in MSM than in MSW [24-26].

HPV is a highly prevalent sexually transmitted disease [24]. Studies have shown that most anal carcinomas (up to 93%) are associated with HPV infection, specifically high risk types such as HPV-16 and HPV-18 [3, 5]. Hr-HPV may not be a necessary for the development of anal cancer, compared to cervical squamous cell carcinoma and adenocarcinoma [27]. HPV-16 is the most frequently detected subtype (87%), while HPV-18 infection is relatively uncommon (6%) [28]. The association is specific to anal squamous cell carcinomas, including basaloid types (this group comprises 70% of all North American anal carcinomas). The incidence of anal carcinoma is also higher in women with vulvar or cervical carcinoma. This is thought to be due to the shared exposure to high risk, oncogenic HPV infections.

There are other risk factors for the development of anal carcinoma. Anal adenocarcinoma (although only accounting for about 10% of anal carcinomas) is frequently associated with chronic fistulae, strictures and proctitis associated with Crohn's disease [5]. Longstanding abscesses and fistulae unrelated to Crohn's also increases the risk of developing anal canal adenocarcinoma [2, 4]. Hr-HPV has been detected in the minority of anal canal adenocarcinomas (8 out of 20 cases and 2 out of

6 cases, respectively) [25, 29]. It is not known if hr-HPV infection is necessary for anal adenocarcinoma development.

Other risk factors for developing squamous cell carcinoma and variants include a history of smoking, an increased number of sexual partners (≥15 partners for men and women), a history of anogenital warts and receptive anal intercourse (both men and women) [5, 6, 25].

Almost all male and female HIV-positive individuals test positive for anal canal HPV infection, and most harbour at least one high risk type. Coinfection by multiple types of HPV is also common [6]. HIV-related immune suppression has less of an effect on the course of HPV-16 infection, than for other HPV types (a broader range of HPV types may develop into high-grade dysplastic lesions in HIV-positive patients) [30]. Histologically proven high-grade squamous dysplasia of the anal canal is present in 20 – 30% of MSM [28].

Immunosuppression due to other reasons such as immune disorders or solid-organ transplant are also risk factors for developing of anal carcinoma [5].

2.4 Screening for anal cancer

Several methods exist for the screening of anal canal carcinoma in high-risk populations (especially HIV-positive individuals and MSM). Methods include digital anorectal examination, anal cytology and high-resolution anoscopy (HRA), which is essentially anal directed colposcopy [6].

Currently, the need to do HPV testing on anal cytology samples still needs to be determined, but detection of hr-HPV DNA and E6 and E7 mRNA with either fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR) is a possibility [4, 31, 32].

There is currently not enough evidence to determine whether anal cancer screening is effective. Moreover, studies on cost-effectiveness have been conflicting because there is limited information on the natural history of anal dysplasia and anal cancer [6]. However, several organisations have recommended screening. The European AIDS Clinical Society recommends digital anorectal examination with or without anal cytology in HIV-positive MSM every one to three years. HRA will follow if the anal cytology is abnormal. The New York State Department of Public Health AIDS Institute recommends yearly examination of all HIV-positive individuals. This includes inspection of the perianal region and digital anorectal examination [6]. There are currently no official British guidelines, but patients are advised to check for and report any lumps in the anal canal [6, 33].

HRA-directed biopsy is considered the gold standard for diagnosing anal pre-cancer and invasive carcinoma [6].

2.5 Histopathologic biomarkers for hr-HPV infection and anal carcinoma

A biomarker is defined as a measurable substance whose presence is indicative of disease, infection or environmental exposure. There is a need for dependable biomarkers in the gastrointestinal tract and elsewhere for dysplastic or cancerous lesions. Ideally, such a marker would decrease the interobserver variability in anal dysplastic lesions and be helpful in predicting progression to invasive carcinoma (i.e. detecting hr-HPV infection).

2.5.1 p16

$p16^{INK4a}$ (p16, a cyclin-dependant kinase inhibitor and the protein product of the gene *CDKN2A*) is a protein which assists with regulation of the cell cycle by preventing phosphorylation of the tumour suppressor, retinoblastoma (pRb) [34]. It has been shown to be the most accurate histopathologic biomarker in formalin fixed paraffin embedded (FFPE) tissue for hr-HPV infection (see below) [4, 35]. It is also highly

sensitive for the diagnosis of CIN II (81.1 – 100%) and CIN III (100%), but less sensitive in CIN I (LSIL) [32].

The majority of anal canal carcinomas are positive for p16 immunohistochemistry (IHC). A special computer assisted technique to quantify p16 IHC staining (AQUA) has been shown to define a subset of patients with anal cancer with a poorer prognosis [36].

2.5.2 Ki-67

This is a nuclear protein which is expressed throughout the cell cycle except G0 phase (see *Lower anogenital squamous terminology [LAST] project*). Normally, IHC expression of Ki-67 (MIB1 clone) is seen along the basal and parabasal regions in surface epithelium. Increased proliferation of squamous cells, for whatever reason, will result in staining within the upper two thirds of the epithelium. This stain is not helpful in distinguishing between LSIL, HSIL and other processes, such as reactive or regenerating epithelium. The stain is most helpful in deciding if surface epithelium is normal or koilocytic atypia is present (LSIL). Two strong staining nuclei of squamous cells should be seen in the upper two thirds of the surface for a diagnosis of squamous dysplasia / condyloma [34].

2.5.3 ProEx^{TM}C

Two monoclonal antibodies make up this cocktail IHC stain, minichromosome maintenance 2 (MCM2) and DNA topoisomerase IIA (TOP2A). It is used as a proliferation marker and has a similar staining pattern to Ki-67. It also has the same shortcomings (i.e. it cannot reliably distinguish between LSIL and HSIL) [34].

2.5.4 HPV L1 capsid protein

L1 protein IHC is not sensitive or specific for any class of cervical neoplasia. It is thought there is low production of viral capsids in high-grade lesions or cancer [34, 37].

2.5.5 Bcl-2

This is an anti-apoptotic protein which regulates the permeability of the mitochondrial membrane. It blocks cytochrome C being released from mitochondria in the intrinsic pathway of apoptosis [2].
It has been found that Bcl-2 expression may be higher in non-keratinising squamous cell carcinoma of the anal canal, compared to keratinising squamous cell carcinoma [38].

2.5.6 Cadherin

Anal canal squamous cell carcinomas show pan-cadherin (an antibody reacting with all known members of the cadherin family) positivity, but decreased pan-cadherin staining may be seen in 70% [39].

2.6 Lower anogenital squamous terminology (LAST) project

In past years, the terminology for a variety of HPV-related anogenital lesions with dysplasia have been unstandardised. Different clinical specialities have historically had different terms for essentially the same lesions. The biology and pathogenesis of HPV-related lesions were also not reflected in the terminology. For example, high-grade squamous intraepithelial lesions (HSIL) of the perianal skin and anal canal may have been designated as Bowen disease/carcinoma in situ (CIS) and AIN II or III, respectively. A similar process in the cervix, would be designated as CIN II or III [4, 35].

To unify terminology across the lower anogenital tract, the LAST project was sponsored by the College of American Pathologists (CAP) and the American Society for Colposcopy and Cervical Pathology. The project consisted of five work groups. Three work groups did extensive literature reviews and developed initial recommendations. One work group was tasked to research the historical background and the last work group continues to implement the LAST recommendations.

The LAST consensus conference was held in San Francisco, California in March 2012 and consisted of work group members, advisors and representatives from government agencies and stakeholder companies [35]. Work group 2 recommended that the nomenclature for all HPV-related squamous lesions in the lower anogenital tract (LAT) be unified. A two-tiered system was recommended for these lesions (LSIL and HSIL) [4, 35]. Work group 4 concluded that there was only enough evidence to recommend biomarker p16 as an adjunct to help differentiate morphologically between precancer (AIN II or III) and mimics of precancer. There was not sufficient evidence to recommend using the biomarkers ProEx^{TM}C and Ki-67 alone or in combination to facilitate this differentiation (see *Biomarkers for hr-HPV infection and anal carcinoma*).

"Block staining" (or block positive) is required to interpret an IHC stain of p16 as positive. Block positive staining is defined as strong nuclear and cytoplasmic staining in squamous epithelia, and extending at least from the basal layer and involving the lower third of the surface epithelium. Full-thickness or upper half staining of the surface epithelium is not required to interpret a specimen as positive.

There were also three other recommendations made by work group 4:

- Performance of p16 IHC is recommended when an equivocal lesion is present; i.e. morphologic features between a low-grade and a high-grade lesion (precancer).
- When a discrepancy is present between the opinion of two pathologists (i.e. one favours a low-grade lesion and the other a high-grade lesion), performance of p16 IHC is recommended
- p16 is not recommended for use when a histological assessment of negative for dysplasia is made, but
    - when there is a risk of a missed high-grade lesion, p16 IHC is recommended

2.7 The cell cycle

The eukaryotic cell cycle is divided into two parts, namely mitosis and interphase. Mitosis corresponds to separation of daughter chromosomes and usually ends with cell division. This process lasts approximately one hour, so interphase comprises most of the cell cycle.

The cell cycle is divided into four discrete phases. During the G1 phase (gap 1 – following mitosis) the cell is metabolically active, but does not replicate DNA. DNA synthesis takes place during the S phase (synthesis) and lastly G2 phase (gap 2) is important for cell growth and protein production prior to mitosis. The M phase corresponds with mitosis.

Different cells have cell cycles of varying durations. Early embryonic cells have a cell cycle of 30 minutes and other cells may cease cell division indefinitely, such as, skin fibroblasts. These cells enter the G0 phase (quiescence or resting) and do not proliferate unless stimulated to do so.

Regulation of the cell cycle is achieved by different extracellular mechanisms and internal control checkpoints. An example of an extracellular mechanism is growth factor binding to specific cell membrane receptors. A major internal checkpoint late within G1 is the restriction point (G1/S checkpoint). The appropriate growth factors enable cells to pass the restriction point and enter S phase. The cell is committed to completion of the cell cycle after it passes the restriction point without any further growth factor stimulation. If the appropriate growth factors are not available during G1 phase, the cell will enter G0 phase. The G2 checkpoint prevents initiation of mitosis prior to the complete replication of DNA. This checkpoint prevents incompletely replicated DNA from being passed on the daughter cells.

DNA damage may cause cell cycle arrest at the G1 and G2 checkpoints as well as slowing the S phase. A functional (wild-type) protein product of *TP53* (p53) will arrest

the cycle at the G1 restriction point, and prevent the cell from entering S phase and replicating damaged DNA.

A checkpoint towards the end of M phase is also present and this monitors correct alignment of chromosomes on the mitotic spindle. Incorrect alignment will result in arrest in metaphase [40].

2.8 The WNT signalling pathway

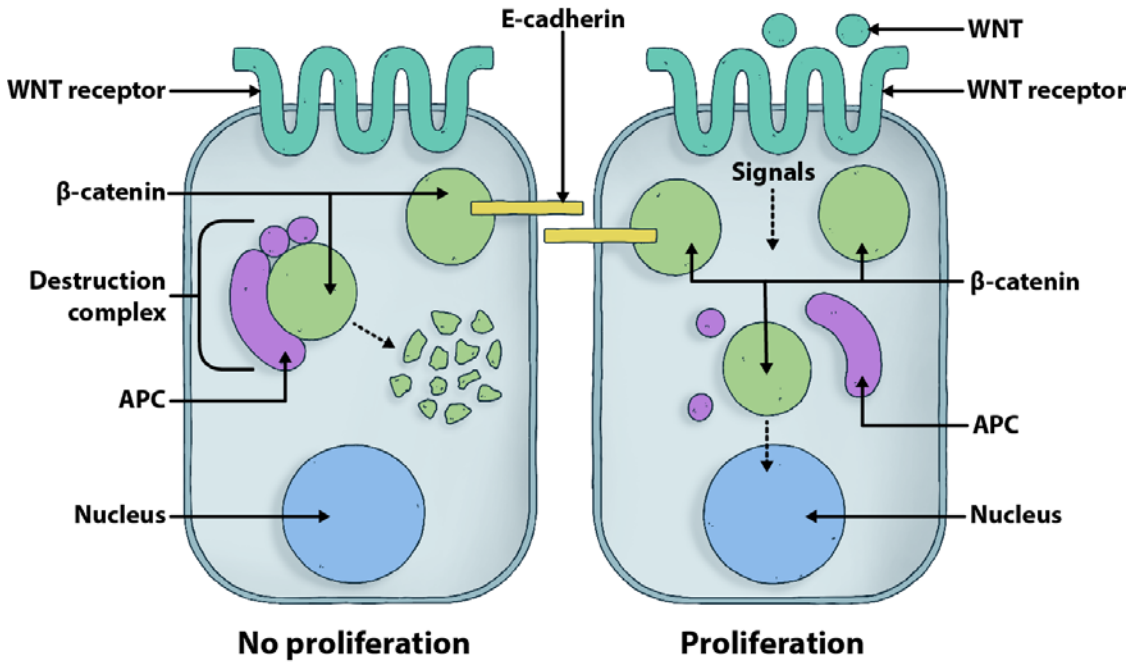

Figure 2.3: The WNT signalling pathway (adapted from Robbins and Cotran Pathologic Basis of Disease, 8th edition [41]).

The WNT signalling pathway (**Figure 2.3**) is an important mechanism that helps control cellular proliferation and cell polarity during embryogenesis and tissue homeostasis. Several WNT proteins participate in this pathway by binding to cell surface receptors of

the Frizzled family and other co-receptors (e.g. lipoprotein receptor-related protein (LRP) –5/6).

In the canonical WNT pathway, β-catenin (protein product of *CTNNB1*) plays a central role in cell signalling. In the absence of WNT ligands binding to cell surface receptors, β-catenin is phosphorylated and targeted for degradation by the axin complex. This complex formed by axin, APC, casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) prevents β-catenin from accumulating in the cytoplasm and translocating to the nucleus of the cell. A GSK3β-independent pathway may also lead to the ubiquitination and degradation of β-catenin. Seven in absentia homologue 1 (Siah1)-interacting protein Skp1-Ebi promotes β-catenin degradation [42]. However, in the presence of certain WNT ligands, GSK3β is inhibited and results in dysfunction of the axin degradation complex. β-catenin accumulates and is translocated to the nucleus where it serves as a coactivator to transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family. Genes, including *MYC* and *CCND1* (cyclin D1 gene) are then transcribed.

β-catenin is a multifunction protein and also plays a role in cell adhesion. It interacts with the cytoplasmic domain of type I cadherin proteins on the cell surface, mostly E-cadherin, by linking with the actin cytoskeleton. Phosphorylation of both E-cadherin and β-catenin enhances the interaction with one another [42-45].

2.9 The Role of Human papillomaviruses

2.9.1 Introduction

The human papillomavirus (HPV) family is a large heterogeneic group of DNA viruses, consisting of more than 100 members. HPVs have icosahedral capsids and are non-enveloped. Their genomes are replicated within the nuclei of cutaneous and mucosal epithelium. Sites where infection may occur include the skin, oropharynx, genital tract and anal canal [46-49]. HPV infection may have four possible outcomes, namely:

clearance of the infection, a productive infection, latency and persistence of the infection [6].

HPVs can be broadly categorised into two groups: low-risk and high-risk HPV. High-risk HPV is linked to the development of premalignant and malignant lesions (e.g. squamous cell carcinoma or rarely adenocarcinoma), whereas low-risk HPVs cause benign warts [48].

Members of the high-risk HPV group include HPV-16, HPV-18, HPV-31, HPV-33 and HPV-45 [48]. Infection by these viruses may cause premalignant dysplastic lesions (AIN). The inability of the immune system to clear this infection allows for persistence and exacerbation of the lesion, and possible development of invasive carcinoma [48].

2.9.2 HPV life cycle

Replication of HPV and completion of the viral life cycle requires integration of the viral genome into the proliferating epithelial cells or keratinocytes. Actively replicating basal cells are the targets for the virus. It is thought that damage to the superficial, differentiating and differentiated epithelia exposes the basal cell layer, and thereby accommodates entry of the viral particles into the host epithelium [47, 48, 50]. Viral DNA is maintained at a low copy number in basal cells in an episomal form, but replicated to high copy numbers in the midzone of the epithelium. Mature HPV particles (viral progeny) are released from superficial cells during shedding [47, 48, 50].

Infection by hr-HPV impairs epithelial differentiation and senescence, thereby ultimately producing mature HPV particles that are released from the most superficial layers of the epithelium [47, 48]. All HPVs have so-called early (*E1* – *E7*) and late (*L1* – *L2*) genes [49]. The L1 and L2 proteins are assembled late in the life cycle of HPV, and form the icosahedral capsids (L2) necessary for virion assembly (L1) [50]. The most important early genes are *E5*, *E6* and *E7*, which act as viral oncogenes and result in increased proliferation of infected cells which do not exit the cell cycle. Integration of

hr-HPV DNA into the host genome, disrupts the expression of the gene which transcribes E2 protein. E2 is a transcriptional suppressor of E6 and E7, and its absence leads to unrestrained expression of E6 and E7 [42]. Uninfected epithelial cells normally undergo senescence (G0) when they leave the basal layer, with loss of superficial nuclei [47, 48, 50].

2.9.3 Early viral genes

Both the E6 and E7 protein products can cause immortalisation of cells and inhibition of their expression halts carcinogenesis. E6 alone may cause immortalisation of human breast epithelial cells, but only when co-expressed with E7 does it result in efficient immortalisation of human keratinocytes [47, 48]. HPV-16 E6 and E7 viral oncoproteins can induce structural and numerical chromosome instability, independently of interaction with p53 and pRb [50, 51].

E6 binds to p53 and forms a complex with a ubiquitin ligase called E6AP. The 26S proteasome consequently degrades this ubiquitinated p53, resulting in a half-life of less than 20 minutes [48]. It is postulated that binding of E6 to E6AP, in itself, causes ubiquitination and degradation of E6AP. Members of the SRC family tyrosine kinases which are targeted by E6AP for degradation, may therefore have increased activity resulting in growth stimulation [47, 48].

High-risk E6 proteins are also able to reactivate human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, thereby reconstituting telomerase activity and avoiding senescence [6, 47, 48]. The pro-apoptotic gene, *BAK*, may also be degraded, leading to resistance to apoptosis [47, 50]. $p16^{INK4a}$ counteracts these functions [47]. E7 oncoproteins associated with the retinoblastoma family of pocket proteins (pRb, p107 and p130) are also able to immortalise epithelial cells [47, 48]. Cell transcription is normally halted by unphosphorylated pRb that binds to the transcription factor E2F. E2F binds to promoter regions of genes involved in apoptosis and S-phase progression. When pRb is phosphorylated by cyclin-kinases during

progression from G1 to S-phase, it is released from E2F. Genes involved in DNA synthesis are consequently transcribed by free E2F. During hr-HPV infection, E7 sequesters pRb away from E2F and mediates pRb degradation through proteolysis, thereby resulting in constitutive activation of E2F [48, 52, 53]. This leads to upregulation of p16 [47]. Cyclin A and cyclin E are also induced by E7 protein. Binding of E7 to the cyclin-dependant kinase inhibitors, p21 and p27, blocks their function, enabling progression through the cell cycle [47, 48]. A study done on HPV-infected cervical tumour cells found that proteasome inhibitors could prevent pRb degredation and restore its function [53].

E5 forms complexes with the epidermal growth factor receptor, platelet-derived growth factor-ß receptor and colony stimulating factor-1 receptor. However, E5 does not play a role in late events in HPV-mediated carcinogenesis.

High risk-HPV can interfere with the WNT signalling pathway. The E6 oncoprotein from hr-HPV subtypes may disrupt cell adhesion and consequent communication between neighbouring cells, resulting in cancer progression. High risk-HPV E6 targets cell adhesion proteins hDlg and hScrib for degradation by binding to their PDZ domains. hDlg and hScrib normally form complexes with APC and maintain adherens junctions important in cell adhesion.

CDH1 (E-cadherin gene) is frequently methylated in cervical squamous cell carcinoma. Loss of E-cadherin results in accumulation of β-catenin within the cytoplasm and translocation to the nucleus, resulting in transcriptional activation [50]. Studies have suggested that HPV infection can upregulate the WNT signalling pathway [42, 45, 54].

2.10 Immunology and control of HPV infection

Studies of the immune response to HPV have historically focused on cervical pathology. Even though the biology of HPV is similar across different types of

epithelia, because of the difference in the anatomy and physiology of the cervix and anus, the natural history of infection may differ between these two sites [6].

The human immune response is needed to control, or ultimately clear HPV infection. Three branches of the immune system have a role in HPV infection.

2.10.1 Innate immune system

This system consists of components which are part of the first line of defence against viral infection. An intact, physical epithelial barrier is of utmost importance to prevent basal cell infection. Other factors, such as, antimicrobrial peptides, toll-like receptors and various cells, such as, lymphocytes, natural killer cells (NK-cells) and phagocytes also play a role.

However, hr-HPV may evade these innate responses and in doing so, cause a delay in the activation of adaptive immune responses. HPV-16 oncogenes may function to evade pro-apoptotic signals, such as, tumour necrosis factor alpha-mediated and inhibit interferon-γ (IFN-γ). This results in hampering of the signalling pathways that are important in the acute inflammatory response and stimulation of adaptive immunity [55, 56]. HPV-16 and HPV-18 can also interfere with the downstream signalling of innate immune receptors and ultimately lead to down-regulation of genes involved in antigen presentation, the inflammasome and numerous cytokines [57].

2.10.2 Adaptive immune system

2.10.2.1 Humoral immunity

Studies have been done to assess the humoral immune response to HPV infection, but the majority of these are based on women with cervical lesions. Natural HPV infection is intraepithelial, and infected cells are shed from mucosal surfaces. There is little or no viraemia, and this infectious cycle in itself is a way to evade the immune system [58].

Serum antibodies to the L1 viral capsid protein is type-specific and low-titre [59]. Seroconversion is incomplete and it is unsure if these antibodies protect against reinfection [60].

A higher prevalence of HPV-16 seroposivity is seen in HIV infected women compared to HIV negative individuals and is possibly due to decreased clearance of infection in immunocompromised patients [6].

Immunoglobulin A (IgA) antibodies towards HPV-16 are produced within cervical mucosa. HIV infection impairs the mucosal production of IgA , but not the serum IgA response to HPV-16 [6]. It is not known whether IgA antibodies towards HPV-16 is produced in the anal mucosa. Serum IgA antibodies towards a peptide antigen derived from the E2 region of HPV-16 have been detected in patients with anal squamous cell carcinoma [61].

2.10.2.2 Cellular immunity

Cell-mediated immunity plays a role in the outcome and control of HPV infection. CD4+ T-cells may infiltrate warts during regression. Cytotoxic T-cell responses to HPV-16 oncogenes E6 and E7 in HPV-16 positive women are more commonly detected in women without CIN than HPV-16 positive women with CIN [6, 62]. This suggests that cytotoxic IFN-γ produced by HPV-16/18 specific CD4+ cells, protects against intraepithelial neoplasia [6, 47].

In HIV infected patients, a CD4+ count below 200 cells/µl is a risk factor for dysplasia and invasive carcinoma [6]. However, factors other than CD4+ count must also play a role, as the quality of mucosal cellular immune response to HPV is poorer in HIV-positive patients on antiretroviral therapy, regardless of CD4+ count compared to HIV-negative patients.

2.10.3 Control of HPV infection in proliferating cells

In addition to immune responses, two modes exist to protect hr-HPV infected proliferating cells from undergoing malignant transformation [47].

The E6 oncoprotein may immortalise cells and inactivate p16. In cells that are not immortalised by E6, p16 inactivates cyclin D1-CDK4 or cyclin D1-CDK6 protein complexes. Cyclin E is not expressed and the cell cycle is stopped. Thus, p16 can functionally interfere with the transforming activity of E6 [63].

E7 inhibits pRb, and in doing so, releases the E2F transcription factor. This results in the upregulation of p16, but E7 in itself inactivates p16. Thus, within cells which are transfected and immortalised by both E6 and E7, *CDKN2A* is still active [47].

Blocking of HPV DNA transcription may also be achieved by paracrine stimulation of epithelial cells by macrophages and tumour necrosis factor-α (TNF-α). There are effects on HPV-immortalised cells, including modification of the transcription factor, AP1, which is important for HPV gene expression. This process is known as the cellular interference factor (CIF) concept [47, 64].

2.11 HPV-16 and the WNT signalling pathway

High-risk HPV infection may immortalise epithelial cells, but does not independently lead to invasive carcinoma in cervical studies. Progression after hr-HPV infection is thus thought to be a multistep process [45].

A study on cervical squamous cell carcinoma was done to evaluate gene alterations in HPV-16 positive tumours. The authors used genome-wide association studies (GWAS) to perform whole genome expression profile in cervical carcinoma and normal cervical tissue. In cervical carcinoma, novel deregulated genes were identified including overexpression of four genes of the WNT signalling [54]. Upregulation of *JUN*, *MYC*, *FZD2*, *RAC1*, *GSK3β* and *CTNNB1* were detected. It has been proposed that the WNT

pathway functions as a promoter of HPV-immortalised human keratinocyte transformation.

Another group found that HPV-16 positive oropharyngeal cancers showed upregulation of β-catenin compared to HPV-16 negative carcinomas [42]. They found that β-catenin accumulated in the nucleus of HPV-16 positive oropharyngeal carcinomas and HeLa cervical cancer cells. Repression of E6 and E7 protein resulted in a substantial reduction in nuclear β-catenin levels and a substantial upregulation of Siah1 mRNA and protein levels. With introduction of a Siah1 expression vector, endogenous β-catenin levels were decreased in cancer cells. Thus, E6 and E7 proteins play a role in decreased degradation of β-catenin protein.

Downregulation and loss of E-cadherin has been associated with invasive and poorly differentiated malignancies (specifically in head and neck squamous cell carcinomas) [65]. A study was performed on keratinocytes co-transfected with HPV-16 E6 and E7 proteins [66]. E-cadherin membrane staining was decreased in these cells and α-, β- and γ-catenin were redistributed from a submembranous location to the cytoplasm. Upregulation of the epidermal growth factor receptor (EGFR) was also seen in these cells. Upregulation of E-cadherin in these cells essentially reversed these findings i.e. downregulated EGFR, restored membrane bound E-cadherin and catenin, and reversed the invasive phenotype. This may be a potential target for treatment (EGFR inhibitors) of HPV-related cancer in the future.

2.12 Langerhans cells, HPV and HIV

2.12.1 Langerhans cells (LCs)

Langerhans cells are the immature dendritic cells found in stratified epithelium and the epidermis, which are normally located within a suprabasal position [67]. The main function of these cells is to capture antigens within the epithelium and transport it to lymph nodes to present to T-cells [67, 68]. LCs possess receptors for antigen uptake, one being CD1a, which is involved in presentation of glycolipids to T-cells [69]. Other

surface antigens expressed by LCs include CD4 antigens, HLA-DR antigens and FcIgG receptors, which enable HIV-1 to bind to and enter the cell [70]. Antigen presentation activates T-cells (CD4+ and CD8+ cells) which in turn enables a cellular immune response to be mounted against infective agents or cancer. LCs may also directly stimulate B-cells [68, 69].

2.12.2 The influence of HPV and HIV on Langerhans cells

A study from France in 2002 demonstrated a significant increase in the number of mucosal LCs in HIV-negative patients with anal condylomata [71]. The same study found the number to be decreased in anal condylomata of HIV-positive patients. A Brazilian study demonstrated that the number of LCs in anal squamous cell carcinoma of HIV-positive patients were lower than in HIV-negative patients with anal squamous cell carcinoma [67]. A similar increase in numbers of LCs have been seen in dysplastic lesions of the cervix and vulva [72, 73].

As mentioned, HIV-1 may bind to and enter LCs, and HIV-positive infected patients demonstrate a reduction in the epidermal LC population, leading to compromised local immunity [70]. Ultrastructural studies have demonstrated damage to LCs in this situation. Studies have also demonstrated that LCs may transmit HIV-1 to T-cells in ex vivo skin explant models [74, 75].

However, contrary to other dendritic cells (DCs), LCs express the receptor langerin, which induces the formation of Birbeck granules [76]. In contrast to other dendritic cells which express the receptor DC-SIGN, langerin prevents transmission of HIV-1 by LCs. The virus is captured by langerin, internalised and degraded in Birbeck granules. If, however, langerin is inhibited or a patient has a very high HIV-1 viral load, the LC can become infected by HIV-1 and transmit the virus.

2.13 Aims and objectives

- To review the pathology of anal carcinomas and anal intraepithelial neoplasia (AIN) diagnosed between 2003 and 2012.

- To determine the frequency of hr-HPV infection and HIV infection in premalignant and malignant epithelial anal lesions.
  - HPV infection will be detected with an L1-capsid immunohistochemical stain.
  - p16 immunohistochemical staining will be used as a surrogate marker to detect hr-HPV infection in dysplastic tissues and carcinomas.
  - p24 immunohistochemistry will be performed on all cases to confirm HIV status.

- To investigate the effect of hr-HPV and HIV infection in the density of Langerhans cells within normal squamous and dysplastic squamous epithelium and invasive carcinoma.

- To investigate the role of cell cycle markers and WNT signalling pathway markers in the pathogenesis of these lesions.
  - Bcl-2 and pRb expression will be immunohistochemically determined.
  - E-cadherin and β-catenin immunoexpression will be performed to study the role of the WNT signalling pathway.

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethics approval

The protocol was approved by the Human Research Ethics Committee (HREC) of the Faculty of Health Sciences, University of Cape Town (HREC reference number: 196/2013).

3.2 Study design

This was a retrospective study. A search of the NHLS DISA database in the division of Anatomical Pathology, Groote Schuur Hospital, for all cases of AIN and all histologic subtypes of anal carcinoma was performed. Clinical and patient information was recorded from the DISA database (age, sex and HIV status). HIV status was recorded by either noting the result of an enzyme-linked immunosorbent assay (ELISA) or from the presence of a HIV viral load test. Funding was received from the National Health Laboratory Service (NHLS) Research Trust.

3.3 Sample selection

All slides from 2003 to 2012 were retrieved from the archives. The diagnoses of all cases were reviewed with my supervisor, and cases with sufficient residual tissue were selected for the study. Fifty-one cases were selected and information on every case was entered into a Microsoft Excel (2010) spreadsheet (**Appendix 1**). Names were removed to ensure patient confidentiality.

3.4 Histochemical and immunohistochemical staining

Where possible, blocks were selected in which representation of normal squamous epithelium, dysplastic squamous epithelium and carcinoma were present. A section for haematoxylin and eosin (H&E) staining was cut on each case. Additional 3µm sections were cut for immunohistochemical staining. Ten immunohistochemical stains were performed on each case (**Table 3.1**) using two kits (**Table 3.2**).

Table 3.1: Primary antibody information.

PRIMARY ANTIBODY	CLONE	CLONALITY / SPECIES	SUPPLIER	ANTIGEN RETRIEVAL	DILUTION	INCUBATION TIME	POSITIVE CONTROL TISSUE
Retinoblastoma	13A10	Monoclonal/Mouse	Novocastra	Citric acid	1:30	1 Hr - RT	Tonsil
p16	JC8	Monoclonal/Mouse	Santa Cruz Biotechnology	EDTA	1:25	1 Hr - RT	SCC of the cervix
p24	Kal-1	Monoclonal/Mouse	Dako	TEDTA	1:20	1 Hr - RT	HIV + lymph node
CD1a	MTB1	Monoclonal/Mouse	Novocastra	TEDTA	1:40	1 Hr - RT	Skin
Langerin	12D6	Monoclonal/Mouse	Novocastra	TEDTA	1:100	1 Hr - RT	Skin
BCL2	124	Monoclonal/Mouse	Dako	TEDTA	1:20	1 Hr - RT	Appendix
Ki-67	MIB-1	Monoclonal/Mouse	Dako	TEDTA	1:100	1 Hr - RT	Appendix
E-Cadherin	36/E-Cadh	Monoclonal/Mouse	BD Bioscience	EDTA	1:200	1 Hr - RT	Prostate
β-Catenin	17C2	Monoclonal/Mouse	Novocastra	TEDTA	1:50	1 Hr - RT	Breast
HPV (L1 capsid)	K1H8	Monoclonal/Mouse	Dako	TEDTA	1:50	ON - RT	SCC of the cervix

Key

TEDTA - Tris EDTA

RT - Room Temperature

ON - Overnight

Supplier Information

Novocastra - Newcastle-upon-Tyne, United Kingdom

Santa Cruz Biotechnologies - Santa Cruz, CA, USA

Dako - Glostrup, Denmark

BD Biosciences - San Jose, CA, USA

Table 3.2: Kits used in the study.

KITS	SUPPLIER
Envision HRP System Labelled Polymer Anti-mouse	Dako - Carpinteria, CA, USA
Liquid DAB + Substrate chromogen system	Dako - Carpinteria, CA, USA

3.5 Immunohistochemical protocol

- Three micron paraffin wax embedded tissue sections were cut on a rotary microtome, picked up onto Histobond slides (Marienfeld – Lauda-Königshofen, Germany) and heat fixed on a hotplate at 60^{o}C for 10-15 minutes.
- Sections were dewaxed through xylene, cleared in ethanol and hydrated in water.
- Endogenous peroxidase activity was blocked by treating the slides with a 3% hydrogen peroxide (H_2O_2) solution for 10 minutes.
- Slides were then washed well in water.
- Antigen retrieval was performed by pressure-cooking slides in either citric acid (pH6), EDTA (pH8) or TEDTA (pH9) for 1 minute 30 seconds at full pressure (**Table 3.1**).
- Slides were allowed to cool and then washed in tap water.
- Thereafter, slides were rinsed with phosphate buffered saline (PBS) solution (pH 7.6) (Oxoid-Hampshire, Basingstoke, England).
- Non-specific binding was blocked by treating slides with a 5% goat serum solution (DAKO).
- Serum was then drained off and sections were incubated with primary antibody at room temperature at specified duration and dilutions (**Table 3.1**). All dilutions were optimised in-house.
- The slides were then washed well with PBS buffer.
- This was followed by incubation with the secondary antibody, DAKO Envision labelled Polymer, HRP (DAKO) for 30 minutes at room temperature.
- Sections were washed well with PBS buffer.

- Positivity was developed by applying the chromogenic substrate 3.3–diaminobenzidine (DAB), (DAKO) for 5-10 minutes.
- Slides were washed in running tap water and counterstained with Mayers haematoxylin for approximately 3 minutes.
- After washing in running tap water, sections were blued in ammoniated water.
- Finally, the slides were then dehydrated through alcohols, cleared with xylene and mounted with Entellan (Merck – Darmstadt, Germany).

Controls:
A negative reagent control together with a positive tissue control (**Table 3.1**) was added to each staining batch. The primary antibody was replaced with PBS in the negative reagent control.

3.6 Scoring of immunohistochemical stains

All ten immunohistochemical stains (**Table 3.1**) were performed on all 51 cases. Where possible, scoring of each stain was performed in areas of normal squamous epithelium, dysplastic squamous epithelium and carcinoma. Scoring was done on an Olympus BX43 light microscope with a 40x objective.

Intensity of staining was scored on a four-tiered system (0 = no staining; 1+ = weak; 2+ = moderate and 3+ = strong).

Proportion was assessed by counting a hundred cells at high-power and determining the percentage of positive staining cells. In areas of heterogeneity, more than one set of a hundred cells were counted and the average calculated.

Density was defined as the number of positive staining cells in one high-power field. In the case of CD1a and Langerin stains, the average density in three high-power fields was calculated.

E-cadherin staining was scored on a four-tiered system (0 = no staining; 1+ = weak, partial membrane staining in more than 10% of cells; 2+ = weak to moderate circumferential staining in more than 10% of cells; 3+ = strong circumferential staining in more than 10% of cells) [77].

Ki-67 staining was scored as a percentage of cells showing nuclear staining.

β-catenin staining was scored according to the protocol devised by Jass *et al*. [78]. A score of 1 to 5 was assigned based on the intensity of staining and the cellular compartment which was stained (1 point for loss of membrane staining, 1 or 2 for weak or marked cytoplasmic staining, respectively, and 1 or 2 for weak or marked nuclear staining, respectively). A score of 4 or more was considered abnormal β-catenin localisation.

p16 staining was considered block positive if ≥70% of cells showed nuclear and cytoplasmic staining [79].

3.7 Statistical analysis

All data were entered into the statistical analysis programme, Statistica™ version 13 (Dell Inc. 2015). A variable was assigned to every immunohistochemical stain for normal squamous epithelium, dysplastic squamous epithelium and cancer. For example, the density of CD1a staining in normal, dysplastic and cancerous tissue was assigned the variable names CD1aDensNorm, CD1aDensDysp and CD1aDensCancer, respectively. Unfortunately, as previously mentioned, and illustrated in **Appendix 1**, only two cases had normal, dysplastic and cancerous tissue. This resulted in "missing data" across the study and certain variables containing values in a limited number of cases.

Most statistical analyses are based on the assumption that the data are normally distributed (central limit theorem). In cases where data was not normally distributed,

the Box-Cox transformation was employed to ensure normality. In the case of Bcl-2 staining of cancerous tissue, the data could not be transformed to normality, and ANOVA tests could not be performed. Summary frequency tables were created with a new variable CodedBCL2PropCancer. This was grouped into cases displaying a value of 0 or more than 0 for BCLPropCancer. Pearson chi-squared (χ^2) tests were performed in this instance.

Levene's test for homogeneity of variance was performed to verify the assumption of constancy of residual variance.

Analysis of variance (ANOVA) was performed on variables to determine if there was a significant influence by factors, such as, HIV status, differentiation and cancer subtype.

A variance components analysis (VEPAC) was performed in which case all data were used, in particular also those pairs of measurements on the same patient (which are statistically dependent). In cases where significance was established, it was followed up by the Fisher's least significant difference test (LSD) to identify significant pairs of tissue types (normal, dysplastic and cancer). Least square means (LS means) of the responses were calculated in these cases.

In all instances, a p-value of less than 0.05 was considered statistically significant (rejecting the null hypothesis of equal true mean values).

CHAPTER 4

RESULTS

4.1 General

Fifty-one cases were selected. The patients' ages ranged from 24 to 81 years with a mean age of 52.5 years. Thirty-one patients were 50 years or older in age and 19 younger than 50 years. Sex ratio was almost equal, with 24 males and 26 females. One case of a well differentiated, non-keratinising squamous cell carcinoma did not have information on sex, age or HIV status.

Twenty-one females and 17 males had invasive carcinoma. There were 25 patients of 50 years or older in age with invasive carcinoma, while only 13 patients younger than 50 years had an invasive carcinoma.

Thirty patients had HIV status information available, 12 were HIV-positive and 18 HIV-negative. The HIV status was not available in the records in 21 cases. **Figure 4.1** is a representation of HIV status by sex.

Forty cases showed evidence of invasive carcinoma on the tissue examined. The majority (36) of these diagnoses were squamous cell carcinomas. There were three adenocarcinomas and one adenosquamous carcinoma. Eleven cases did not have invasive carcinoma, and only demonstrated squamous cell dysplasia **(Figure 4.2**). Ten cases of squamous cell carcinoma also demonstrated squamous dysplasia.

Twenty-one cases demonstrated squamous dysplasia, and 18 of these were HSIL. Of these 18 cases, 8 (44.4%) were from HIV-negative patients and 5 (27.8%) were from HIV-positive patients.

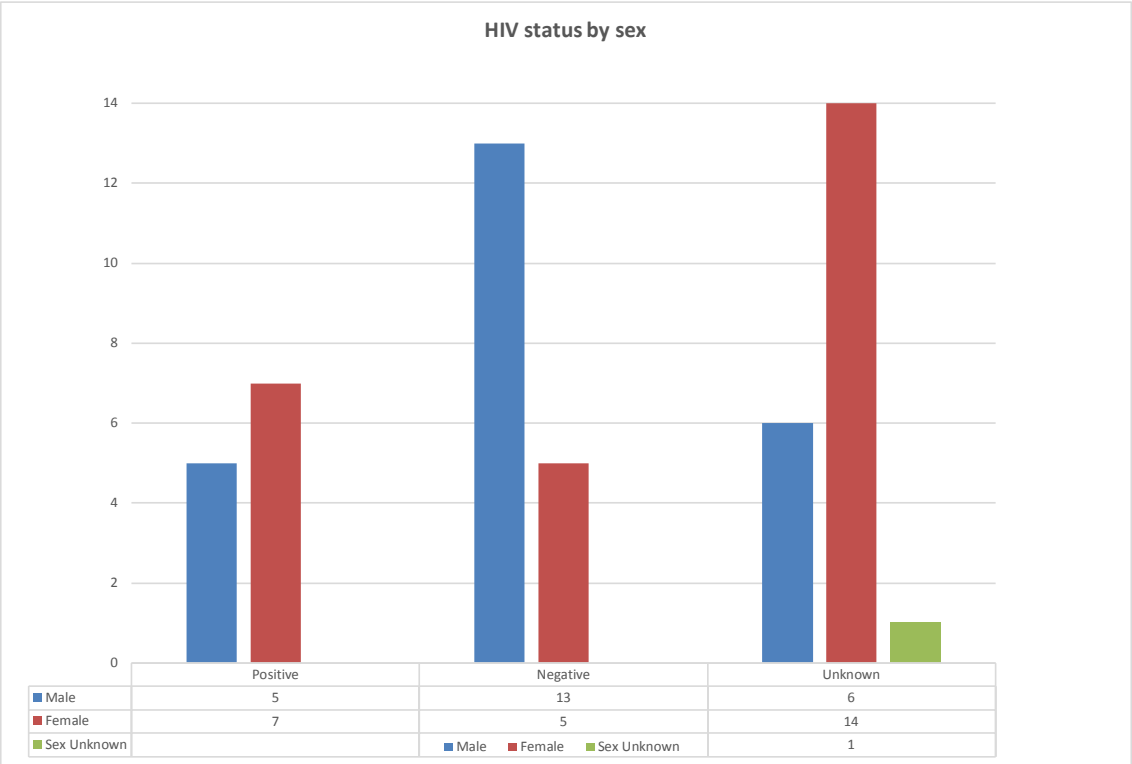

Figure 4.1: HIV status by sex.

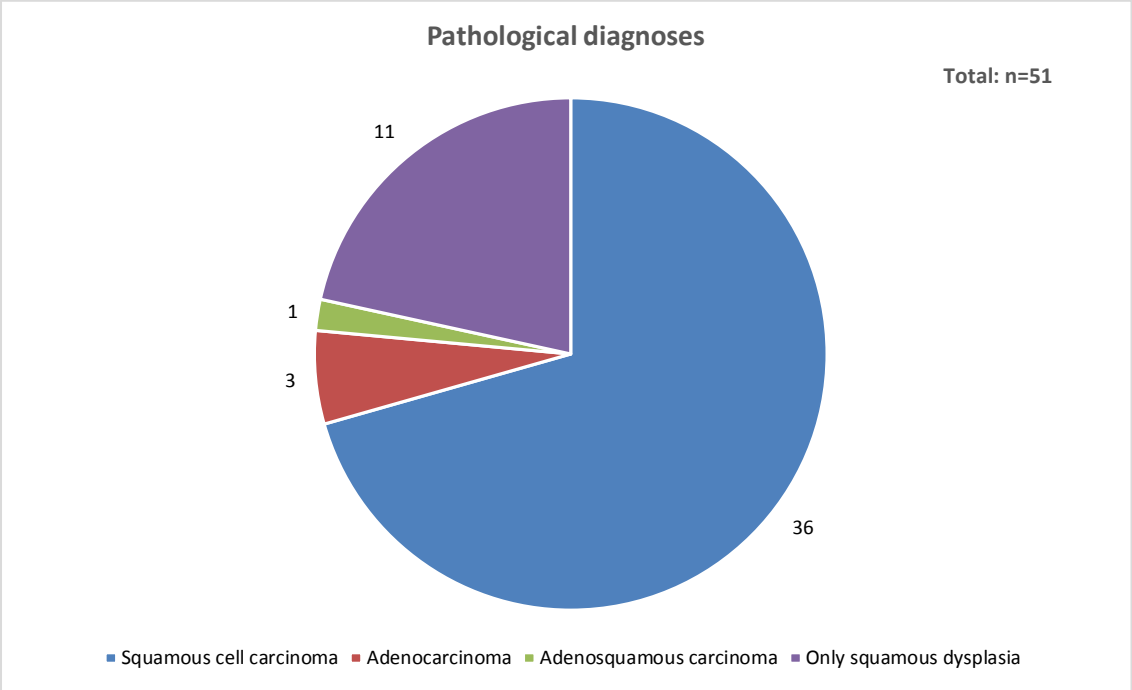

Figure 4.2: Pathological diagnoses.

Squamous cell carcinomas were further subdivided into three subtypes: basaloid, keratinising and non-keratinising (**Figure 4.3**). Keratinising and non-keratinising squamous cell carcinomas were graded as well, moderately and poorly differentiated. Basaloid carcinoma, by definition, is poorly differentiated.

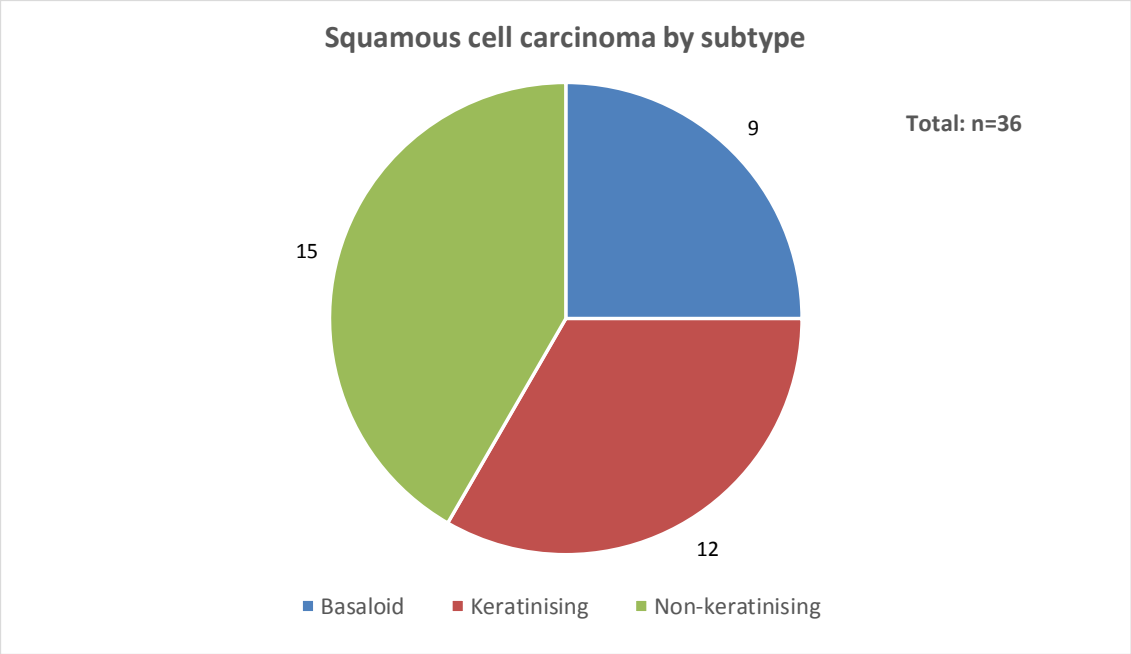

Figure 4.3: Squamous cell carcinoma by subtype.

4.2 Immunohistochemical stain analysis

4.2.1 p24

All cases, except three, showed no p24 staining. Two HIV-positive cases out of 12 known HIV-positive cases stained positive for p24 (16.7%). The known HIV-positive cases were a 42 year old female (**Figure 4.4**) and 50 year old female respectively. Occasional lymphocytes adjacent to squamous epithelium (normal and dysplastic) and cancer stained. The third case was a 47 year old male whose HIV status could not be determined from DISA records. Only lymphocytes adjacent to the cancer stained. LCs did not show p24 staining.

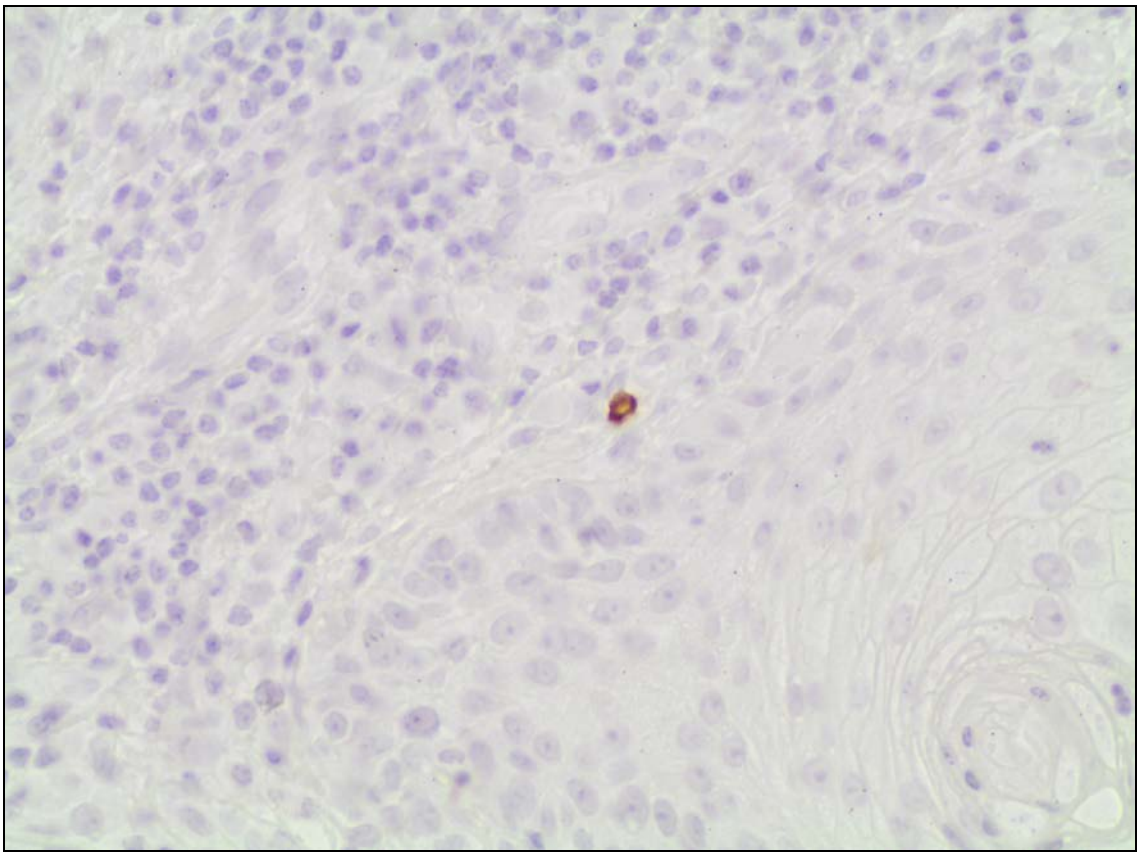

Figure 4.4: Strong p24 staining in a lymphocyte adjacent to normal squamous epithelium (x400).

4.2.2 p16

p16 immunohistochemistry was scored according to the intensity of staining and proportion of cells stained. None of the adenocarcinomas or the adenosquamous carcinoma showed block positive staining. All squamous cell carcinomas, except one case, showed block positive staining (97.2%) (**Figure 4.5**). Six out of the 21 cases with dysplastic squamous epithelium available did not show block positive staining. Of all the HSIL cases (AIN II and AIN III), 14 out of 18 cases demonstrated block positive staining. There were no cases with normal squamous epithelium which showed block positive staining. **Figure 4.6** shows p16 expression in normal tissue, high grade squamous dysplasia and squamous cell carcinoma.

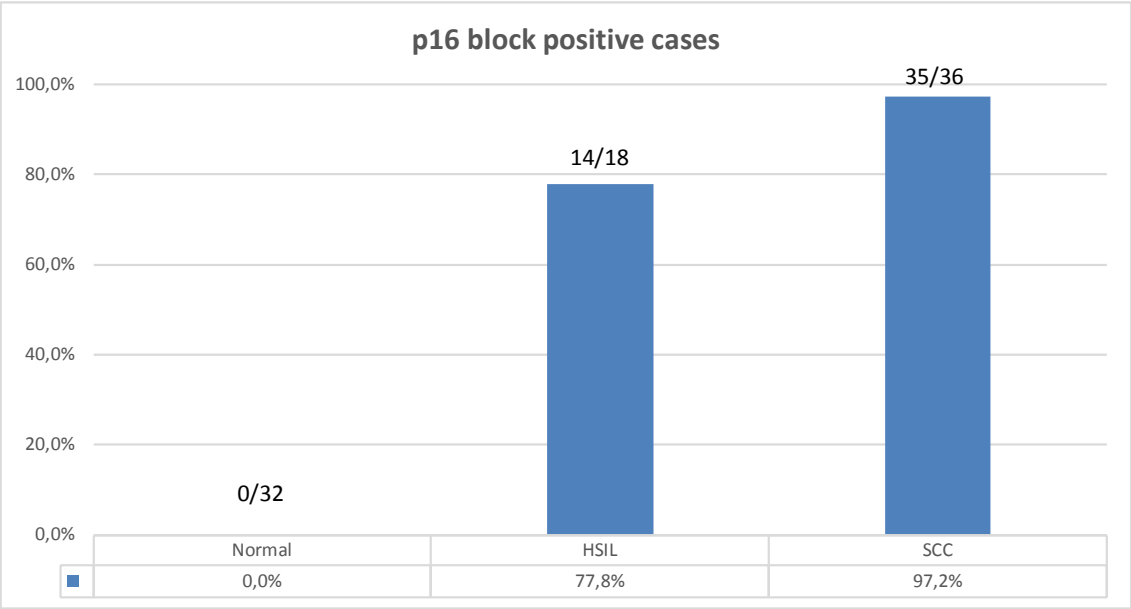

Figure 4.5: Percentage of cases showing block positive p16 staining.

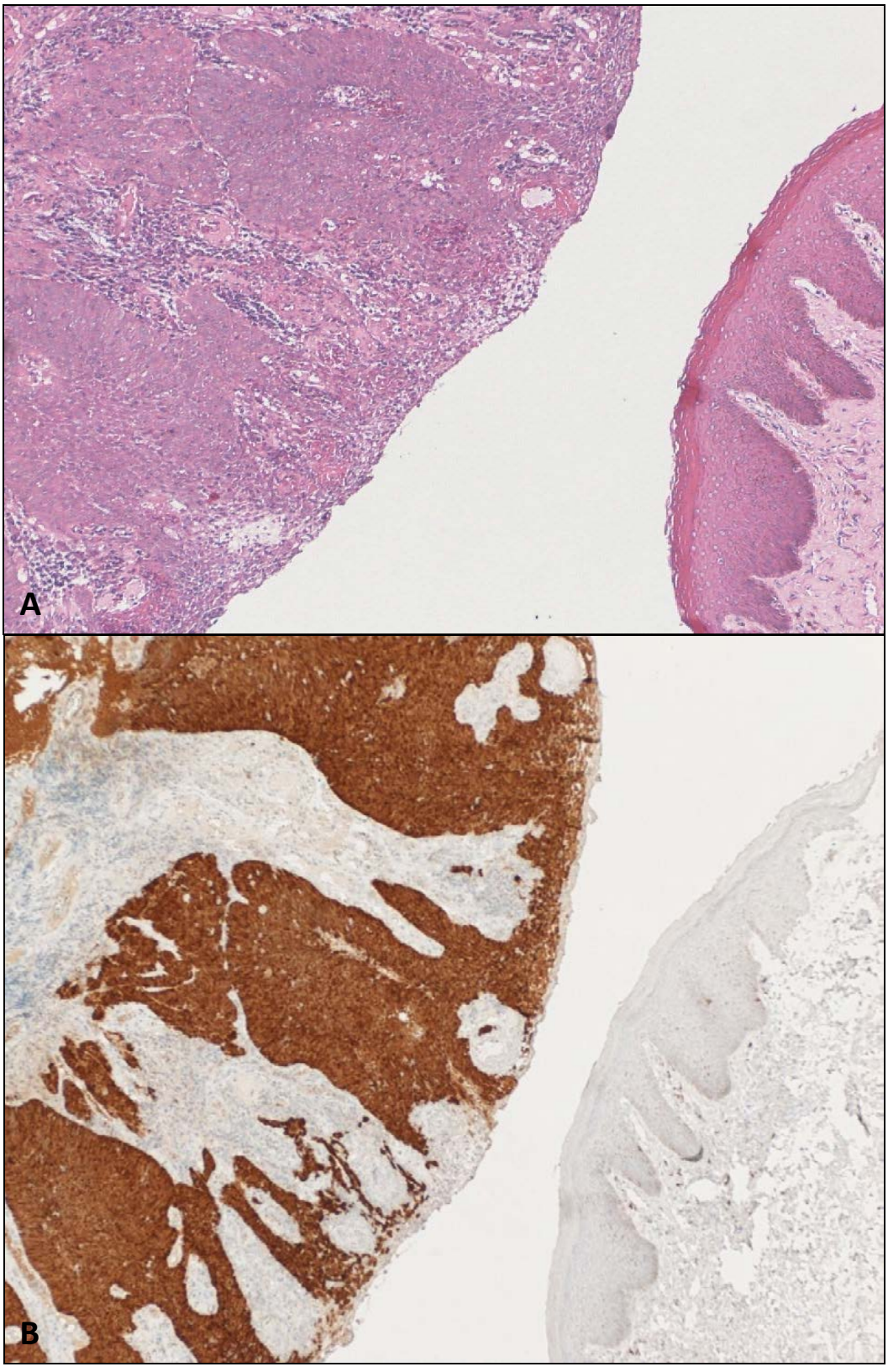

Figure 4.6: A: Normal squamous epithelium (right) and invasive squamous cell carcinoma (left) (H&E, x40). B: Strong block positive p16 staining in squamous cell carcinoma (left), and no staining of the normal squamous epithelium (right) (x40).

There were no significant differences between p16 expression and differentiation and subtype of squamous cell carcinoma (p=0.36 and p=0.32, respectively).

4.2.3 pRb

The proportion of staining appeared to decrease from normal to squamous cell carcinomatous tissue. A proportion of ≥90% was seen in six out of 32 (18.8%) cases with normal squamous epithelium and four out of 40 (10%) cases with squamous cell carcinomatous tissue. One case with dysplastic squamous epithelium out of 20 (5%) showed a proportion of ≥90%.

Only two basaloid squamous cell carcinomas did not show any staining (5.5% of SCCs). One of these basaloid squamous cell carcinoma cases had an area of dysplastic squamous epithelium (AIN III), and this also did not stain with pRb. The normal squamous epithelium in this case showed a high proportion of cells staining (80%) and other immunohistochemical markers showed staining of the dysplastic and carcinomatous tissue. The pRb stain was not repeated for this case. All other cases with dysplasia demonstrated staining with pRb within dysplastic cells.

In many cases peritumoural lymphocytes showed nuclear positivity for pRb. In dysplastic and normal epithelium, epithelial cells showed stronger and denser staining towards the superficial layers of stratified squamous epithelium (**Figure 4.7**).

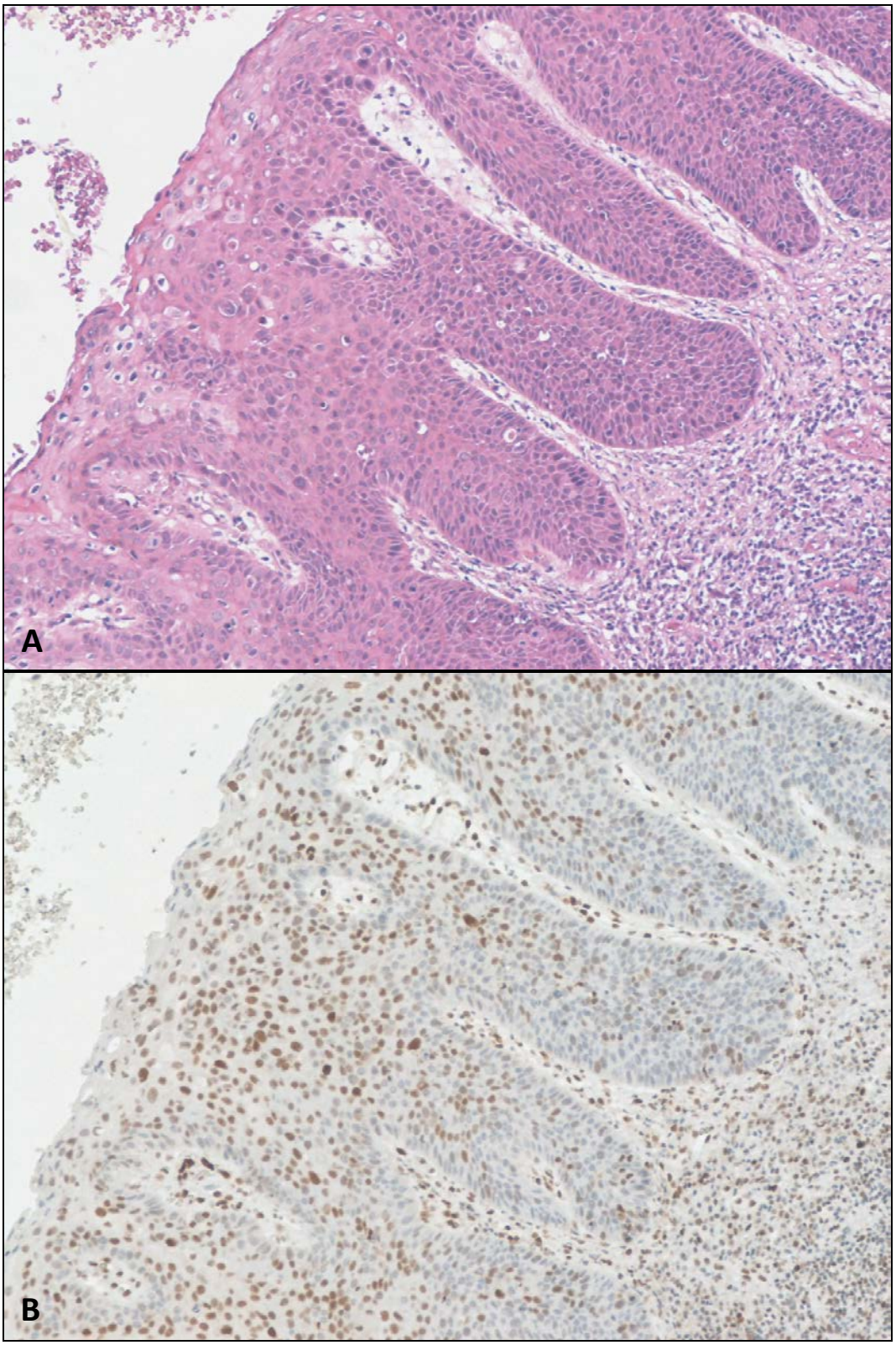

Figure 4.7: A: Dysplastic stratified squamous epithelium (AIN II) (H&E, x200). B: Nuclear staining of dysplastic epithelial cells with pRb. Note relative decrease in staining within the basal layer (x 200).

There was a significant difference in pRb expression when compared with differentiation of the carcinoma (a decrease in the proportion of positive cells from well to poorly differentiated squamous cell carcinomas; p=0.03) (**Table 4.1 and Figure 4.8**). HIV status did not seem to play a role (HIV status alone or in combination with differentiation; p=0.11 and p=0.79, respectively). The means for each type of differentiation are illustrated in **Figure 4.8**.

Table 4.1: ANOVA (univariate) of pRb – Factors: HIV status, differentiation.

Effect	Univariate tests of significance for pRBPropCancer		
	SS	Degr. Of freedom	p
Intercept	5.14	1	0.00
HIV	0.34	2	0.11
Differentiation	0.55	2	0.03
HIV*Differentiation	0.12	4	0.79

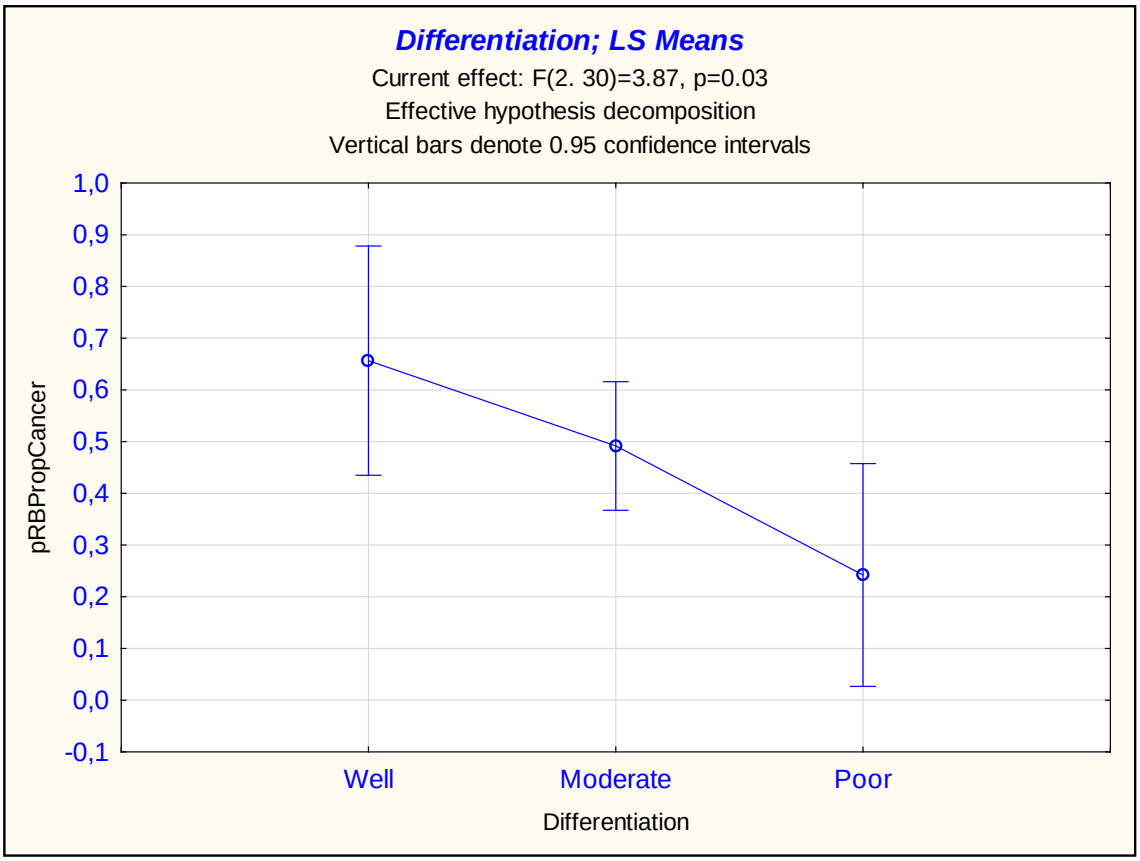

Figure 4.8: LS means for pRb proportion in well, moderately and poorly differentiated squamous cell carcinomas.

There was no significant correlation between proportion of pRb staining and subtype of carcinoma (p=0.09). HIV status in combination with the subtype, also did not play a role (p=0.71).

4.2.4 E-cadherin

All cases (51) showed staining of at least 2+ (most 3+) in areas of normal squamous epithelium. In one basaloid squamous carcinoma, staining was totally lost (negative). Four out of nine basaloid squamous carcinomas demonstrated 1+ staining. The other four basaloid squamous carcinomas showed 2+ and 3+ staining (two 2+ and two 3+).

Six out of 11 poorly differentiated squamous cell carcinomas showed 1+ or negative staining (**Figure 4.9**). Out of five well differentiated squamous cell carcinomas, four showed 3+ staining and one 2+ staining.

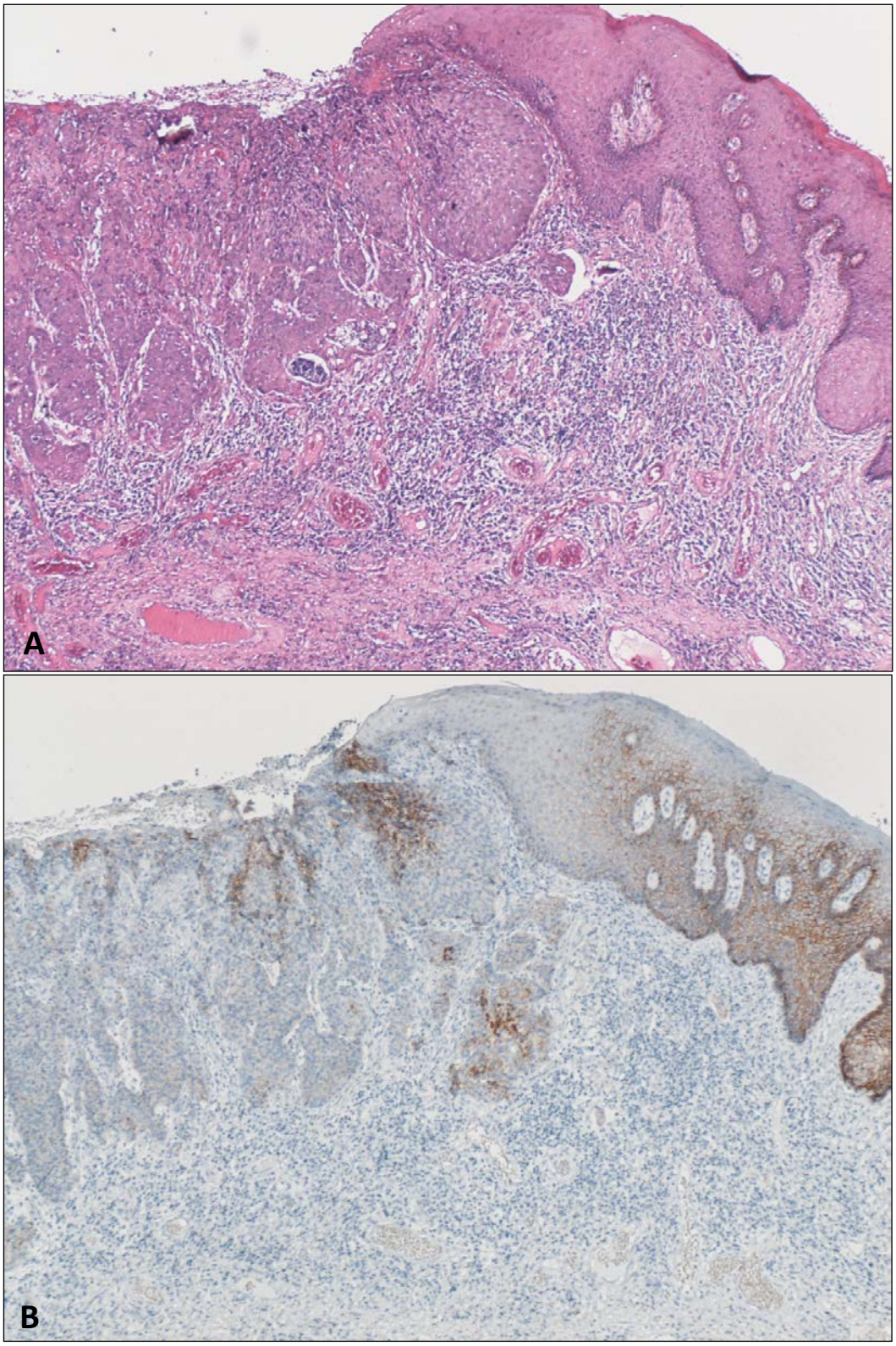

Figure 4.9: A: Normal anal squamous epithelium (right) (H&E, x40) and adjacent invasive squamous cell carcinoma (left). B: Loss of E-cadherin staining in poorly differentiated squamous cell carcinoma (x40).

HIV did not seem to influence E-cadherin expression, but differentiation of the cancer had a significant effect on the score (p=0.52 and 0.04, respectively) (**Table 4.2**). There was a correlation between differentiation and score, meaning that the more well differentiated a carcinoma was, the higher the E-cadherin score. The means for each type of differentiation are illustrated in **Figure 4.10**.

Table 4.2: ANOVA (univariate) of E-cadherin – Factors: HIV status, differentiation.

Effect	Univariate Tests of Significance for E_CadherinCancer		
	SS	Degr. of Freedom	p
Intercept	106.31	1	0.00
HIV	1.05	2	0.52
Differentiation	5.52	2	0.04
HIV*Differentiation	2.63	4	0.51

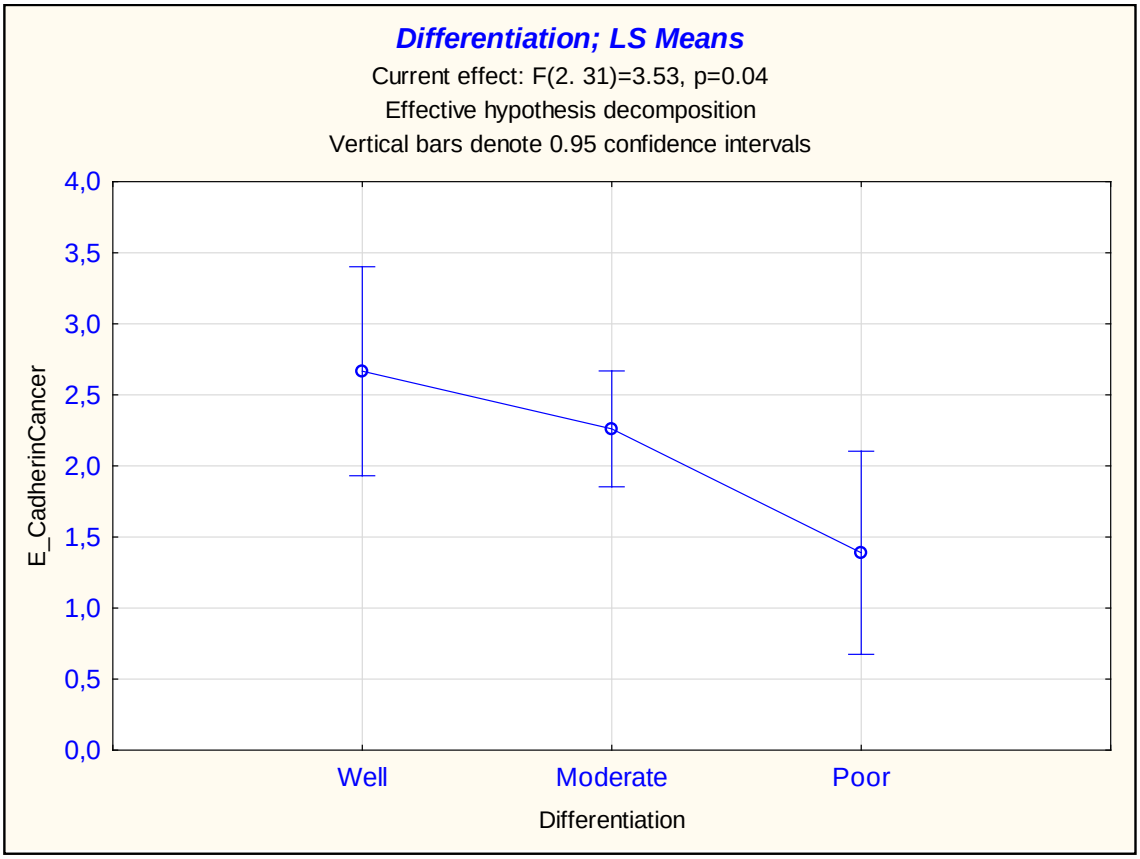

Figure 4.10: LS means for E-cadherin score in well, moderately and poorly differentiated carcinomas.

E-cadherin expression in basaloid squamous cell carcinoma, compared to other squamous cell carcinoma subtypes, did not reach statistical significance (p=0.05) (**Table 4.3**).

Table 4.3: ANOVA (univariate) of E-cadherin – Factors: HIV status, subtype.

Effect	Univariate Tests of Significance for		
	SS	Degr. Of Freedom	p
Intercept	111.17	1	0.00
HIV	1.78	2	0.35
Subtype	5.39	2	0.05
HIV*Subtype	1.78	4	0.70

VEPAC analysis and LSD test showed that there was a significant difference in the E-cadherin score between normal tissue and squamous cell carcinoma, and dysplastic tissue and squamous cell carcinoma (p=0.002 and p=0.004, respectively). There was no significant difference in the score between normal and dysplastic tissues (p=0.75) (**Table 4.4**). **Figure 4.11** is a graphical representation of the LS means of the E-cadherin score in the three different types of tissue in HIV-positive patients, HIV-negative patients and patients with an unknown HIV status.

Table 4.4: LSD test for comparing E-cadherin scores in tissues with one another.

Comparisons	LSD test; variable E_Cad Stain		
	1st	2nd	p
Normal - Dysplastic	E_CadherinNormal	E_CadherinDysp	0.75
Normal - Cancer	E_CadherinNormal	E_CadherinCancer	0.002
Dysplastic - Cancer	E_CadherinDysp	E_CadherinCancer	0.004

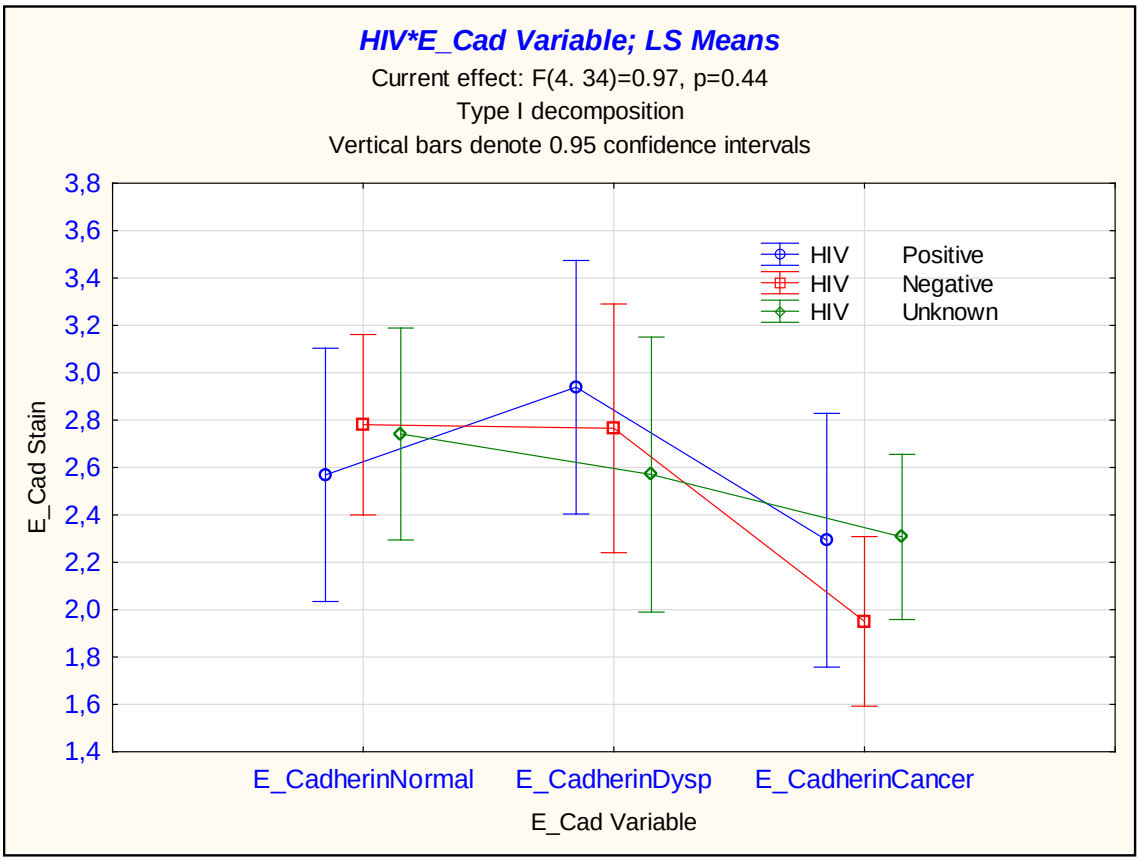

Figure 4.11: LS mean scores of E-cadherin in three different types of tissue according to HIV status (HIV-positive, -negative and unknown).

4.2.5 CD1a

The range of density between normal, dysplastic and carcinomatous tissue was similar (**Figure 4.12**). Normal tissue showed a range from 0 to 44, dysplastic tissue two to 48 and carcinomatous tissue 0 to 45.

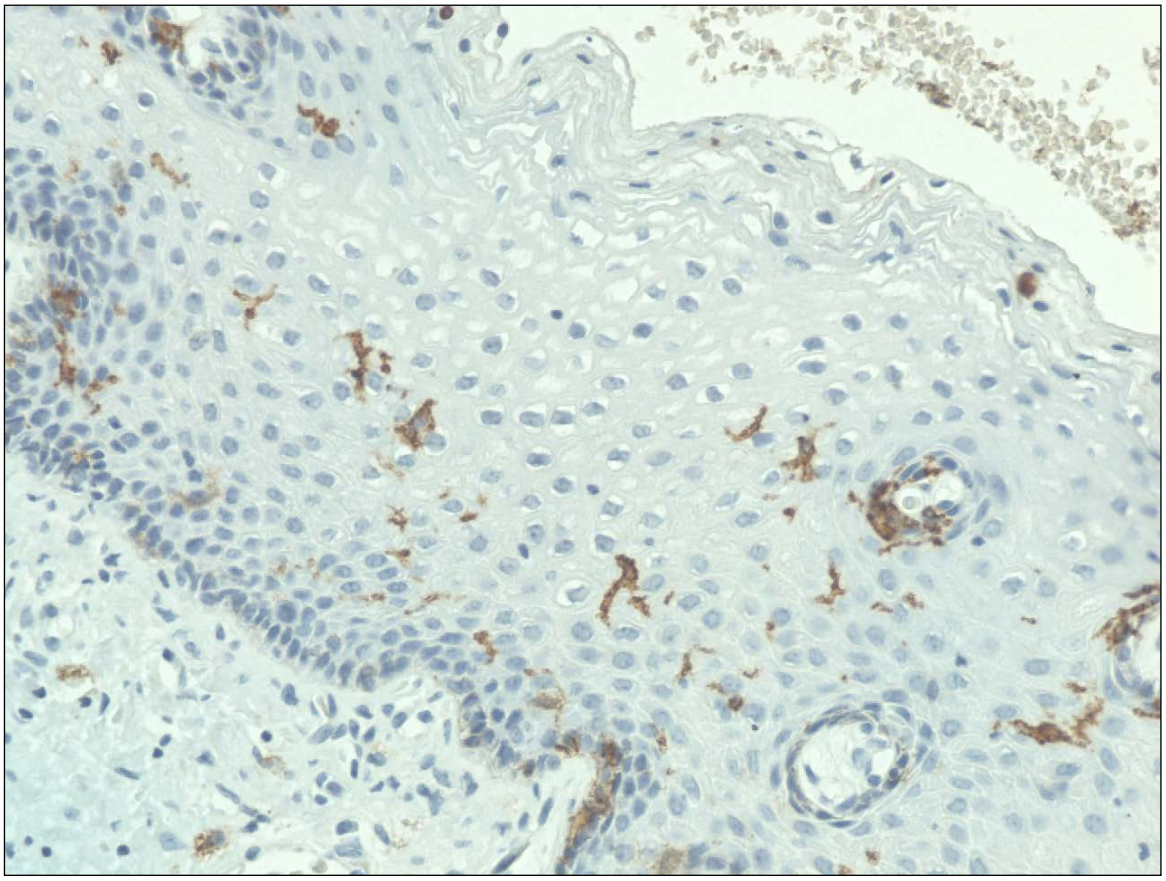

Figure 4.12: Langerhans cells in normal squamous epithelium staining with CD1a. Note dendritic processes intertwined between epithelial cells (x400).

Probability plots (p-plots) demonstrated deviation of the data from normality (**Figure 4.13** and **Figure 4.14**) and Box-Cox transformation of the data was performed. Differentiation, subtype of squamous cell carcinoma and HIV status did not play a role in the density of LCs (even after transformation of data; p=0.59, p=0.37 and 0.17, respectively).

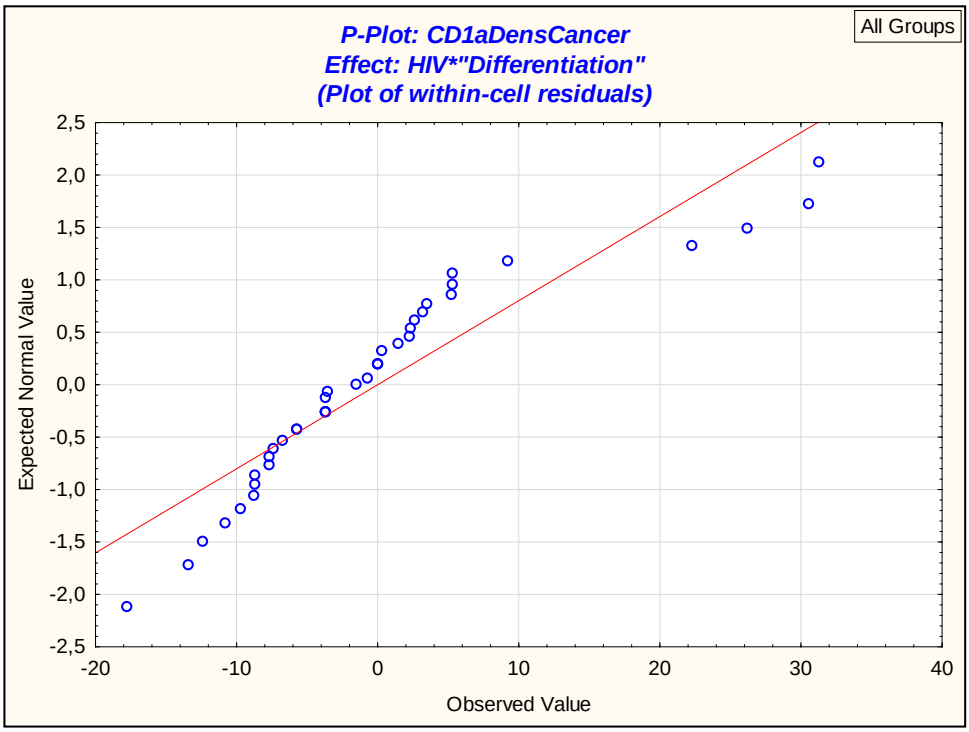

Figure 4.13: Probability plot of density of CD1a-positive cells in cancer deviates from normality (taking into account HIV status and differentiation of squamous cell carcinomas).

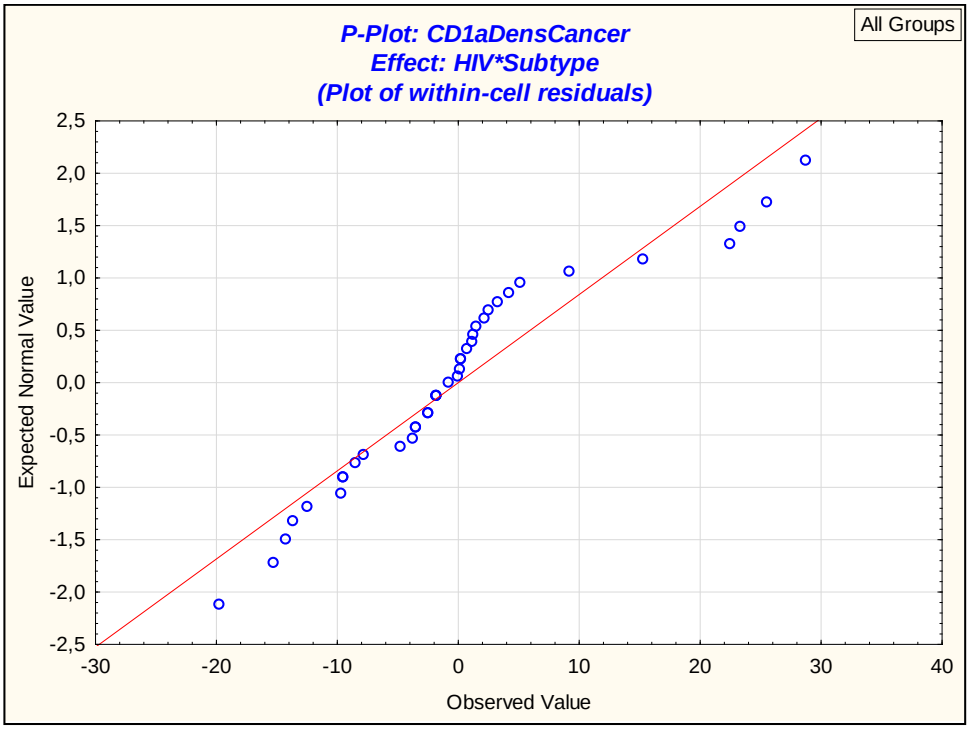

Figure 4.14: Probability plot of density of CD1a-positive cells in cancer deviates from normality (taking into account HIV status and subtype of squamous cell carcinomas).

VEPAC analyses were done and no significant difference in the density of CD1a-positive cells was demonstrated among normal, dysplastic and squamous cell carcinoma tissue when HIV status was taken into account (p=0.58), or independent of HIV status (p=0.18).

4.2.6 Langerin

Differentiation, subtype of squamous cell carcinoma and HIV status did not play a significant role in the density of Langerin-positive cells in cancerous tissue (0.41, 0.26 and 0.13, respectively). There was also no significant difference in the densities between the three types of tissue when HIV status was not taken into account, and ranged from 0 to 24 (normal), one to 25 (dysplastic) and 0 to 30 (carcinomatous) (p=0.22). HIV status appeared to have an effect in the Langerin-positive cell density in the three types of tissue (increased density in tissues of HIV positive patients). However, this did not reach statistical significance (p=0.054) (**Table 4.5**).

Table 4.5: VEPAC of Langerin density – Factors: HIV status, Langerin density in the three different tissues.

Effect	Fixed Effect Test for LangerinDens		
	Num. DF	Den. DF	p
HIV	1	28	0.054
Variable	2	23	0.97
HIV*Variable	2	23	0.28

4.2.7 Bcl-2

No staining of cells was observed within normal tissue and only one case with AIN III showed staining. Fifteen out of 40 carcinomas showed staining of carcinoma cells (**Figure 4.15**).

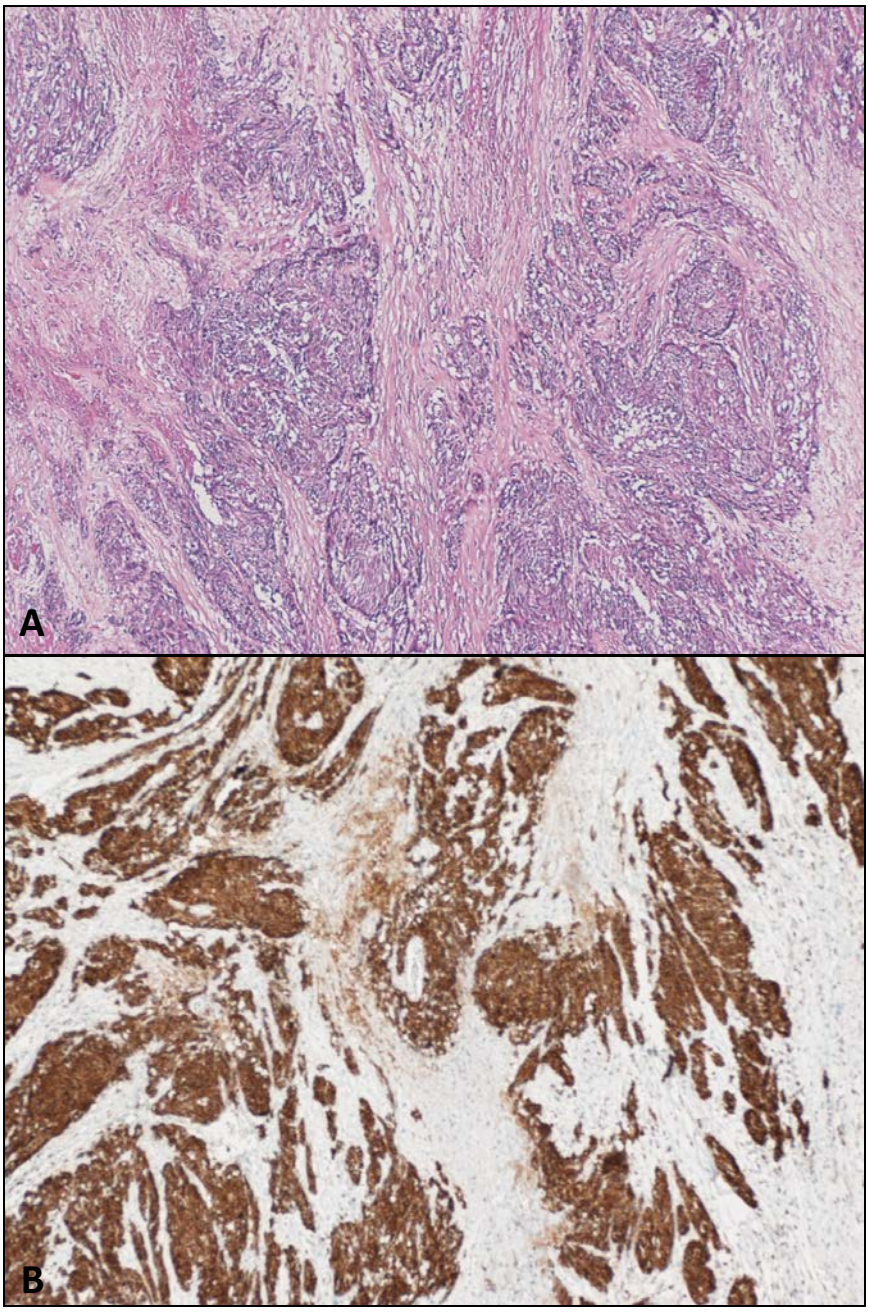

Figure 4.15: A: Basaloid squamous cell carcinoma. (H&E, x40) B: Bcl-2 stain of same basaloid squamous carcinoma showing strong and diffuse cytoplasmic positivity (x40).

The differentiation, subtype of squamous cell carcinomas and HIV status, did not have a significant effect on the Bcl-2 expression (p=0.12, p=0.54 and p=0.94, respectively).

VEPAC analyses were done and a significant difference in Bcl-2 expression was demonstrated among normal, dysplastic and cancerous tissue (Bcl-2 staining increases

from normal to cancerous tissue; p=0.02) (**Table 4.6**). The LS means for the proportion of Bcl-2-positive cells showed an upward trend from normal and dysplastic tissue to carcinomatous tissue (**Figure 4.16**). HIV-negative cases showed a range of proportion in carcinomatous tissue from 0 to 100% (Figure 4.17). HIV-positive cases showed a range from 0 to 75%. HIV status did not have a significant effect on the proportion of Bcl-2-positive cells in the three types of tissue (p=0.81) (**Table 4.6**).

An LSD test was performed and HIV status was ignored. There was a significant difference between the proportion of Bcl-2-positive cells in normal and cancerous tissue (p=0.01). The same significant differences were not seen when comparing the proportions of normal and dysplastic tissue, and dysplastic and cancerous tissue (p=0.69 and p=0.07, respectively; **Table 4.7**).

Table 4.6: VEPAC of Bcl-2 proportion – Factors: HIV status, Bcl-2 proportion in three different tissues.

Effect	Fixed Effect Test for BCL2Prop Stain		
	Num. DF	Den. DF	p
HIV	2	48	0.81
BCL2Prop Variable	2	32	0.02
HIV*BCL2Prop Variable	4	32	0.73

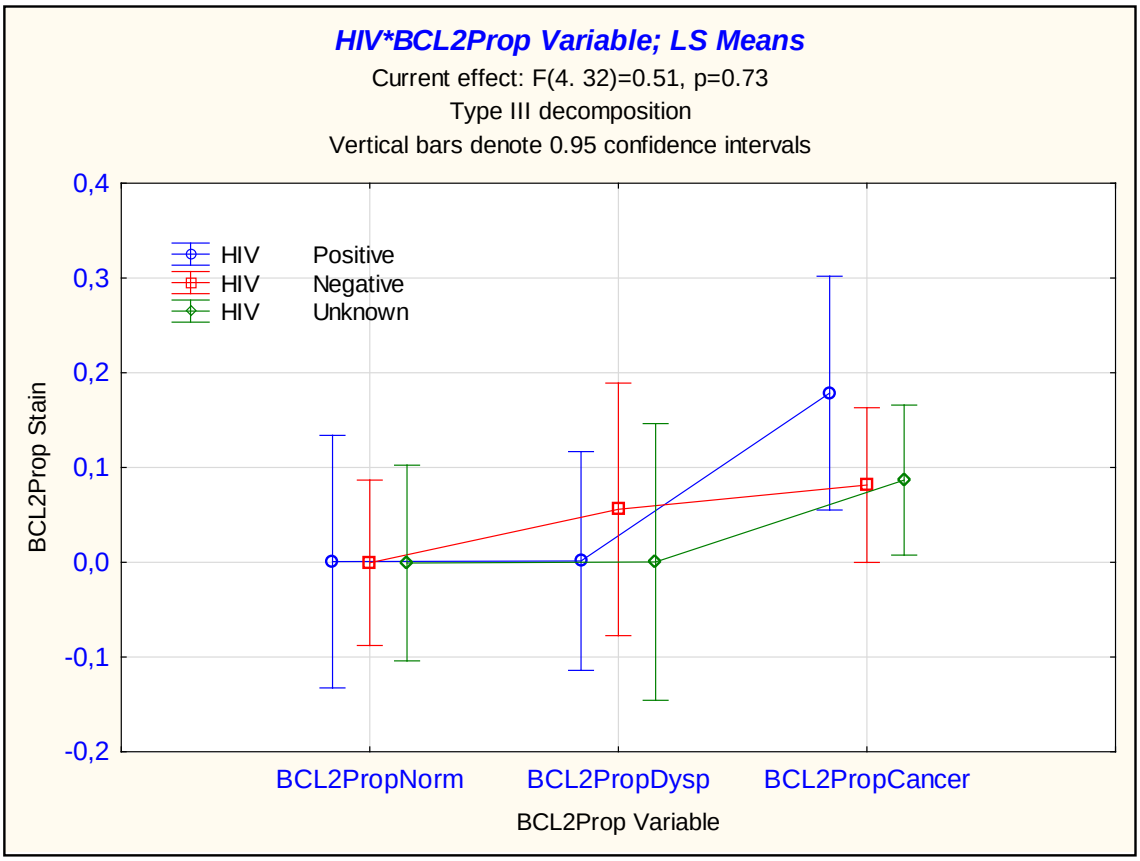

Figure 4.16: LS means for proportion of Bcl-2-positive cells in three different types of tissue according to HIV status (HIV-positive, -negative and unknown).

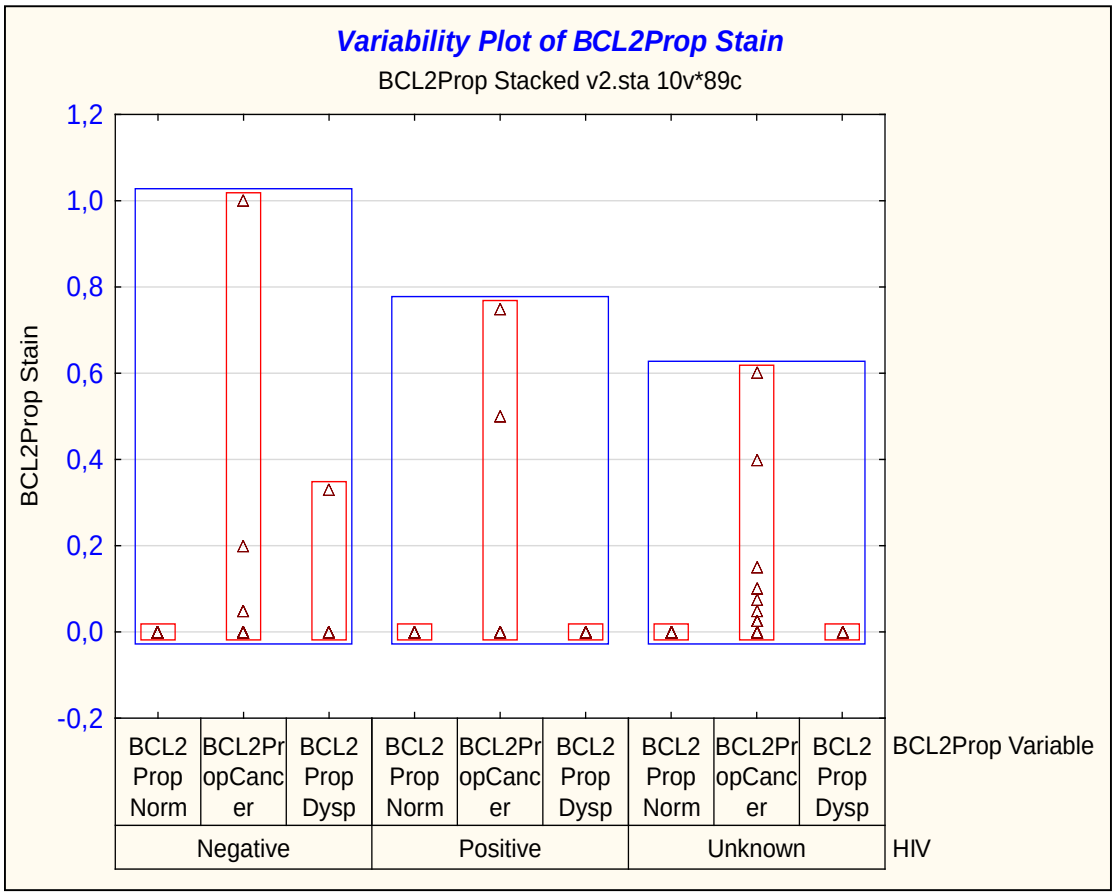

Figure 4.17: Variability plot of proportion of Bcl-2-positive cells.

Table 4.7: LSD test for comparing Bcl-2 expression in normal, dysplastic and carcinomatous tissues.

Comparisons	LSD test; variable BCL2Prop Stain		
	1st	2nd	p
Normal - Dysplastic	BCL2PropNorm	BCL2PropDysp	0.69
Normal - Cancer	BCL2PropNorm	BCL2PropCancer	0.01
Dysplastic - Cancer	BCL2PropDysp	BCL2PropCancer	0.07

4.2.8 Ki-67

The proliferation index was measured in normal, dysplastic and cancerous tissues. Basal staining was observed in normal stratified squamous epithelium. In mildly dysplastic regions (AIN I), staining occurred predominantly in the lower third but included cells above the lower third of epithelium (**Figure 4.18**).

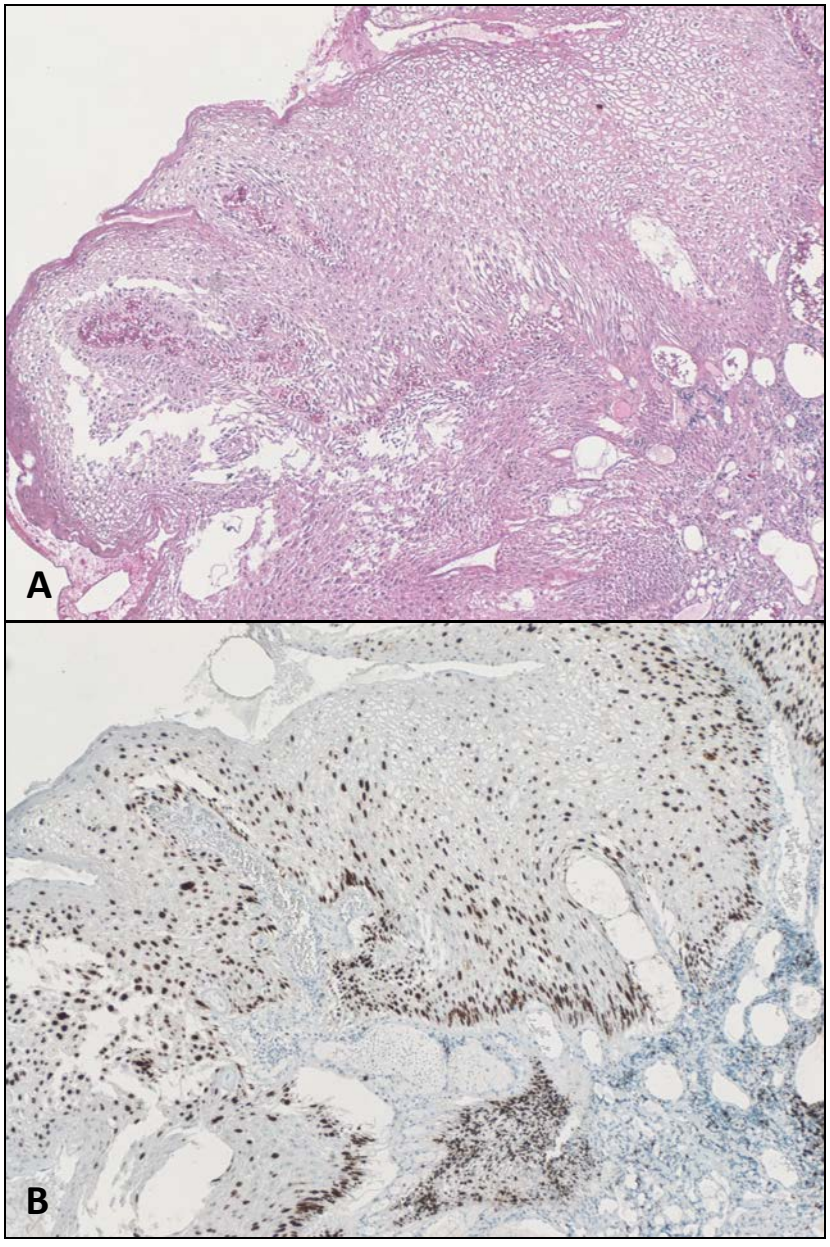

Figure 4.18: A: Mild squamous dysplasia (AIN I) (H&E, x40). B: Ki-67 nuclear staining throughout the full thickness of the epithelium, but predominantly in the lower third (x40).

The proliferation index in HSIL cases ranged from 33% to 95% and generally demonstrated stronger staining in the upper two thirds of the epithelium. Carcinomatous tissue also demonstrated a range of proliferation from 33% to 95%.

There was no significant difference in the proliferation index among the different subtypes of squamous cell carcinoma (p=0.23) and the degrees of differentiation (p=0.23). HIV status did not have a significant effect on the proliferation index of carcinomas (p=0.44).

4.2.9 HPV L1 capsid

Only two carcinomas out of 40 (5%) showed weak staining (intensity 1+) in a small proportion of the cancer cells (3% and 5%, respectively). Both of these were squamous cell carcinomas.

Only nine cases out of 26 (34.6%) showed staining of dysplastic tissues, and this was only within mild and moderately dysplastic (AIN I and AIN II) cells (**Figure 4.19**). None of the 12 cases harbouring AIN III showed positive staining.

Normal squamous epithelium did not show staining for HPV L1 capsid.

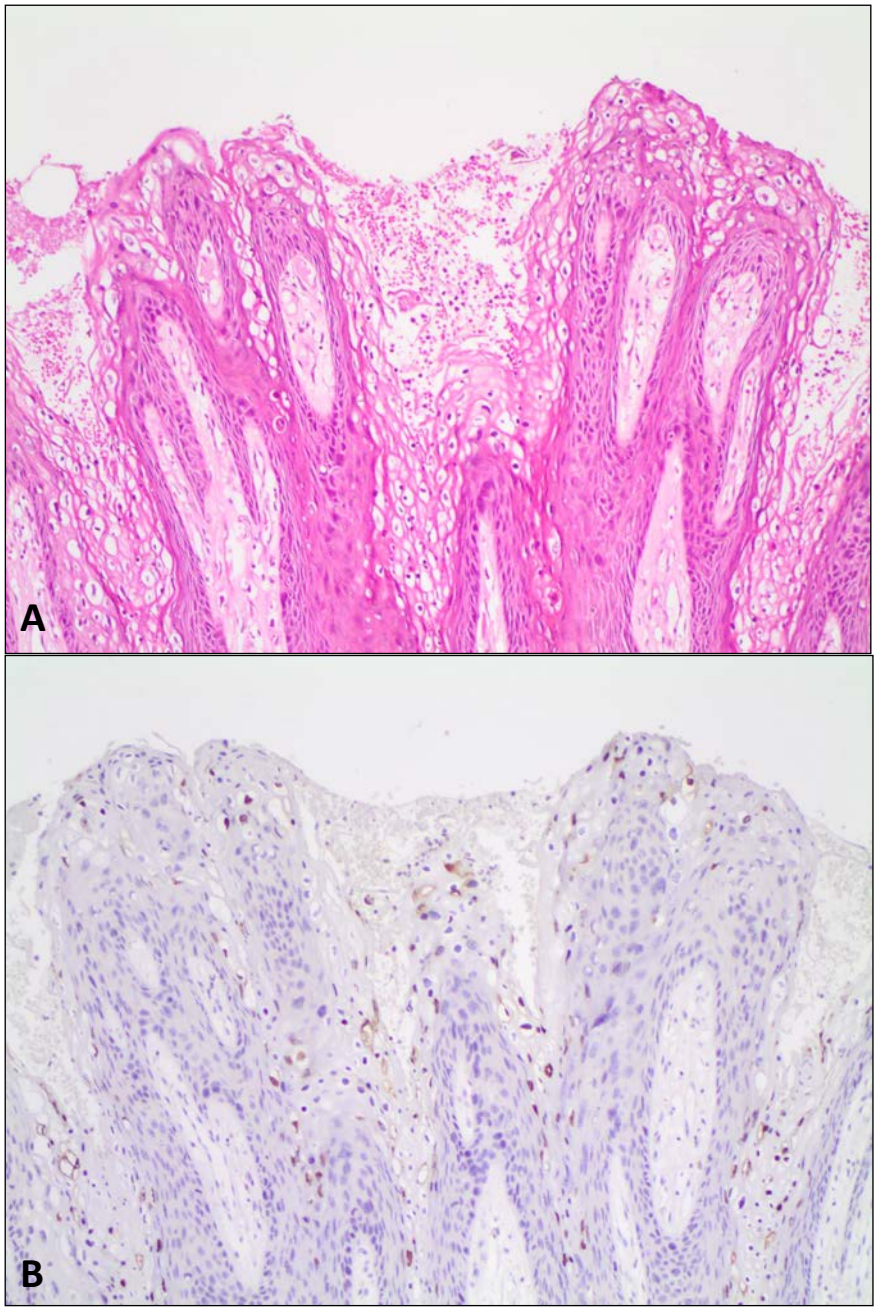

Figure 4.19: A: Condylomatous region with koilocytic atypia (AIN I) (H&E, x100). B: The same region showing HPV nuclear L1 capsid staining (x100).

4.2.10 β-catenin

There was no case which demonstrated a score of 4 or more, using Jass' criteria, in any type of tissue. This indicates that there was no abnormal localisation of β-catenin in any of the cases.

However, 24 carcinomas out of 40 (60%) demonstrated loss of membrane staining and 11 (27.5%) showed weak cytoplasmic staining (**Figure 4.20**). One case (2.5%) showed strong cytoplasmic staining. There were no cases with β-catenin nuclear staining.

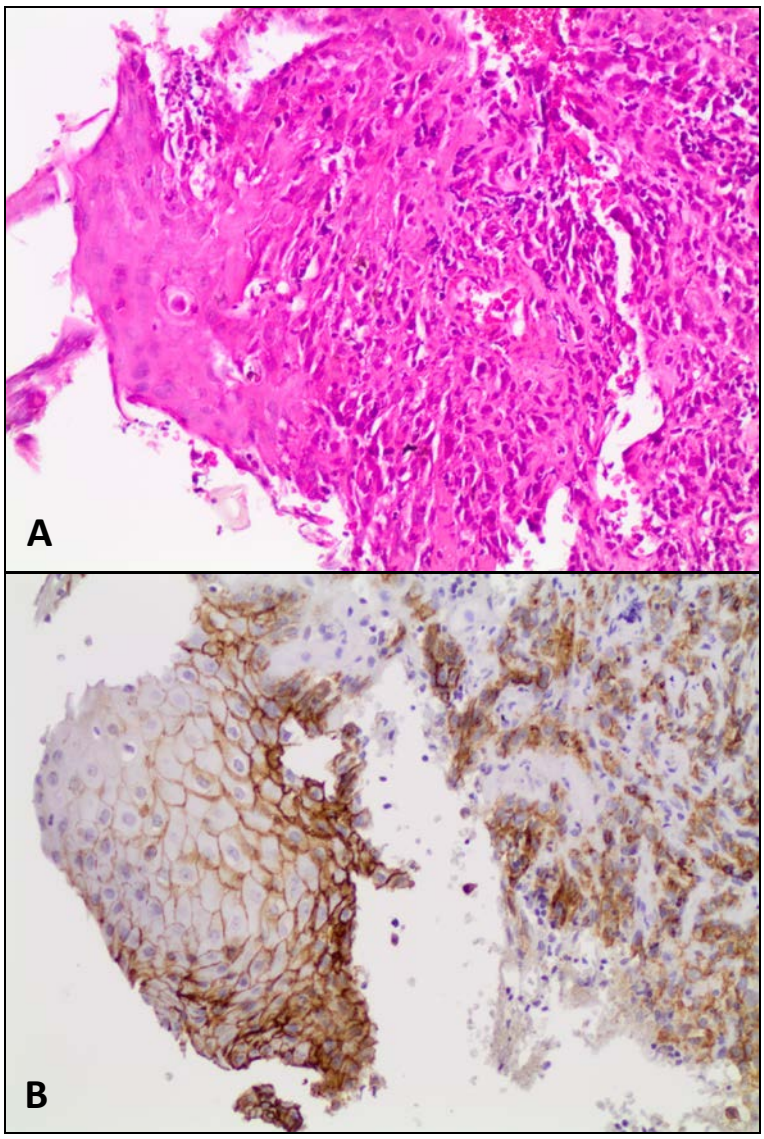

Figure 4.20: A: Normal squamous epithelium (left) with an adjacent invasive squamous cell carcinoma (right) (H&E, x200). B: Strong membranous staining for β-catenin in normal and carcinoma cells. There is also additional weak cytoplasmic staining in some carcinoma cells (x200).

CHAPTER 5

DISCUSSION

5.1 Patient demographics

There were more female patients than male patients with invasive anal carcinoma. The male to female ratio was 0.92. This is similar to the reported incidence of invasive squamous cell carcinoma in males and females in the USA; male to female ratio of 0.81) [3-5]. There was almost double the number of patients with invasive carcinoma over 50, compared to those younger than 50. This is also consistent with the literature [4, 5, 18, 20, 80]. Similar information on the sex distribution for dysplasia is not available as most studies focused on MSM and did not document anal dysplasia in women.

No information regarding HIV status was available on the DISA lab system in 41,1% of our cases. This is surprising as it is known that immunosuppression by HIV infection amongst other reasons is a risk factor for developing anal squamous cell carcinoma. It is feasible that many patients were already aware of their HIV status and shared this information with their surgeons or that the HIV tests were done at another laboratory. This is a possible reason for ELISA test results not being available on the Groote Schuur Hospital DISA laboratory information system.

Sexual orientation and practices of patients were not documented in patient records. Thus, no comment can be made in our study regarding the possibility of these factors portending an increased risk for anal cancer development.

5.2 Pathological diagnoses

The majority of invasive carcinomas were squamous cell carcinomas. Anal canal adenocarcinomas are rare, and account for about a tenth of carcinomas of this region [81]. In our study, 7.5% of invasive carcinomas were adenocarcinomas.

Our study had more cases of HIV-negative patients with HSIL than HIV-positive patients, but unfortunately the HIV status of five cases which demonstrated HSIL was not known. In a meta-analysis by Machalek *et al.* there was no significant difference between the prevalence of HSIL in HIV-positive and HIV-negative MSM [28]. A study by Xi *et al.* found that some HPV-16 variants are associated with an increased risk for HSIL, irrespective of HIV status in MSM [82]. HIV status may not be the most important factor for hr-HPV causing HSIL.

5.3 p24

A study by Moonim *et al.* in 2010 identified the usefulness of p24 immunohistochemical staining to detect HIV infection in lymphoid and non-lymphoid organs [83]. The non-lymphoid tissue which demonstrated staining of cells in lymphoid infiltrates consisted of anal tissue, lung tissue and brain tissue. Cells that were identified as expressing p24 were follicular dendritic cells, lymphocytes (intrafollicular, interfollicular and mantle zone), macrophages and multinucleated giant cells. Plasma cells, stromal cells or epithelial cells did not stain. No mention was made about p24 staining of Langerhans cells in this study.

Although HIV is known to infect Langerhans cells in surface epithelia, there is almost no literature available on p24 immunohistochemical staining of these cells. A study by Becker *et al*. on the immunohistochemical detection of structural HIV proteins in oral mucosa of HIV-infected patients, detected a single p24-positive cell which morphologically resembled a LC (one case out of seventeen biopsies) [84].

A very low percentage of HIV-positive patients showed positive p24 staining in our study (16.7%). This is in contrast to the study by Moonim [83]. Their study used the same clone of p24 than ours (and had a dilution of 1:40). During the selection of cases for this study, blocks with tissue containing lymphocytic infiltrates were not actively sought, and this could be a contributing factor for the poor correlation. In our study, 27 of the cases demonstrated lymphocytic infiltrates. Of the 12 HIV-positive cases, eight showed infiltrates and four did not. Both p24-positive cases showed lymphocytic infiltrates. Similar to Moonim's study, there was no staining of stromal cells or epithelial cells [83].

Interestingly, there were also no LCs that stained positive in the surface squamous epithelium or amongst cancer cells in HIV-positive cases. This would support findings that functioning Langerin is a natural barrier for HIV infection of LCs and transmission [76].

The findings of this study suggest that p24 is a poor immunohistochemical marker for HIV infection in non-lymphoid tissues.

5.4 p16

Most of the squamous cell carcinomas in this study showed block positive staining for p16 in cancer cells. Although p16 is widely regarded as a surrogate marker for transcriptionally active hr-HPV infection, other unknown factors such as oncogenic stress may result in increased p16 expression [79]. Previous authors who have studied factors which may portend a better prognosis for anal squamous cell carcinoma have, in conjunction with p16 immunohistochemistry, relied on PCR techniques to identify hr-HPV infection [79, 85, 86]. It is assumed in our study, based on the general acceptance of p16 as a surrogate marker of hr-HPV infection, that 97.2% of squamous cell carcinomas that showed block positive staining were related to hr-HPV infection. The percentage of 97.2% was higher than expected, but noting that up to 93% of anal carcinomas in USA are associated with hr-HPV infection, this figure may be a true

reflection of hr-HPV-associated anal squamous cell carcinomas [3]. One particular study by Lu *et al*. in USA found that 100% of their cases of anorectal squamous cell carcinoma (29 cases) stained diffusely with p16 [87]. PCR techniques employed in this study also confirmed that all cases harboured hr-HPV DNA (HPV-16 in 85% of cases) . A large study in the UK (151 cases of anal squamous cell carcinoma) found that 95% of cases harboured HPV infection (89% of these were HPV-16) [88]. Seven percent of patients from one centre in the UK study were HIV-positive . In Germany, Rödel *et al*. found 95.8% (91 out of 95) of their cases of anal squamous cell carcinoma to harbour HPV DNA [86]. There was mono-infection by HPV-16 in 78.9% of cases . The fact that the prevalence of adult HIV infection in 2013 was 19.2% in South Africa, may very well be the reason for the slightly higher rates of hr-HPV infection in our study [7]. This is in stark contrast to a much lower prevalence in USA (0.38% -2012) [9] and UK (0.17% - 2013) [8].

A large study consisting of 135 patients with American Joint Committee on Cancer (AJCC) stage I to III anal carcinomas (squamous cell carcinomas and verrucous carcinomas) found p16 to be an independent prognostic factor for overall survival (OS) and disease-specific survival (DSS) [79]. Diffuse expression of p16 (>70% of cells) resulted in improved OS and DSS. Studies done on head and neck squamous cell carcinomas (HNSCC) have found that hr-HPV infection and diffuse expression of p16 alone in tumour cells, predict a better prognosis [89-91]. In contrast, one study of anal squamous cell carcinoma found p16-positive/hr-HPV-negative cancers to have an overall worse prognosis [79]. The authors of the latter study suggest that p16 immunohistochemistry should not be done without concurrent molecular tests to identify the presence of hr-HPV DNA [79]. These findings raise the possibility that there is an inherent difference in the tumour biology between HNSCC and anal SCC.

The relative affordability and availability of immunohistochemistry in South Africa makes p16 staining an easy way for pathologists to provide prognostic information on anal squamous cell carcinoma. However, PCR techniques for identifying hr-HPV infection, is not routinely offered. More studies on the outcome of anal squamous

carcinoma may need to be done to confirm the absolute necessity for hr-HPV identification in conjunction with p16 immunohistochemical staining.

A study in 1991 by Koulos *et al*. detected HPV-18 DNA present within two out of six anal adenocarcinomas [92]. Seven colorectal adenocarcinomas were all negative for hr-HPV DNA. These authors did not perform p16 immunohistochemical staining on these cases. The negative p16 staining of our adenocarcinomas makes it less likely that these cases were related to hr-HPV infection.

5.5 pRb

Cordon-Cardo *et al*., who investigated the nuclear expression of pRb in normal tissues, found that in stratified epithelia, maturing cells demonstrated the strongest staining, while basal/progenitor cells showed weak to absent staining [93]. Maturing haematopoietic cells also showed the strongest staining compared to other cells in this lineage.

We found similar pRb staining of stratified squamous epithelium in our cases to that described by Cordon-Cardo *et al* [93]. There was a relative lack of staining in basal areas of normal and dysplastic squamous epithelium.

Only 5.5% of squamous cell carcinomas showed complete loss of staining in our study. This is contrary to the findings of a similar study in USA, where 69% of cases showed no staining (20 out of 29 cases) [87]. Most of our SCCs showed diffuse p16 staining (97.2%), and one would expect that if these were hr-HPV related, that the E7 oncoprotein would result in the degradation of pRb. Diffuse p16 staining occurred regardless of carcinoma differentiation, however, even well and moderately differentiated carcinomas showed a mean proportion of pRb staining of above 50%. The decrease in the proportion of cells staining with pRb from well to poorly differentiated carcinoma, showed that the differentiation of a carcinoma was significantly associated with pRb expression.

5.6 The WNT signalling pathway

Similar to what has been found in HNSCC, E-cadherin expression is decreased in poorly differentiated squamous cell carcinomas [65]. It must be noted, however, that E-cadherin expression in the cited study was evaluated with FISH. Only one basaloid squamous carcinoma in our study showed absence of E-cadherin staining. Most poorly differentiated squamous cell carcinomas in our study demonstrated 1+ staining, which implies a loss of expression with decreasing differentiation.

Behrendt *et al*. published a study in 2001 on the expression of p53, cytokeratins and pan-cadherin in tumours of the anal canal compared to those of the anal margin [39]. They found that pan-cadherin expression was significantly lower in tumours from the anal canal compared to those from the anal margin. Our study was retrospective in nature and the precise anatomic locations of tumours were not recorded.

In 1995 Vessey *et al*. published a study on altered E-cadherin expression in cervical intraepithelial neoplasia (CIN) and invasive cervical squamous cell carcinoma [94]. There was a significant correlation between the presence and localisation of E-cadherin with the grade of CIN. Higher grades of CIN demonstrated an increase in cytoplasmic and decrease of membranous staining. In our study, there was no significant difference in E-cadherin expression in normal squamous epithelium compared to dysplastic epithelium. However, there was a significant difference in the E-cadherin expression of both normal and dysplastic epithelia compared to invasive squamous cell carcinoma, suggesting that loss of E-cadherin plays a role in progression of squamous cell carcinoma [65].

The study by Vessey *et al*. found that there was a decrease in E-cadherin membrane staining with worsening differentiation of the invasive carcinoma [94]. Our findings also showed that differentiation of the carcinoma influenced membranous E-cadherin expression.

Due to uncertainty of the exact number of carcinoma cases related to hr-HPV infection in our study, it is not possible to accurately determine what effect hr-HPV infection has on the WNT signalling pathway. If it is assumed that all p16-positive cases were infected by hr-HPV, there does not seem to be any correlation between hr-HPV infection and loss of E-cadherin or β-catenin nuclear translocation in our study.

This finding is surprising, as nuclear translocation of β-catenin by hr-HPV oncogenes E6 and E7 has been described in oropharyngeal squamous cell carcinoma [42]. This is either due to a difference in the tumour biology of oropharyngeal compared to anal SCC, or a fewer number of our carcinoma cases are actually related to hr-HPV infection. Future detection of hr-HPV DNA in our carcinoma cases through PCR or in situ hybridisation methods may be helpful in clarifying this issue.

A study by Myklebust *et al*. found β-catenin and plakoglobin immunoexpression to be valuable prognostic markers in anal carcinoma [12]. They noted that carcinomas with low membranous β-catenin and high membranous plakoglobin expression have a poor prognosis. These authors also did not find any nuclear staining of β-catenin in their carcinoma cases (0 out of 56 case).

5.7 CD1a and Langerin

There was an increase in the mean density of CD1a-positive and Langerin-positive cells in HIV-positive patients, compared to HIV-negative patients. The mean density of CD1a-positive and Langerin-positive cells were also higher in normal squamous epithelium than in invasive carcinoma. Mean CD1a-positive cell density in dysplastic lesions in HIV-positive versus HIV-negative was almost identical, but mean Langerin-positive cell density in dysplastic lesions was slightly higher in HIV-positive compared to HIV-negative patients. HIV-negative patients showed a lower mean number of CD1a-positive and Langerin-positive cells in dysplastic tissue compared to normal squamous epithelium. Unfortunately, HIV-status or the type of tissue did not appear to be statistically significant factors influencing the mean number of positive cells.

Langerin and CD1a staining cells were counted manually and the distribution was variable on different tissue sections. This could account for the discrepancy between the number of Langerhans cells when counting Langerin and CD1a staining cells, respectively.

These findings are contrary to what previous studies have found when investigating the effect of HIV infection on the number of anal intraepithelial LCs [67, 71]. These studies enrolled HIV-positive patients, most of whom were on antiretroviral therapy, and found decreased numbers of intraepithelial LCs in HIV-positive compared to HIV-negative patients. Information on antiretroviral treatment in our study was not readily available. The unknown HIV status in a large number of cases in our study has affected the overall results.

5.8 Bcl-2

There was no significant association between squamous cell carcinoma differentiation or subtype and Bcl-2 expression. This is in contrast to a previous study that found Bcl-2 expression higher in non-keratinising squamous cell carcinoma [38]. Our study, however, revealed that there was a significant increase in Bcl-2 expression in carcinoma cells compared to normal squamous epithelium.

This information may be useful with regards to prognosis of these tumours. Tumours which are positive for Bcl-2 by IHC may respond better to radio- and chemotherapy than tumours which are Bcl-2-negative [95]. This finding could be explained by overexpression of Bcl-2 increasing the half-life of the pro-apoptotic protein Bax and that Bcl-2 has been found to act as an antiangiogenesis factor in non-small cell lung cancer [96, 97]. Various anti-Bcl-2 drugs, which have been found to be useful in treatment of lymphomas and solid tumours (small cell lung cancer), are currently in pharmacological trial phases [10, 11].

Information regarding Bcl-2 staining patterns of carcinomas (including anal squamous cell carcinomas) may become critical for oncological treatment of cancer patients.

5.9 Ki-67

The proliferation index of dysplastic lesions varied widely. Positive-staining cells were seen within the upper third of mildly dysplastic squamous epithelium (AIN I) in addition to the lower two thirds. Normal squamous epithelium similarly showed a wide variation of proliferation index, but positive staining cells were confined the basal regions. A study by Bean *et al*. found a significant correlation between the proportion of Ki-67-positive cells and the level of surface epithelial staining and degree of dysplasia [98]. However, there was no clearly identifiable way of using Ki-67 staining in our study to differentiate between different grades of dysplasia, confirming its qualitative rather than quantitative diagnostic utility by the LAST project and other studies [34, 35].

A study published in 2000 by Indinnimeo *et al*. found that the Ki-67 proliferation index correlated significantly with depth of invasion and lymph node metastasis in anal canal carcinomas. However, there was no correlation between the proliferation index and neoplastic relapse [99].

5.10 HPV

In our study, approximately one third of cases with dysplastic tissue stained with the antibody against the HPV L1 capsid protein, confirming the low sensitivity of HPV infection and intraepithelial neoplasia, previously demonstrated in cervical tissue [34, 37].

Only two squamous cell carcinomas were positive (weak staining). This is similar to a previous study that found no staining in anal SCCs [100]. These authors suggested that HPV nuclear staining is lost in the progression from intraepithelial neoplasia to

carcinoma, and that this may be prognostically helpful to identify intraepithelial neoplasia with an increased risk of malignant (invasive) potential. The findings of our study suggest a similar trend of losing HPV L1 capsid staining as the degree of dysplasia worsens. However, unlike these authors, we did not use molecular techniques to confirm the presence of HPV infection. This lessens the validity of our findings.

5.11 HPV vaccines and anal cancer

Vaccines protecting against HPV-16 and HPV-18 (Cervarix and Gardasil) have been proven in the PATRICIA trial to be very efficient in protecting against cervical intraepithelial neoplasia grade III (CIN III), invasive squamous cell carcinoma and adenocarcinoma in situ (AIS) associated with HPV-16/18 (both 100% protection) [13]. Other studies have demonstrated effective implementation of HPV vaccination amongst young school girls [101] and that it would be cost-effective in South Africa to introduce HPV vaccination to the current cervical screening programme [102].

A trial to determine the efficacy of the quadrivalent HPV vaccine (Gardasil) to prevent anal HPV infection and AIN amongst young MSM (16 to 26 years of age), found it to be 77.5% effective in preventing AIN associated with HPV-6, HPV-11, HPV-16 and HPV-18 [14]. This may very well cause a significant reduction in the incidence of anal squamous cell carcinoma.

The Centers for Disease Control and Prevention (CDC) currently recommends that all children aged 11 or 12 years should receive a 3-dose HPV vaccination series to protect against HPV. Women and MSM are recommended to receive the vaccination up to age 26 years and MSW up to 21 years of age [103].

The South African National Department of Health (DOH) implemented a programme in 2014 where the Cervarix vaccine is offered to all girls in grade 4 in public schools. It may be wise to consider including young boys in these programmes to try and ensure herd immunity amongst young people. Not only will this protect women against

developing CIN and cervical SCC, but also men and women against AIN and anal carcinoma.

5.12 Limitations of the study

Fifty-one cases were selected for the study, but there were 89 cases in total that were identified as either squamous dysplasia and/or carcinoma. Unfortunately, 38 of these cases were not suitable due to the amount of residual tissue in the paraffin wax blocks. Many cases were small biopsies and it was foreseen that there would not be enough tissue to perform all immunohistochemical stains. The exclusion of these cases may have altered the epidemiological information gathered in this study.

Only three adenocarcinomas and one adenosquamous carcinoma were selected. Information on the DISA laboratory information system and morphological analysis was used to confirm origin from the anal canal. Immunohistochemistry was not employed to try and distinguish anal canal adenocarcinoma (with a colorectal phenotype: CK7- and CK20+) from those originating from the anal glands (CK7+ and CK20-) [5]. In any case, too few cases were available to draw any significant conclusions.

Many cases did not have representation of all three tissue types (normal squamous epithelium, dysplastic squamous epithelium and invasive carcinoma) available in a single section. This led to “missing data” as described under statistical analysis. Cutting deeper into wax blocks for immunohistochemical staining, also resulted in tissue type being cut away and limiting the evaluation of certain stains. These factors limited the number of values for certain variables, and most likely limited the statistical analysis (e.g. HIV status playing a role in LC density in anal epithelium).

A difficulty that was recognised during scoring of immunohistochemical stains, was that anal squamous epithelium contains melanocytes and that marked melanin pigmentation and incontinence were noted. The immunohistochemical chromogen

used was DAB which gives a brown signal. Distinction of true staining from background melanin pigment posed some difficulty. In retrospect, using a red chromogen may have alleviated difficulties in scoring certain immunohistochemical stains.

The HIV status of the majority of patients was not known. The study aimed to determine the role of HIV infection in pre-cancerous conditions and invasive carcinoma of the anus. HIV status was known in 30 patients, which was insufficient to draw statistically significant conclusions.

There was limited follow up and clinical information available. This restricted the clinicopathological analysis.

CHAPTER 6

CONCLUSION

Fifty-one cases from different patients were selected for the study, and the majority of invasive carcinomas were squamous cell carcinomas. There was a wide age distribution amongst cases and there were slightly more female patients than males. HIV status was not known in approximately 40% of cases; this was a major limitation in the study.

p24 staining was not helpful in determining HIV status in cases where this was not known. Less than 20% of HIV-positive cases showed positive staining. p24 does not appear to be a useful stain to determine HIV status in non-lymphoid tissues.

Almost all squamous cell carcinomas showed block positive p16 staining. p16 is known to be a surrogate marker for hr-HPV infection, and this suggests the majority of squamous cell carcinomas were associated with hr-HPV infection. This project can possibly be expanded by identifying hr-HPV DNA in tissues with PCR techniques. This will enable us to hopefully confirm the good correlation between p16 positivity and hr-HPV infection.

Staining of pRb was found to be significantly correlated with differentiation of squamous cell carcinomas; there was a decrease in the proportion of pRb-positive tumour cells from well to poorly differentiated squamous cell carcinoma.

E-cadherin staining was significantly decreased in cells of squamous cell carcinoma tissue compared to cells of normal and dysplastic tissue. There was also a significant correlation of E-cadherin expression with the differentiation of a squamous cell carcinoma.

There was no abnormal (nuclear) β-catenin localisation in any of the anal carcinomas.

The mean density of CD1a- and Langerin-positive cells was increased in HIV-positive patients, compared to HIV-negative patients.

There were significantly more Bcl-2-positive cells in squamous cell carcinoma than in normal squamous epithelium. In the future, treatment of anal carcinoma with anti-Bcl-2 drugs may become a reality. Information on the expression of Bcl-2 in tumours will be vital for oncologists who plan to use these drugs in their treatment regimen.

HPV L1 capsid IHC had a low sensitivity of detecting HPV infection, anal intraepithelial neoplasia and invasive squamous cell carcinoma of the anus. This finding is similar to that of previous studies that have been done and confirms the limited use of this antibody to detect HPV infection of tissues [34, 37].

Hr-HPV vaccines have been proven to reduce the risk of cervical precursor lesions and invasive carcinoma [13]. The CDC recommends preteen girls and boys to be vaccinated against the virus [103]. Seeing as the vast majority of anal squamous cell carcinomas are associated with hr-HPV infection, studies on anal carcinoma in South Africa such as ours may help pave the way for the DOH to include these vaccinations into the expanded programme on immunisation (EPI).

The usefulness of p16 immunohistochemical staining in hr-HPV-associated lesions is well established. This study has found that other markers may be potentially useful in the diagnosis of hr-HPV related anal lesions.

In conclusion, this study revealed new information and knowledge on preinvasive lesions and carcinomas of the anal region. However, molecular investigation may provide further insights into the pathogenesis and pathology of these lesions.

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Case No	Age	Gender	HIV	Diagnosis	Differentiation	Subtype	AIN	Block	Tissue	P24		P16		pRB		E-Cadherin	CD3a		Langerin		BCL2		Ki-67	HPV		Beta-Catenin
										Intensity	Density	Intensity	Proportion	Intensity	Proportion		Intensity	Density	Intensity	Density	Intensity	Proportion		Intensity	Proportion	
1	66	F	Unknown	Adenocarcinoma	Moderate	No subtype		A1	Normal	0	0	0	0%	2	95%	3	2	12	2	4	0	0%	60%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	15%	3	100%	3	1	1	0	0	0					2
2	48	M	Negative	Adenocarcinoma	Moderate	No subtype		A1	Normal	0	0	0	0%	2	90%	3	3	17	3	6	0	0%	70%	0	0%	1
									Dysplastic																	
									Cancer	0	0	0	0%	3	100%	3	2	1	2	1	0	0%	40%	0	0%	0
3	43	F	Unknown	Squamous cell carcinoma	Poor	Basoloid		A1	Normal	0	0	0	0%	2	66%	2	2	2	3	1	1	0%	40%	0	0%	1
									Dysplastic			3	100%													
									Cancer	0	0	3	100%	1	60%	2	2		2		2	40%	45%	0	0%	2
4	68	F	Unknown	Squamous cell carcinoma	Moderate	Keratinising	AIN III	A3	Normal	0	0	0														
									Dysplastic	0	0	2	100%	3	50%	1	3	9	2	5	0	0%	50%	0	0%	2
									Cancer	0	0	2	50%	3	40%	1	3	9	2	5	0	0%	50%	0	0%	2
5	62	M	Negative	Squamous cell carcinoma	Moderate	Keratinising		A1	Normal	0	0	0	0%	2	70%	2	3	21	3	6	0	0%	40%	0	0%	0
									Dysplastic																	
									Cancer	0	0	2	100%	1	40%	2	3	11	3	3	0	0%	66%	0	0%	2
6	63	M	Negative	Squamous cell carcinoma	Moderate	Keratinising		A6	Normal	0	0			3	80%	2	3	10	1	3	0	0%	40%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	100%	2	30%	1	3	5	2	3	0	0%	60%	0	0%	2
7	67	F	Negative	Squamous cell carcinoma	Moderate	No subtype	AIN III	A1	Normal																	
									Dysplastic	0	0	2	70%													
									Cancer	0	0	3	100%	1	10%	1	3	3	3	3	0	0%	80%	1	5%	2
8	45	F	Unknown	Squamous cell carcinoma	Moderate	Keratinising		A9	Normal																	
									Dysplastic																	
									Cancer	0	0	3	100%	2	50%	1	2	10	3	10	2	5%	50%	0	0%	2
9	61	F	Unknown	Squamous cell carcinoma	Moderate	No subtype	AIN III	A19	Normal	0	0	0	0%	3	33%	2	3	4	1	1	0	0%	60%	0	0%	0
									Dysplastic	0	0	3	100%	2	25%	2	3	6	1	2	0	0%	60%	0	0%	0
									Cancer																	
									Normal																	
10	63	F	Unknown	Squamous cell carcinoma	Poor	Basoloid		A1	Dysplastic																	
									Cancer	0	0	2	90%	1	40%	3	3	17	2	4	0	0%	66%	0	0%	2
									Normal	0	0			2	66%	3	2	13	3	7	0	0%	80%			0
11	38	M	Unknown	Squamous cell carcinoma	Well	Keratinising		A1	Dysplastic			1	20%													
									Cancer	0	0	0	0%	3	90%	3	3	11	3	4	0	0%	80%	1	3%	0
									Normal	0	0	0	0%	3	80%	3	1	2	1	1	0	0%	60%	0	0%	0
12	73	F	Negative	Squamous cell carcinoma	Moderate	No subtype		A1	Dysplastic			2	80%													
									Cancer	0	0	3	100%	2	85%	3	2	3	1	0	0	0%	60%	0	0%	1
									Normal	0	0	2	60%	2	70%	3	3	10	3	6	0	0%	40%	0	0%	0
13	71	F	Unknown	Adenocarcinoma	Moderate	No subtype		A1	Dysplastic																	
									Cancer	0	0	0	0%	3	95%	3	2	0	1	0	1	3%	85%	0	0%	3
									Normal	0	0	0	0%	2	66%	3	3	7	1	3	0	0%	20%			2
14	58	M	Negative	Adenosquamous carcinoma	Moderate	No subtype		B1	Dysplastic	0	0	2	50%											1	10%	
									Cancer	0	0	2	66%	2	33%	1	0	0	0	0	1	5%	70%	0	0%	2
									Normal	0	0	0	0%	3	90%	2	2	5	1	2	0	0%	5%	0	0%	0
15	50	M	Negative	Squamous cell carcinoma	Poor	Basoloid	AIN II	B1	Dysplastic	0	0	2	80%	2	50%	3	2	5	1	2	0	0%	55%	3	1%	1
									Cancer	0	0	3	100%	1	25%	0	1	1	1	1	0	0%	60%	0	0%	2
									Normal																	
16	75	F	Unknown	Squamous cell carcinoma	Poor	Basoloid		A1	Dysplastic																	
									Cancer	0	0	2	50%	0	0%	1	1	1	0	0	0	0%	85%	0	0%	1
									Normal	0	0	0	0%	2	75%	2	2	10	2	8	0	0%	33%	0	0%	0
17	45	M	Positive	Squamous cell carcinoma	Poor	Basoloid		A1	Dysplastic																	
									Cancer	0	0	3	100%	2	20%	1	1	6	1	3	0	0%	75%	0	0%	1
									Normal	0	0	0	0%	3	90%	3	1	4	3	5	0	0%	20%			0
18	64	F	Negative	Squamous cell carcinoma	Poor	No subtype		A1	Dysplastic			3	100%													
									Cancer	0	0	3	100%	1	10%	1	2	3	2	3	2	20%	60%	0	0%	1
									Normal																	
19	56	F	Unknown	No cancer			AIN III	A1	Dysplastic	0	0	0	0%	3	95%	3	2	2	2	3	0	0%	70%	0	0%	0
									Cancer																	
									Normal																	
20	50	M	Negative	Squamous cell carcinoma	Moderate	No subtype	AIN III	A1	Dysplastic	0	0	3	100%	3	50%	2	3	11	2	4	0	0%	50%	0	0%	0
									Cancer	0	0	3	100%			2	3	5	2	3	0	0%	50%	0	0%	0
									Normal	0	0	0	0%	3	80%	3	3	22	2	11	0	0%	40%	0	0%	0
21	71	M	Negative	Squamous cell carcinoma	Poor	Basoloid	AIN III	A1	Dysplastic	0	0	3	100%	0	0%	2	1	6	1	5	0	0%	33%	0	0%	0
									Cancer	0	0	3	100%	0	0%	1	2	5	1	4	2	5%	33%	0	0%	1
									Normal																	
22	51	F	Unknown	No cancer			AIN II	A1	Dysplastic	0	0	2	90%	2	75%	2	3	8	2	5	0	0%	60%	0	0%	0
									Cancer																	
									Normal	0	0	0	0%	3	80%	3	3	12	3	9	0	0%	50%	0	0%	0
23	49	F	Negative	Squamous cell carcinoma	Well	Keratinising		A1	Dysplastic																	
									Cancer	0	0	3	100%	2	50%	3	2	2	2	3	0	0%	70%	0	0%	0
									Normal																	
24	35	F	Positive	No cancer			AIN II	A1	Dysplastic	0	0	2	100%	2	60%	3	3	25	3	9	0	0%	50%	3	20%	0
									Cancer																	
									Normal																	
25	68	M	Negative	Squamous cell carcinoma	Moderate	No subtype		A16	Dysplastic																	
									Cancer	0	0	3	100%	2	40%	3	3	5	1	2	0	0%	80%	0	0%	0
									Normal																	
26	54	M	Unknown	Squamous cell carcinoma	Poor	Basoloid		A1	Dysplastic																	
									Cancer	0	0	3	100%	1	66%	3	2	2	3	2	1	8%	75%	2	5%	0

Appendix 1: All data collected in study

Case No	Age	Gender	HIV	Diagnosis	Differentiation	Subtype	AIN	Block	Tissue	P24		P16		pRB		E-Cadherin	CD1a		Langerin		BCL2		Ki-67	HPV		Beta-Catenin
										Intensity	Density	Intensity	Proportion	Intensity	Proportion		Intensity	Density	Intensity	Density	Intensity	Proportion		Intensity	Proportion	
27	81	F	Unknown	Squamous cell carcinoma	Poor	Basaloid		A1	Normal	0	0	0	0%		2 90%	3	3	32	2	11	0	0%	20%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	100%	1	20%	1	3	45	3	16	2	15%	40%	0	0%	1
28	57	F	Negative	Squamous cell carcinoma	Poor	Basaloid		A10	Normal	0	0	0	0%	3	90%	3	3	9	2	2	0	0%	15%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	100%	2	25%	2	3	38	3	15	3	100%	60%	0	0%	0
29	30	F	Positive	No cancer			AIN I	A1	Normal	0	0	0	0%	2	75%	2	3	29	3	11	0	0%	10%	0	0%	0
									Cancer	0	0	2	80%	2	80%	3	2	3	2	1	0	0%	50%	3	15%	0
30	53	M	Positive	Squamous cell carcinoma	Moderate	No subtype		C2	Dysplastic																	
									Cancer	0	0	2	100%	2	20%	3	2	4	3	6	2	75%	50%	0	0%	2
31	54	F	Unknown	Squamous cell carcinoma	Moderate	Keratinising		A1	Normal	0	0	0	0%	2	75%	3	3	7	2	1	0	0%	5%	0	0%	0
									Dysplastic																	
									Cancer	0	0	1	80%	1	66%	2	3	15	2	2	0	0%	50%	0	0%	1
32	53	F	Unknown	Squamous cell carcinoma	Moderate	Keratinising		A1	Normal	0	0	0	0%	2	75%	2	0	0	0	0	0	0%	25%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	100%	2	80%	3	2	2	1	1	1	3%	33%	0	0%	1
33	30	F	Positive	No cancer			AIN III	A1	Normal	0	0	2	100%	1	60%	3	2	15	2	7	0	0%	95%	0	0%	1
									Dysplastic																	
									Cancer	0	0	0	0%	3	80%	3	3	24	3	16	0	0%	60%	0	0%	0
34	47	M	Unknown	Squamous cell carcinoma	Poor	No subtype		A1	Normal	0	0	0	0%	3	80%	3	3	7	2	2	1	60%	80%	0	0%	0
									Dysplastic																	
									Cancer	1	2	3	100%	1	40%	3	3	7	3	4	0	0%	80%	0	0%	0
35	52	M	Negative	No cancer			AIN II	A1	Normal	0	0	0	0%	2	85%	3	3	7	3	4	0	0%	50%	0	0%	0
									Dysplastic	0	0	0	0%	2	80%	3	3	3	3	22	0	0%	50%	0	0%	0
									Cancer																	
36	37	F	Positive	Squamous cell carcinoma	Well	Keratinising		A1	Normal	0	0	0	0%	3	80%	3	3	26	3	17	0	0%	40%	0	0%	0
									Dysplastic																	
									Cancer	0	0	3	100%	3	80%	3	3	36	3	19	0	0%	75%	1	1%	1
									Normal																	
37	24	M	Unknown	No cancer			AIN I	A1	Dysplastic	0	0	2	50%	2	85%	3	3	32	3	20	0	0%	70%	3	5%	0
									Cancer																	
									Normal	3	2	0	0%	3	75%	2	2	6	2	3	0	0%	90%	0	0%	0
38	42	F	Positive	Squamous cell carcinoma	Moderate	Keratinising	AIN II	A1	Dysplastic	0	0	3	90%	3	65%	2	2	27	3	10	0	0%	75%	3	5%	1
									Cancer	3	2	3	100%	2	66%	2	2	31	3	16	0	0%	60%	0	0%	1
									Normal	0	0	0	0%	3	90%	3	3	29	3	17	0	0%	50%	0	0%	0
39	42	M	Negative	Squamous cell carcinoma	Moderate	No subtype		A6	Dysplastic																	
									Cancer	0	0	3	100%	2	85%	3	2	14	2	4	0	0%	85%	0	0%	0
									Normal	0	0	0	0%	2	85%	3	3	29	2	14	0	0%	80%	0	0%	0
40	?	?	Unknown	Squamous cell carcinoma	Well	No subtype		A1	Dysplastic																	
									Cancer	0	0	3	100%	2	66%	2	2	11	1	6	0	0%	85%	0	0%	1
									Normal																	
41	30	M	Positive	No cancer			AIN II	A2	Dysplastic	0	0	3	50%	3	85%	3	3	28	3	14	0	0%	66%	3	2%	0
									Cancer																	
									Normal	0	0	0	0%	2	75%	3	3	7	2	4	0	0%	20%	0	0%	0
42	30	M	Positive	No cancer			AIN I	B3	Dysplastic	0	0	2	40%	2	80%	3	3	13	3	12	0	0%	85%	3	5%	0
									Cancer																	
									Normal																	
43	57	M	Unknown	No cancer			AIN III	A1	Dysplastic	0	0	2	50%	3	85%	3	3	48	3	25	0	0%	50%	3	1%	0
									Cancer																	
									Normal																	
44	41	F	Positive	Squamous cell carcinoma	Moderate	No subtype	AIN III	A1	Dysplastic	0	0	2	100%	2	80%	3	2	26	3	17	0	0%	40%	0	0%	0
									Cancer	0	0	3	100%	2	70%	1	2	25	2	14	0	0%	50%	0	0%	1
									Normal																	
45	44	M	Unknown	Squamous cell carcinoma	Moderate	Keratinising		A5	Dysplastic																	
									Cancer	0	0	3	100%	2	10%	3	3	12	2	2	2	10%	60%	0	0%	0
									Normal																	
46	61	M	Negative	Squamous cell carcinoma	Well	No subtype	AIN III	A3	Dysplastic	0	0	2	70%	2	33%	3	3	30	3	10				0	0%	1
									Cancer	0	0	3	100%	2	33%	3	2	9	3	2	0	0%	75%	0	0%	1
									Normal	0	0	1	10%	2	75%	3	2	7	3	6	0	0%	50%	0	0%	0
47	56	F	Unknown	Squamous cell carcinoma	Moderate	No subtype		A1	Dysplastic																	
									Cancer	0	0	3	100%	2	80%	3	3	32	3	15	1	3%	80%	0	0%	0
									Normal	0	0	0	0%	2	80%	3	3	18	2	11	0	0%	10%	0	0%	0
48	63	M	Negative	No cancer			AIN III	A1	Dysplastic	0	0	3	100%	2	20%	3	2	11	2	4	2	33%	80%	0	0%	1
									Cancer																	
									Normal	0	0	0	0%	2	66%	3	3	26	3	12	0	0%	50%			0
49	50	F	Positive	Squamous cell carcinoma	Moderate	No subtype		A1	Dysplastic	1	3	0	0%	1	10%	3	2	27	2	13	1	50%	90%	3	1%	2
									Cancer	1	3	3	100%	1	10%	3	2	27	2	13	1	50%	90%			1
									Normal	0	0	0	0%	3	70%	3	3	32	2	24	0	0%	40%	0	0%	0
50	56	M	Negative	Squamous cell carcinoma	Moderate	Keratinising	AIN II	A1	Dysplastic	0	0	2	70%	2	60%	3	2	34	3	20	0	0%	40%	0	0%	0
									Cancer	0	0	3	100%	2	30%	3	2	40	3	30	0	0%	50%	0	0%	0
									Normal	0	0	0	0%	3	70%	3	3	44	3	19			80%	0	0%	0
51	40	M	Positive	Squamous cell carcinoma	Well	No subtype		A1	Dysplastic																	
									Cancer	0	0	3	100%	2	75%	3	3	39	3	25	0	0%	75%	0	0%	0

Appendix 1 (cont): All data collected in study