HUMAN IMMUNODEFICIENCY VIRUS/HUMAN PAPILLOMAVIRUS CO-INFECTION AND HOST MOLECULAR GENETICS OF CERVICAL CARCINOMA

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Preface and Declaration

All experimental works described in this thesis were carried out in the Division of Medical Virology and the Division of Human Genetics, University of Cape Town, and at the Laboratory for Tissue Immunology and the Haematology Laboratory, CD4 unit, National Health Laboratory Science (NHLS), Groote Schuur Hospital, Cape Town, South Africa, from June 2016 to August 2018, under the supervision of Dr. Eveline Kaambo, Dr. Gloudi Agenbag, Dr. Alltallents Murahwa, and the NHLS team.

I, **Ramadhani Salum Chambuso**, declare that this thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma at any University. Where use has been made of the work of others, it is duly acknowledged in the text and reference. Permission of use of figures and images from other peer-reviewed journals has been granted by the University of Cape Town, provided they are duly acknowledged in the text and reference.

Plan of This Thesis

This thesis is divided into six chapters. The first chapter provides a background of cervical cancer and the role of human immunodeficiency virus type 1 (HIV-1) and human papillomavirus (HPV) co-infection. Furthermore, the role of host genetic factors is elaborated. The second chapter (**Study I**) is a literature review for the host molecular genetic variations and HIV-1/HPV co-infection on cervical cancer progression.

However, chapters 3 to 5 which represent the actual research (Studies II, III and IV) are linked together and listed according to the specific objectives of this thesis. The subject recruitment involved 200 women histologically diagnosed with cervical disease and 200 controls.

Chapter 3 (**Study II**) represents the investigation of 181 patients for the characterization of HPV genotypes within cervical tumour biopsies, and assessment of the relationships with cervical disease stage, age, HIV-1 status, absolute CD4 count, and CD4 percentage, and further studying the predictive power of these variables for cervical disease stage in a cohort of South African women. In Chapter 4 (**Study III**), the association of Human Leukocyte Antigen class II (*HLA* II) -DRB1 and -DQB1 alleles, with cervical cancer, in HIV-1/HPV co-infected women is investigated by using 56 women histologically diagnosed with cervical disease and 200 controls, matched for age and ethnicity.

In Chapter 5 (**Study IV**), a total of 164 matched-DNA samples from cervical tumour biopsies and buccal swabs were investigated for chromosomal loss of heterozygosity (LOH) and microsatellite instability (MSI) at the *HLA* II locus in 74 HIV-1/HPV co-infected women, as well as in 90 HIV-1-seronegative women.

The final chapter is the general discussion, conclusions with the strengths, weaknesses and the future perspectives pertinent to this thesis.

ABSTRACT

A subgroup of women who are co-infected with human immunodeficiency virus type 1 (HIV-1) and human papillomavirus (HPV) progress relatively rapidly to cervical disease regardless of the number of absolute CD4 count. During infection, viral peptides are recognized by the host immune system. It is reasonable to propose that the development of viral-associated cancers, like cervical cancer, requires interference with specific immune-response genes. This thesis investigates this proposition with consideration of host molecular genetic alterations and variations of the human leukocyte antigen class II (*HLA* II) genes as one of the groups of immune-response genes that are involved in directing CD4 T-cell responses during infection, in the instance of cervical cancer progression in HIV-1/HPV co-infected women.

Study I, reviewed the available literature on host molecular genetics and HIV-1/HPV co-infection on cervical cancer progression. This study suggests that the dual pro-oncogenic effects of HPV oncoproteins E6/E7 and the HIV-1 oncoprotein Tat, may exacerbate and accelerate the rate of cervical disease progression in a subgroup of HIV-1-positive women. Additionally, HIV-1-positive cervical cancer has three important carcinogenesis steps: firstly, HPV integration into the host genome, secondly, dual pro-oncogenic effects of HPV oncoproteins E6/E7, and the HIV-1 Tat oncoprotein in the host genome and, thirdly, the accumulation of repeated, unrepaired genetic mutations and genetic alterations within the host chromosomal DNA. Genetic variations or mutations that affect the following host gene categories were suggested to be responsible for cervical cancer susceptibility and disease progression; (i) genes for the immune-response against oncogenic HPV infection, (ii) oncogenes, (iii) tumour-suppressor genes, (iv) apoptosis-related genes, (v) DNA damage-repair genes, and (vi) cell cycle-regulatory genes.

However, studies II, III and IV are linked together and listed according to the specific objectives of this thesis. **Study II**, characterized the distribution of HPV genotypes within cervical tumour biopsies from a cohort of 181 HPV-unvaccinated South African women and studied the relationships with HIV-1 infection, age of patients, absolute CD4 count, CD4 percentage and the stage of cervical disease, and identified the predictive power of these

variables for cervical disease stage. Distribution of HPV genotypes was related to the stage of cervical disease in HIV-1-positive women. Older age was a significant predictor for invasive cervical cancer (ICC) in both HIV-1-seronegative ($p<0.0001$, $q<0.0001$) and HIV-1-positive women ($p=0.0003$, $q=0.0003$). Sixty-eight percent (59/87) of HIV-1-positive women with different stages of cervical disease presented with CD4 percentage below or equal to 28 and a median absolute CD4 count of 400 cells/µl (IQR 300-500 cells/µl). Of the HIV-1-positive women, 75% (30/40) with ICC, possessed ≤28% CD4 cells versus 25% (10/40) who possessed >28% CD4 cells (both $p<0.001$, $q<0.001$). Furthermore, 70% (28/40) of women with ICC possessed absolute CD4 count >350 cells/µl compared to 30% (12/40) who possessed absolute CD4 count ≤ 350 cells/µl (both $p<0.001$, $q<0.001$).

Study III, was the first case-control study to investigate the association of HIV-1/HPV co-infection with specific host *HLA* II-DRB1 and -DQB1 alleles in cervical cancer. Two hundred and fifty-six (256) women of the same ethnicity were recruited, comprising 56 cases and 200 age-matched controls. A total of 624 *HLA*-DRB1 and -DQB1 class II genotypes were studied. *HLA* II-DQB1*03:01 and -DQB1*06:02 alleles were associated with cervical cancer in HIV-1/HPV co-infected women ($p=0.001$ and $p<0.0001$, respectively) while *HLA* II-DRB1*13:01 and -DQB1*03:19 were rare or absent in women with cervical disease when compared to the control population ($p=0.012$ and 0.011, respectively).

Study IV, aimed to investigate the host genetic alterations that may be involved in rapid tumour progression in HIV-1/HPV co-infected women. The frequency of loss of heterozygosity (LOH) and microsatellite instability (MSI) at the *HLA* II locus on chromosome 6p was analysed in cervical tumour biopsy DNA, with regard to HIV-1/HPV co-infection in 164 women. Seventy-four women were HIV-1-positive and ninety women were HIV-1-seronegative. Tumour DNA from HIV-1/HPV co-infected women demonstrated a higher frequency of LOH/MSI at the *HLA* II locus at 6p21.21 than tumour DNA from HIV-1-seronegative women (D6S2447, 74.2% versus 42.6%; $p=0.001$, $q=0.003$), D6S2881 at 6p21.31 (78.3% versus 42.9%; $p=0.002$, $q=0.004$), D6S1666 at 6p21.32 (79% versus 57.1%; $p=0.035$, $q=0.052$), and D6S2746, at 6p21.33 (64.3% versus 29.4%; $p<0.001$, $q<0.001$), respectively.

This thesis provides novel insights and adds to the existing knowledge on the relationships between HIV-1/HPV co-infection, CD4 immune status, host *HLA* II allele variations and genetic alterations at chromosome 6p in association or likely protection to cervical disease in the studied cohort of South African women. Identification of host molecular genetic susceptibility to disease with regard to viral infection is important for individualized molecular targeted prevention of cervical cancer.

PLAGIARISM DECLARATION

"This thesis/dissertation has been submitted to the Turnitin module (similarity and originality checking software) and I confirm that my supervisor has seen my report and any concerns revealed by such have been resolved with my supervisor."

"I further confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include publications in my thesis, and where co-authorships are involved, my co-authors have agreed that I may include the publications"

Name of student: Ramadhani Salum Chambuso

Student number: CHMRAM001

Signature: Signed by candidate

Date: August, 2019.

DEDICATION

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……

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their study Roman numerals.

Ethical approval: All procedures performed in this PhD study involving human participants were in accordance with the ethical standards of the institutional and/or national research committees, and with The Declaration of Helsinki (1964) and its later amendments or comparable ethical standards.

The publication strategy statement: All four (4) studies were published and listed below from the basic science (literature review) to the actual research according to the PhD study specific objectives.

Study I

Chambuso R, Gray CM, Kaambo E, Rebello G and Ramesar R (**2018**). Impact of Host Molecular Genetic Variations and HIV-1/HPV Co-infection on Cervical Cancer Progression: A Systematic review. **Oncomedicine**. 2018; 3:82-93. **doi: 10.7150/oncm.25573**.

Study II

Chambuso R, Ramesar R, Kaambo E, A. Murahwa, Mohammed A, De Sousa M, Denny L, Williamson A-L, Gray CM (**2020**). Age, Absolute CD4 count and CD4 Percentage in Relation to the HPV infection and the Stage of Cervical Disease in HIV-1-Positive Women. Original research. **Medicine**, 2020; Vol: No (e19273).**dx.doi.org/10.1097/MD-D-19-04693**.

Study III

Chambuso R, Ramesar R , Kaambo E, Denny L, Passmore J-A, Williamson A-L, Gray CM (**2019**). Human Leukocyte Antigen (*HLA*) class II -DRB1 and -DQB1 alleles Association between HIV-1/HPV Co-infection and Cervical Disease in South African Women. Original research. **Journal of Cancer**, 10(10):2145-2152. **doi:10.7150/jca.25600**.

Study IV

Chambuso R, Kaambo E, Denny L, Gray CM, Williamson A-L, Migdalska-sek M, Agenbag G, Rebello G, and Ramesar R (**2019**). Investigation of Cervical Tumour Biopsies for Chromosomal Loss of Heterozygosity (LOH) and Microsatellite Instability (MSI) at the *HLA* II Locus in HIV-1/HPV Co-infected Women. Original research. **Frontiers in Oncology**; **doi.org/10.3389/fonc.2019.00951**.

Reprints of published articles were made with permission from the respective publishers.

Compliance with Ethical Standards;

The author contributions statement:

R.C led A.W, C.G and R.R of the presented idea and developed the theory. L.D and others collected the specimens. E.K, G.A and A.M contributed in the laboratory works of the research. Together R.R, C.G, A.W, L.D and J.P encouraged R.C to investigate further, to carry out specific experiments. R.R, C.G and A.W supervised the final results and findings of this work. R.C performed the computations and M.A verified the statistical analysis methods. R.C led all authors to discuss the results and the final preparations of the manuscripts.

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Informed consent: Informed consent was obtained from all individual participants included in this PhD study.

‘Opinions, findings and conclusions or recommendations expressed in all publications and in this thesis generated by the listed organizations supported research is that of the authors alone, and that the organizations accept no liability whatsoever in this regard’.

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ABBREVIATIONS

ADC	Adenocarcinoma
ART	Antiretroviral therapy
ASR	Age-standardised incidence rate
Bp	Base pairs
BV	Bacterial vaginosis
CA	Capsid protein
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD45	Cluster of differentiation 45
CD8	Cluster of differentiation 8
CE	Capillary electrophoresis
CGH	Comparative genomic hybridization
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma i*n situ*
CNAs	Copy number altered regions
Cn-LOH	Copy-neutral loss of heterozygosity
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
EBV	Epstein bar virus
EDTA	Ethylenediaminetetraacetic acid
FANC	Fanconi anemia complementation group
FCR	Flow count rate
FDR	False discovery rate
FISH	Fluorescence *in situ* hybridization
G1 phase	Gap one phase
GLOBOCAN	Global cancer incidence, mortality and prevalence
Gp	Glycoprotein
GWAS	Genome-wide association study
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HPV	Human papillomavirus
Hr	High-risk
HREC	Human research ethics committee

HSIL	High-grade squamous intraepithelial lesions
HSV-2	Herpes simplex virus 2
IARC	International agency for research on cancer
ICC	Invasive cervical cancer
IBM	International business machines corporation
L1	Major capsid protein
L2	Minor capsid protein
LD	Linkage disequilibrium
LEEP	Loop electrosurgical excision procedure
LOH	Loss of heterozygosity
Lr	Low-risk
LSIL	Low-grade squamous intraepithelial lesions
LTI	Laboratory for Tissue Immunology
MHC	Major histocompatibility complex
MSI	Microsatellite instability
MSI-H	Microsatellite instability high
MSI-L	Microsatellite instability low
MSS	Microsatellite stable
Ng	Nano gram
NGS	Next generation sequencing
NHLS	National health laboratory service
OC	Oral contraceptives
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
Pap	Papanicolaou
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Phr	Probable high risk
PI	Protease inhibitor
PIC	Polymorphism information content
PLG	PanLeucogate
PT	Predictive testing
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
S phase	Synthesis phase
SANCR	South African National Cancer Registry
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesions
SIVcpz	Simian immunodeficiency virus of chimpanzee

SIVgor	Simian immunodeficiency virus of gorilla
SNP	Single nucleotide polymorphisms
SPSS	Statistical package for social science
SSO	Sequence-specific oligo
STR	Short tandem repeats
TBE	Tris/borate/edta buffer
TE	Tris-edta
TSG	Tumour suppressor gene
URR	Upstream regulatory region
WHO	World health organisation
µl	Microliter

CHAPTER 1: Background of Cervical Cancer, the Role of HPV, and HIV-1 Co-infection and Host Molecular Genetics.

1.1 Introduction to cancer

Cancer is a genetic disease caused by physiologically uncontrolled cell growth and cell division [1]. It is a genetic disease not because it can be inherited, but because genetic changes must take place over a period of time for cancer to occur [2]. New uncontrolled growth of cells that is not under physiologic control is called neoplasia [3]. An abnormal mass of tissue is called a neoplasm or a tumour. A tumour that usually does not contain any liquid in it is called a solid tumour. A solid tumour can be benign or malignant. A malignant tumour is a synonym for cancer [3]. Benign solid tumours start by forming small abnormal cell lesions which are commonly called precancerous lesions. In some instances, a precancerous lesion, if not treated, may develop into a malignant tumour [4]. A malignant solid tumour penetrates the protective basement membrane to destroy and invade adjacent normal organs and may spread to distant organs. This spread of cancer cells to distant organs is called metastases [5]. In contrast, benign tumours remain contained within the protective layer of the basement membrane, and by definition cannot spread or invade adjacent organs.

Cancers can be classified according to the type of cells or tissue they originate from. Cancers originating in the epithelium and of relevance to the present study, are referred to as carcinomas. Carcinoma development involves three major steps: (i) dysplasia, (ii) carcinoma *in situ* (CIS), and (iii) invasive carcinoma. Dysplasia can be classified as low-grade or high-grade. In low-grade dysplasia, only a few atypical cell changes are seen but do not affect all cells. In high-grade dysplasia, multiple atypical changes are seen in the majority of cells, accompanied by abnormal cell growth [6]. Carcinoma *in situ* (CIS) (Latin meaning “in its place”) means that cancer cells have not invaded the surrounding tissue or penetrated the basement membrane. CIS has a higher risk of transforming into invasive carcinoma than dysplasia [7]. Invasive carcinoma if left untreated, spreads beyond the tissue in which it originates to adjacent and distant organs [8].

Cancer develops in a multi-step process whereby a normal functional cell undergoes one or several different genetic changes to evolve into a cancer cell. However, a single genetic mutation or alteration is generally not enough to change a normal cell into a cancer cell [9]. Usually, a number of independent repeated and physiologically uncorrected genetic changes or mutations are required for cells to be transformed into cancer cells [9] . The cancer cells are usually self-sufficient in growth factors, insensitive to growth-inhibiting signals, avoid body programmed cell death (apoptosis), by-pass the body's natural defence mechanisms and replicate excessively beyond their normal programmed limit [10].

This thesis focuses on a particular carcinoma in women, commonly known as cervical cancer, which is caused by a human papillomavirus (HPV) infection [11, 12]. Using a case-control study, and cross-sectional studies approach, this research sought to investigate how host molecular genetic alterations and variations of the Human Leukocyte Antigen class II genes (*HLA* II) influence cervical disease development with regard to HPV and human immunodeficiency virus type 1 (HIV-1) co-infection in a cohort of South African women.

1.2 Cervical cancer

In 1984, Professor Harald zur Hausen, a German Virologist, working at the German Cancer Research Centre, (Deutsches Krebsforschungszentrum, DKFZ) discovered that HPV is causative of cervical cancer [11] for which, he was awarded the Nobel Prize in Physiology or Medicine in 2008 [13]. Anatomically, the cervix refers to the lower part of the uterus or womb (see Figure 1.1 A). Any cancer that originates in the cervix is called cervical cancer, as shown in **Figure 1.1 B**.

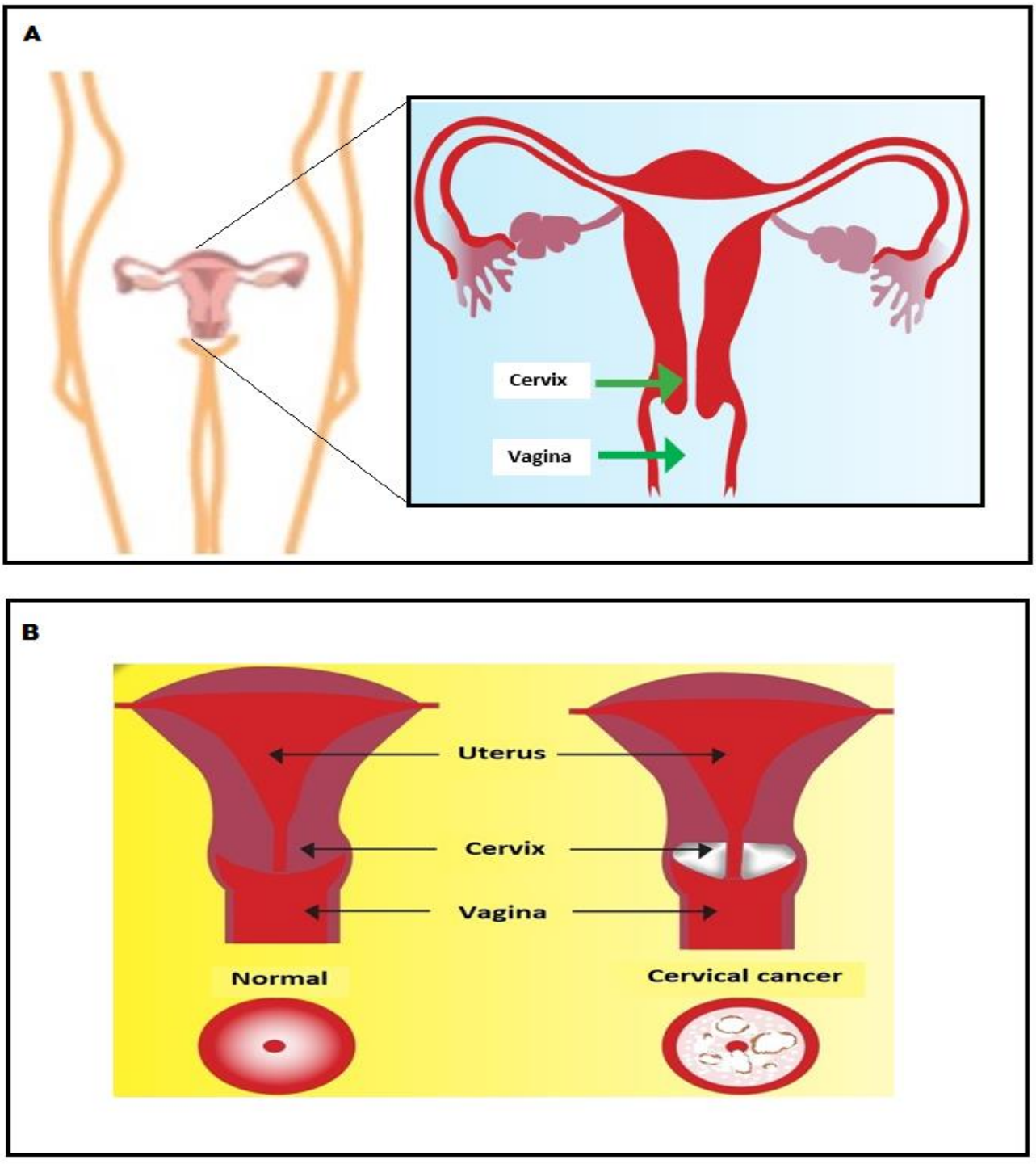

Figure 1. 1 Illustration of female reproductive organs.

(**A**) the location of cervix, relative to gross anatomy, and the uterus, (**B**) normal uterus, cervix, vagina and the localisation of cervical cancer (*in situ*).

The cervix consists of two types of epithelial cells, namely squamous and columnar epithelial cells. Squamous epithelial cells form part of the layers in the cervical epithelium. The columnar cells form the glandular epithelium of the cervix. The junction where these two layers of cervical epithelial cells meet is called the squamo-columnar junction or the transformation zone [14]. This is a junction/zone where columnar epithelium transforms into squamous epithelium during embryological cell differentiation. Dysplasia, the first stage of carcinoma development, starts at this junction/zone [14]. Cervical cancer can be divided into two major types according to its epithelial origin. One arises from cervical squamous cells and is called cervical squamous cell carcinoma (SCC). The other stems from the glandular epithelial cells and is called cervical adenocarcinoma (ADC) [14]. SCC is the most common type and accounts for more than 80% of all cervical cancer cases worldwide. ADC and the combination of the two types, called adeno-squamous cell carcinomas, represent 15-20%, where other rare types comprise less than 1% [15].

1.2.1 Epidemiology of cervical cancer

Cervical cancer is reported to be the fourth most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths among women worldwide [16]. , Globally, in the year 2018, there were an estimated 570,000 new cases of cervical cancer and about 90% of 311,000 affected women in low and middle income countries died from the disease [17]. Approximately 85% of all newly diagnosed cervical cancer cases and 87% of global cervical cancer deaths occur in the less developed countries [18] . Cervical cancer remains the most common cause of cancer-related morbidity and mortality in women in Sub-Saharan Africa. Internationally, the highest incidence rates of cervical cancer are reported in Sub Saharan Africa, India and China, whilst the lowest rates are in Australia/New Zealand and Western Asia, Europe and North America [19] (**Figure 1.2**). The global burden of cervical cancer is projected to increase to nearly 700,000 cases and 400,000 deaths in 2030 [17].

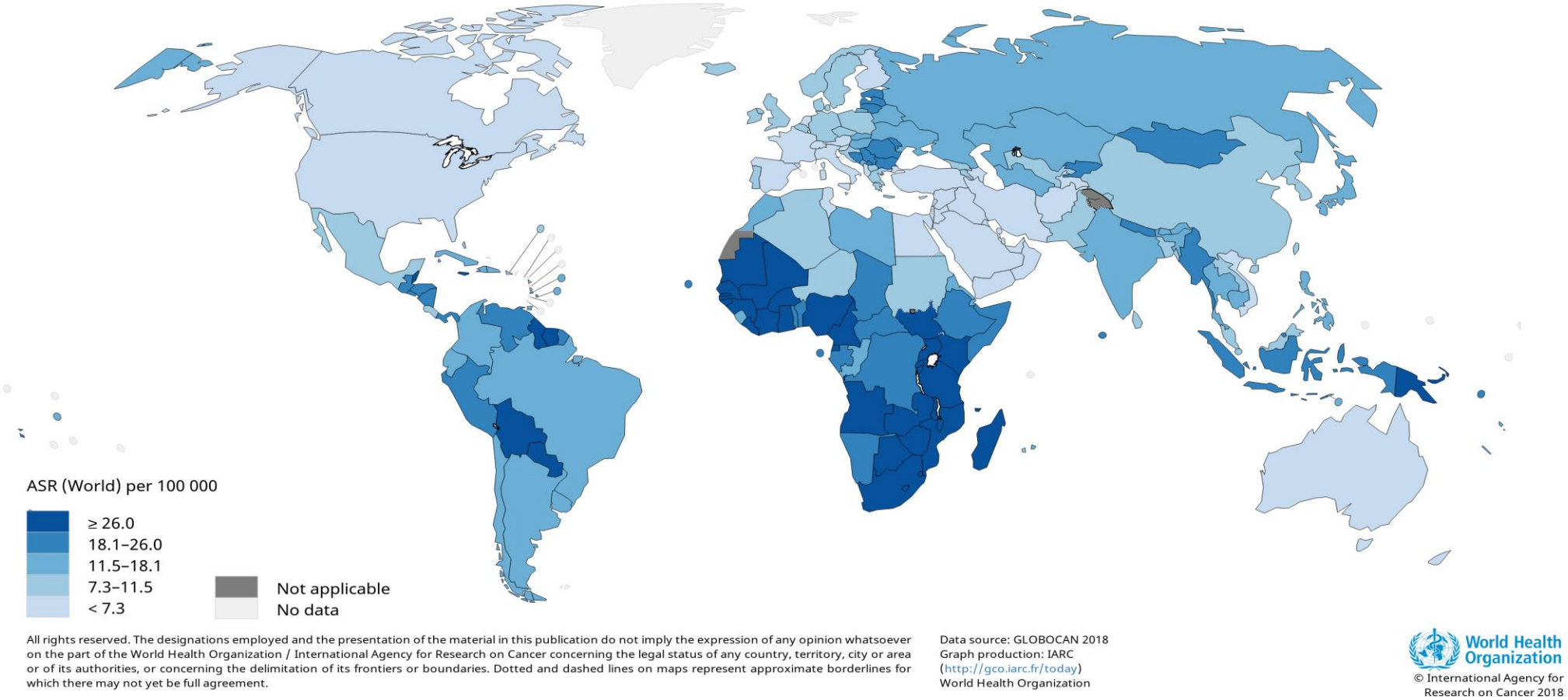

Figure 1. 2 Estimated age-standardised incidence rates (ASR) per 100,000 for cervical cancer all ages in the world.

(Figure from the International Agency for Research on Cancer website, GLOBOCAN, available at www.iarc.com, with copyright considerations as indicated on the website).

In South Africa, cervical cancer is the second most common cause of deaths due to cancer in overall mortality, and is the leading cause of cancer deaths among women, accounting for 17% of cancer deaths among women [20]. In a 16-year study period (1994–2009), 75,099 cervical cancer cases were reported, indicating that approximately one in forty South African women develops cervical cancer [21]. The average number of new cervical cancer cases per annum was reported to be 5,033 in 2011 and 5,735 in 2014 [22]. Despite increasing interventions such as cervical cancer screening programs, there has yet to be significant change in the overall epidemiology of the cervical cancer burden in South Africa. Cervical cancer rates continue to increase among young women, although there is variation of the disease burden among different ethnic groups or populations [21].

According to the South African National Cancer Registry (SANCR), there are rare reported cases of cervical cancer amongst teenage girls, but there is a marked increase in the incidence rate among women 25-29-year-old and which rises rapidly before peaking amongst 45-49-year-old women [23]. (**Figure 1.3**).

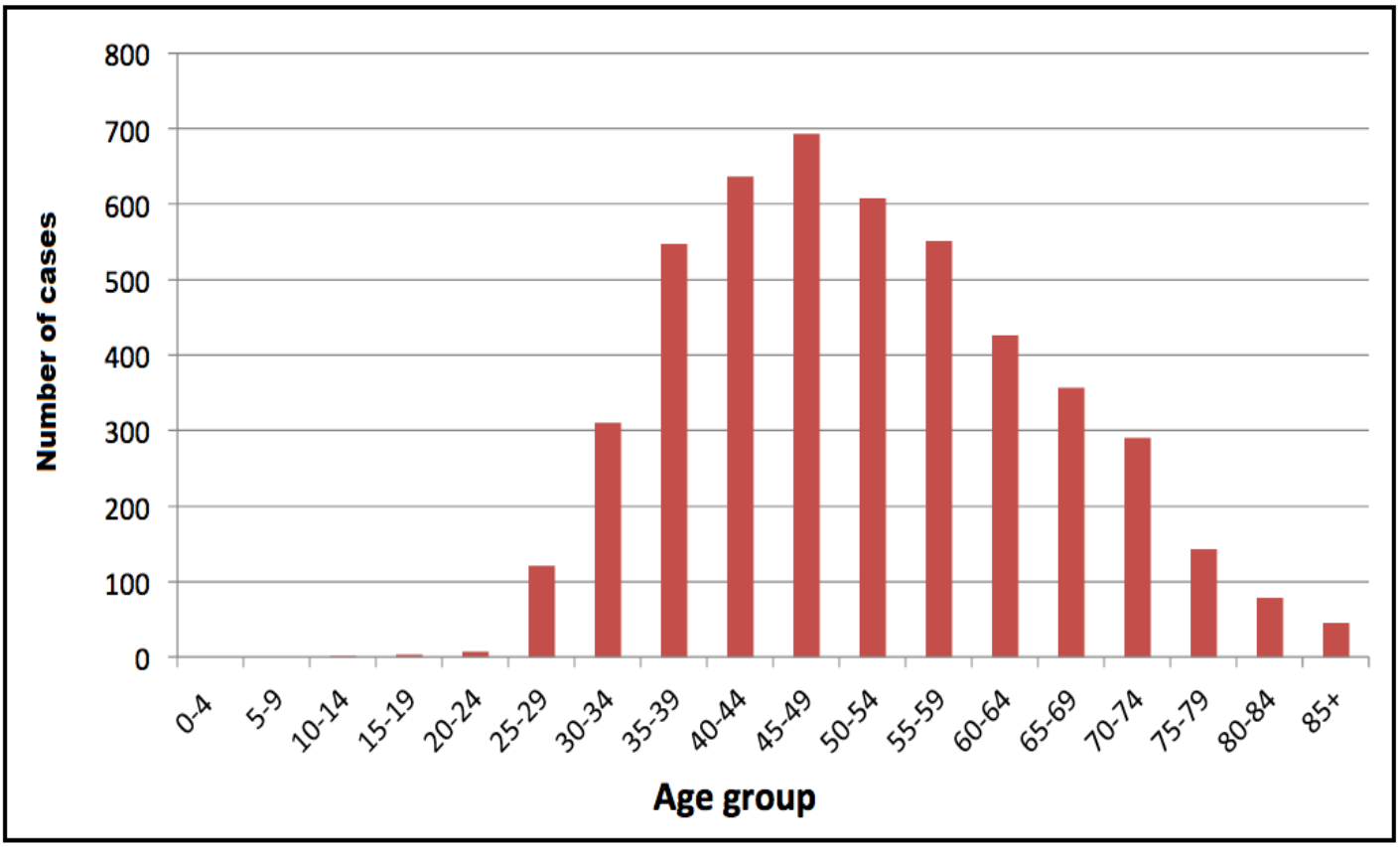

Figure 1. 3 Estimated number of cervical cancer cases according to age groups in the year 2011 in South Africa.

(Image from South African National Cancer Registry, 2011, with slight modification. National Health Laboratory Service with copyright considerations as indicated on the website www.ncr.ac.za).

1.2.2 Risk factors for cervical cancer

Anything which increases the chance of developing a certain disease is referred to as a risk factor for that disease. However, sometimes, other co-factors have to be present for the disease to occur. Briefly, genital HPV types have been subdivided into low-risk (Lr-HPV), which are found mainly in genital warts, and high-risk types (Hr-HPV), which are frequently associated with invasive cervical cancer [24] . Persistent genital infection with Hr-HPV is the major risk factor for cervical cancer [25]. Nonetheless, the majority i.e. 70-90%, of Hr-HPV infected women, will clear the infection naturally. Only a small fraction of HPV-infected women develop abnormal cervical lesions and, if these lesions remain untreated, progress into invasive cervical cancer [26]. However, the fact that only some Hr-HPV-infected women develop disease means that while HPV is a necessary risk factor, there must be other co-factors within the environment or within some women which contribute to the development of cancer [27].

Environmental risk factors have been associated with both HPV infection and cervical cancer. For example, the number of sexual partners is presumed to influence the risk of acquisition of multiple HPV infections: this is because the HPV infection is transmitted through sexual/genital contact [28]. Tobacco smoking is known to increase the risk of cervical squamous cell carcinoma. The risk increases with the number of cigarettes smoked per day and for the number of years that an individual has been a regular smoker [29].

Known internal risk factors include co-infections with other sexually transmitted infections or just vaginal infections such HIV-1, genital herpes simplex virus 2 (HSV-2), *Epstein-Barr* virus (EBV), *Chlamydia trachomatis*, *bacterial vaginosis* (BV) as well as early age sexual debut (before 18 years old) [28, 30]. In addition, there are other known risk factors which are combinations of internal and external conditions, such as parity (number of children), low socioeconomic status, poor genital hygiene, sex with uncircumcised men, and long term use of combined hormonal oral contraceptives (OC) [28].

There is also evidence that genetic inheritance is a significant risk factor for cervical cancer [31-33]. Individuals who have biological relatives who had cervical cancer, are twice as likely to develop cervical cancer compared to individuals who have no family history of the disease [34, 35]. Magnusson *et al.* [36], have shown that shared genes are more significant than shared familial environments in determining the probability of cervical cancer development. It mainly reflects the differences in host reactions to HPV exposure, infection acquisition, the persistence of the infection and response to viral oncoproteins E6/E7 [27].

In addition, genetic studies have identified associations between cervical cancer development and variations in human leukocyte antigen (*HLA*) class II genes [37-39]. The functions of the *HLA* are viral antigen presentation to the immune system and immune recognition of the viral peptides in order to initiate cell-mediated immunity [40]. *HLA* I (*HLA*-A, B and C) molecules present antigens to CD8 T cells and *HLA* II (*HLA*.DR, DQ and DP) present phagocytosed extracellular antigens to CD4 T cells. In other words, the immune system can only initiate an immune reaction to a virus if the *HLA* system presents the viral peptides to CD8 T or CD4 T cells. Furthermore, it has been suggested that many other immune response genes such as *TNF, TAP1,TAP279, IL-1080, CCR-281, IFN, KIR, FAS, STING, MICA, TP53, RB1, EX01, LAMP3* and *MYC* in different pathways, influence HPV persistence and cervical cancer development [27, 41-43]. **Figure 1.4** shows a diagram representing a variety of risk factors for the development of cervical cancer. This present study confines itself to consideration of the host molecular genetic factors, especially the genetic instability and variations of *HLA* II in determining the rapid transformation of HPV cervical lesions into invasive cervical carcinoma in HIV-1/HPV co-infected women.

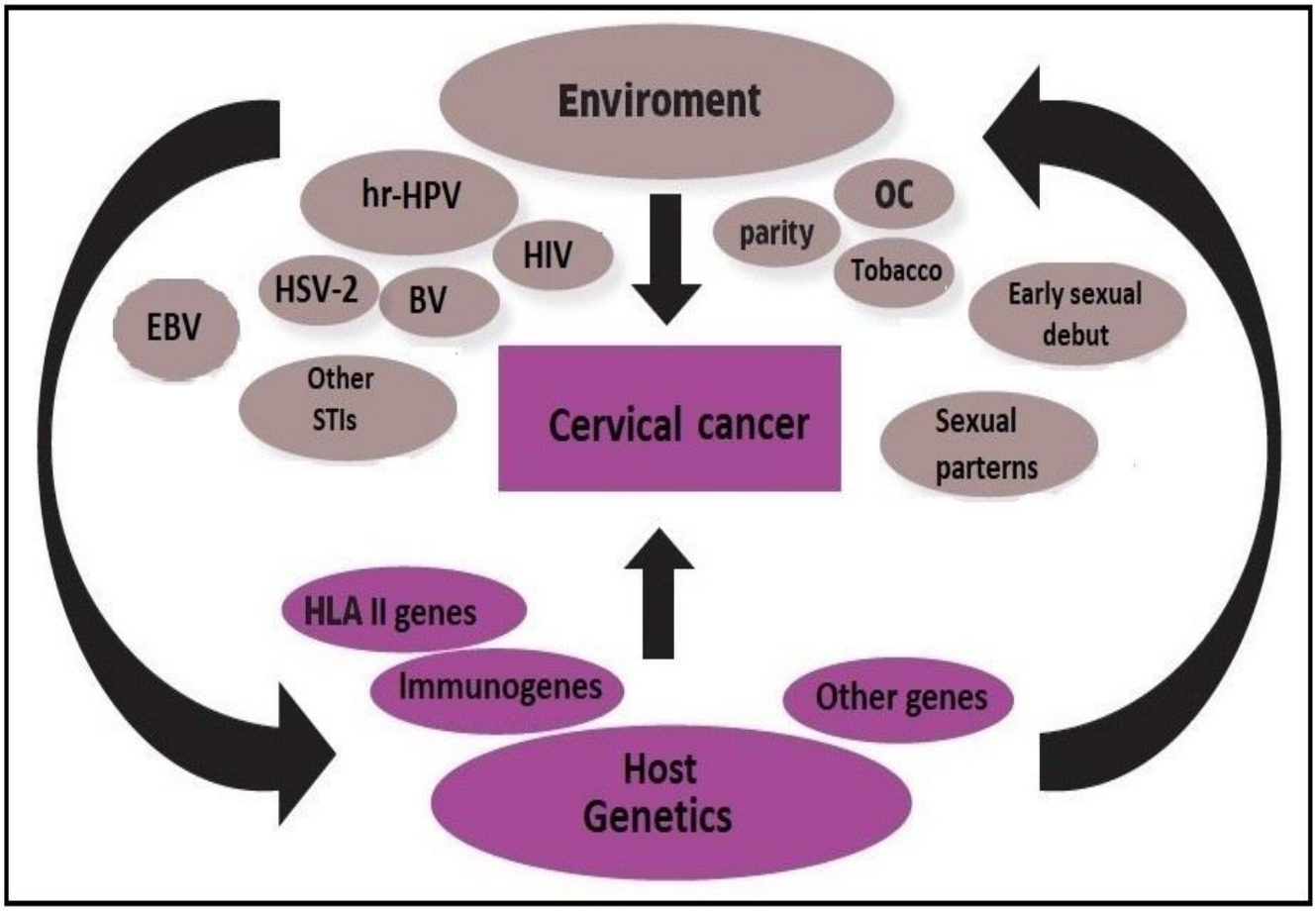

Figure 1. 4 Illustration of the interactions of various risk factors for cervical cancer development.

1.2.3 Human papillomavirus (HPV)

HPV is a double-stranded deoxyribose nucleic acid (DNA) virus which belongs to the family Papillomaviridae [11]. It has a circular genome which has approximately 55 nanometres in diameter, 8,000 base pairs in length. The viral particle is made up of 10% DNA and 90% protein. It has two structural proteins and cellular histones which condense the DNA in the virion [44]. The actual viral genome and the products it codes for is illustrated in **Figure 1.5**.

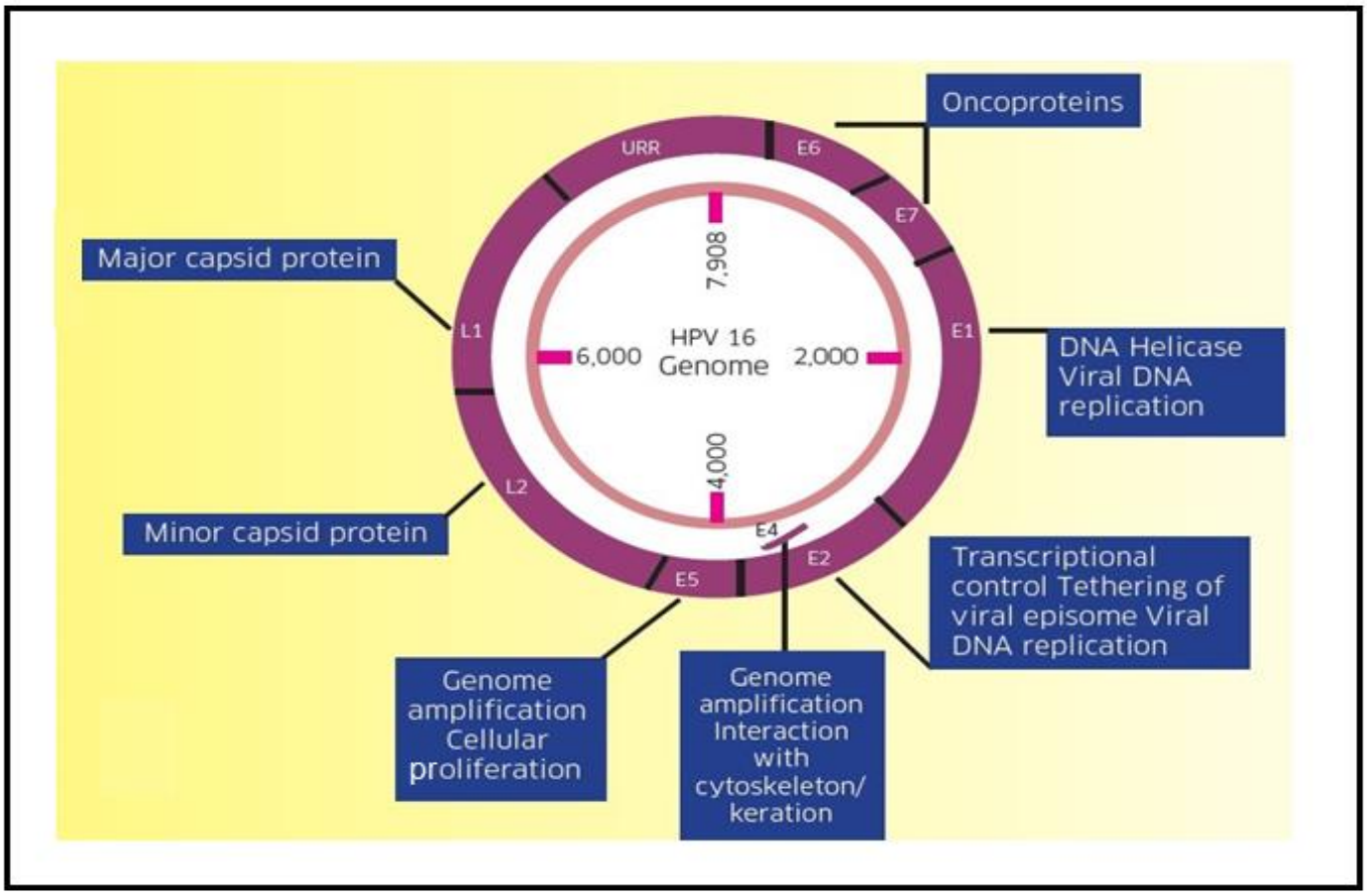

Figure 1. 5 A schematic diagram showing the human papillomavirus genome.

More than 200 HPV genotypes have been reported in humans and other mammals. About 30 to 40 HPV types can infect the anogenital area and the cervicovaginal mucosa of human beings either during sexual intercourse or just skin contact of the genitals [45]. These viruses are classified according to the molecular similarity/divergence of their genetic material and are assigned a genotype number [46]. Genital HPV types are categorised into three types:
(i) Low risk (Lr), (ii) probable high risk (PHr), and (iii) High-risk (Hr). Lr HPV genotypes include; HPV-6, -11, -40, -42, -54, -55, -61, -62, -64, -69, -71, -72, -81, -83, -89 (HPV-CP6108) and –IS39 [47]. HPV-6 and -11 mainly cause cutaneous and anogenital warts [48]. Lr-HPV is usually associated with benign cervical lesions [49]. PHr-HPV genotypes include; HPV-26, -53, -66, -67, -68, -70, -73 and -82. Hr-HPV genotypes include; HIV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59. The International Agency for Research on Cancer (IARC) confirmed that Hr-HPV genotypes are carcinogenic in humans [50]. Among them, HPV-16 and -18 are responsible for over 70% of all invasive cervical cancer cases worldwide [51] . Generally, HPV infects basal keratinocytes of the epidermis through the disruption of the skin or mucosal surface and it remains latent in low copy numbers in the cell as a circular episome [46].

Infections with multiple HPV genotypes or mixed HPV infections can occur and are associated with a high risk of developing cervical cancer, with poor survival [52]. HPV cannot be cultured in laboratories using cell culture. HPV DNA can be detected mainly by the following commonly-used laboratory tests for HPV screening; Cobas® HPV test (Roche Molecular Systems, Pleasanton, CA, USA), Linear Array® (Roche Molecular Systems, CA, USA) and Hybrid Capture® 2 assay (Qiagen Corporation, Gaithersburg, MD, USA) [53].

The HPV genome consists of 3 general regions; (i) an upstream regulatory region (URR) containing sequences that control viral transcription and replication, (ii) a second early region containing open reading frames (ORFs; e.g. E1, E2, E4, E5, E6 and E7) encoding proteins that are involved in multiple functions like trans–activation of transcription, transformation, replication, and viral adaption to different cellular environments, and (iii) a third region,

called the late region that codes for the L1 and L2 capsid proteins that form the structure of the virion and make the viral DNA packaging and maturation possible [54]. HPV requires the presence of proliferative epidermal and mucosal epithelial cells in order to successfully infect the host. Infection of the supra-basal layer and formation of the structural proteins is the next step, where the virus expresses the late genes by initiating replication of the circular viral genome. As the virus approaches the upper layers of the mucosa or epidermis, complete viral particles are assembled and released. The Hr-HPV oncoproteins, E6 and E7 are consistently overexpressed in cervical malignant tissues [54].

Although rare, there have been some cervical cancers reported to be HPV negative [55-57]. However, progressive loss of the HPV L1 region during the progression to cervical cancer has been associated with these findings. The polymerase chain reaction (PCR)-based HPV DNA tests detect the L1 region of the virus which could already be lost at the time of invasive cancer diagnosis [58]. HPV is regarded as a necessary but not sufficient cause of cervical cancer.

1.2.4 Human Immunodeficiency Virus type 1 (HIV-1) infection, absolute CD4 count and CD4 percentage

The sexual route is the main route for HIV-1 transmission. HIV-1 infection occurs through interaction with receptors and co-receptors present in the host cells. The primary entrance of HIV-1 into the host system is through CD4 cells. The major co-receptors on the CD4 cells include the chemokine receptors, CXCR4 and CCR5 [59].

HIV-1 infection is regarded as pathogenic in the host. The virus replicates and attacks host CD4 cells [60]. CD4 cells are types of blood cells that are part of the host immune system, they are a type of white blood cells (lymphocytes). CD4 cells are sometimes called T-helper cells or T-cells. An absolute CD4 cell count for an adult healthy person ranges from 800 to

1050 cells/µl [61]. Upon considering laboratory variations and standard variations, the normal CD4 count range within 500 to 1400 cells/µl, depending on the ethnic and geographical diversity [61, 62]. In HIV-1-positive women, CD4 count decreases over time and at counts below 200 cells/µl, patients become susceptible to opportunistic infections, including abnormal cervical lesions and cervical cancer [63].

In some women, immunosuppression (low absolute CD4 cell count) is associated with persistence of Hr-HPV types, growth of cervical neoplastic cells, as well as, the cervical disease progression [64]. However, another clinical considerable measure for host immune competence is called CD4 percentage of lymphocytes, which shows the relationship of CD4 cell count in consideration with all white blood cell count and lymphocyte differential in the body (including CD45) [65, 66]. In South Africa, the normal range for CD4 percentage of lymphocytes for females is suggested to fall between 31.6-58% [62]. Sometimes a single CD4 count is unexpectedly high or low, depends on the time of the measurements. In this situation, the CD4 percentage of lymphocytes is regarded as a more stable immune marker than the CD4 count [67]. In some instances, absolute CD4 count do not always correlate with CD4 percentage. The discordance between absolute CD4 count and CD4 percentage suggests that other lymphocytes apart from CD4 cells, play a role in host immunity [65, 66]. HIV-1 may incorporate different host proteins such as *HLA* I and II proteins into its lipoprotein layer during the process of budding [68]. Although they are not the primary targets of HIV-1 invasion, epithelial cells of the female genital tract are found to be permissive to HIV-1 infection, *in vitro* [69].

The HIV-1 genome consists of two identical single-stranded RNA molecules that are enclosed within the core of the virus particle [70]. In addition to the structural proteins, the HIV-1 genome codes for several regulatory proteins: trans activator protein and RNA splicing-regulator are necessary for the initiation of HIV-1 replication, while the other regulatory proteins: negative regulating factor, viral infectivity factor, and, virus proteins have an impact on viral replication, virus budding, and pathogenesis [70].

The HIV-1 genome is packaged in an unusual conical core particle at the centre of the infectious virion [71]. The HIV-1 core is composed of a complex of genomic RNA and the nucleocapsid protein surrounded by a shell of the capsid protein (CA) [71] (see **Figure 1.6**). The oncogenic potential of HIV-1 involves Tat proteins, which are early nonstructural proteins necessary for viral replication [72]. The oncogenic activity of HIV-1 Tat proteins includes degradation of host cellular genes, including tumour-suppressor genes which keep cell growth in check (But, which, if muted could promote cell proliferation and carcinogenesis [73].

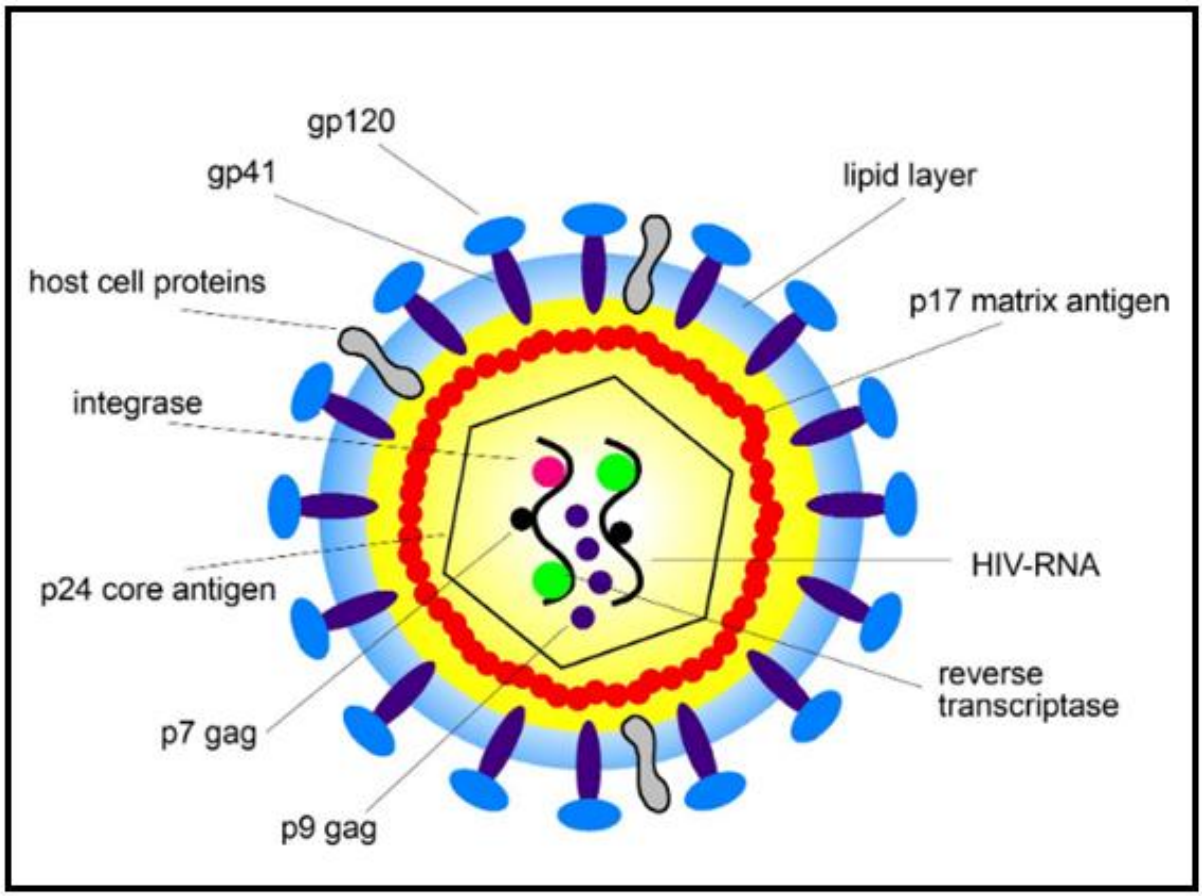

Figure 1. 6 A diagram showing the structure of an HIV-1 virion particle (Picture from Rebbert *et al.*, (2011) [60]).

1.2.5 Oncogenes and tumour-suppressor genes

Cancers can be hereditary or sporadic. 'Hereditary' refers to a cancer predisposition that is passed from a parent to a child through inheritance of a key defective gene, i.e. where the mutation is in the germ-line. 'Sporadic', refers to a cancer that is also the result of a genetic change, but which occurs in somatic tissues; this can occur by chance without any implication for the inheritance of the mutated gene. About 80% of all cancers are sporadic [74]. In sporadic cancers, there are three categories of genes that undergo repeated genetic alterations or mutations, namely: oncogenes, tumour suppressor genes and DNA damage-repair genes [74]. Oncogenes are genes that have the potential to cause cancer. These genes control cell proliferation or apoptosis, or both. Oncogenes can be affected by chromosomal rearrangements, point mutations or gene amplification, causing alteration in (onco)gene structure or affecting its expression [75]. On the other hand, tumour-suppressor genes (TSG) are genes which protect and prevent cells from events leading to cancer development through regulation of the cell cycle [75]. Both alleles of a TSG must be mutated to interfere with its normal function. Mutations in TSGs increase the risk of cancer development [76].

1.3 Loss of heterozygosity on chromosome 6p in cervical cancer

Loss of heterozygosity (LOH) is a commonly observed type of genetic alteration in solid tumours. The whole functional TSG or part of the chromosome containing a functional TSG can be deleted due to chromosomal mutations [77]. However, LOH analysis uses polymorphisms that occur near a suspected TSG in a gene of interest, as surrogate markers for the gene itself [77]. In clinical settings, LOH assay is an important tool for disease diagnosis, prognosis, and treatment. For example, the loss of regions on chromosomes 1p/19q are classical molecular markers for oligodendroglioma (brain cancer) assessment [78]. Interpretation of the clinical significance of molecular genetic alterations in cancers, requires that health professionals and biomedical scientists understand the theoretical fundamentals of molecular diagnostic techniques.

It has been widely reported that LOH mutations and microsatellite instability (MSI) at multiple genomic loci, including the *HLA* region, are the most common host genetic alterations occurring in cervical cancer tissues in all studied populations [79, 80]. This possibly indicates that the integrity of the *HLA* region is compromised during the process of carcinogenesis [79, 81, 82]. LOH and MSI are caused by genetic alterations such as the physical deletion of a chromosomal region, (including a tumour-suppressor gene), or chromosomal non-disjunction during mitotic recombination. It has been hypothesized that cervical cancer progression is influenced by the extent of LOH/MSI and *HLA* variations in immune response towards oncogenic HPV clearance. The combined pro-oncogenic effects of the HIV-1/HPV co-infection are thought to exacerbate the situation [32, 35, 83, 84]. Host genetic variations in the *HLA* that influence the primary immune response and the severity of LOH/MSI at the *HLA* genomic loci may, therefore, determine which lesions are at the highest risk for rapid progression to invasive cervical cancer in HIV-1/HPV co-infected women [85, 86].

LOH can further occur during the following chromosomal genetic events. (**Figure 1.7**).

a) Mitotic chromosome non disjunction, which results in whole chromosome loss to form a copy-number loss of heterozygosity (cn-LOH) or duplication of only one parental chromosome to form a copy-number neutral loss of heterozygosity (cn-neutral LOH) [87].

b) DNA double-strand break and repair to form either an interstitial deletion or an unbalanced translocation [88].

c) Homologous recombination repair to form gene conversion whereby a copy of a segment of one parental chromosome is repeated on the other parental chromosome [88].

d) Chromothripsis to form a complex rearrangement and interstitial deletions [88]

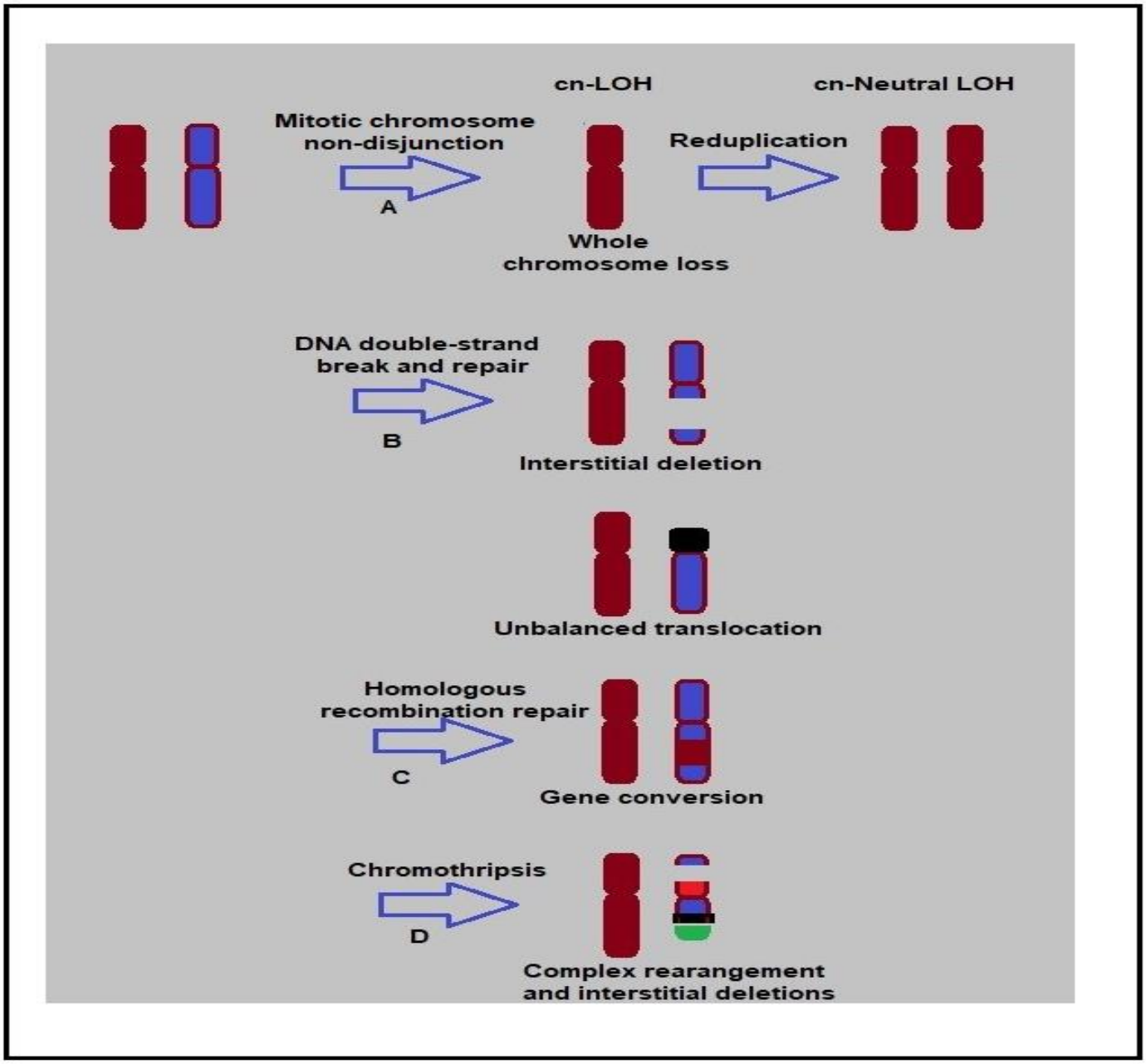

Figure 1. 7 A diagram to illustrate different chromosomal mechanisms for loss of heterozygosity.

(cn=copy number).

1.3.1 Clinical significance of LOH in cervical cancer

LOH can be a useful biomarker in cervical cancer to identify risks of disease progression and survival probability, and tumours that may respond to particular therapies [89]. It is an important assay to aid in the discovery of novel tumour suppressor genes in cervical cancer progression [89]. Another application of the LOH assay occurs when a cancer patient has two tumours at different sites, and where there is a question of a second primary tumour, or whether one tumour is a metastasis of the other. Differentiating between these scenarios can be difficult, especially when the histology is similar. The clinical implications can be significant, because a second primary tumour can be treated with the intention to cure, but a recurrence or distant metastasis equates to advanced-stage disease, generally with limited treatment options. Frequency of LOH using PCR-based microsatellite analyses can be used to measure the association and likelihood of cervical cancer progression.

Biomarker assays of early neoplasia still use microsatellite analyses due to the small quantity and low quality of DNA extracted from small lesions micro-dissected from formalin-fixed paraffin-embedded tissue blocks. LOH can be used as a predictor of cervical cancer recurrence. Chromosome loss identified in LOH could change treatment choice in invasive cervical cancer, where patients can be individualised and benefit from the addition of chemotherapy or radiotherapy. The advantages of using the PCR-based LOH assays include the ability to detect small deletions and the ability to enrich for tumour cells through microdissection [77]. Minor changes in small populations of cells will not usually be identified by most PCR-based assays. Therefore, PCR-based LOH assays are best used to detect clonal DNA damage [77].

The pitfalls that can affect the reliability and sensitivity of LOH analyses, as with any other molecular test include:

i) Contaminated tumour samples. Histologic sections of tumours usually contain a mixture of tumour cells, inflammatory cells, stromal cells, and other cellular contaminants. This tissue heterogeneity can cause problems in molecular analyses of all types, especially in amplification-based procedures. For example, LOH can be masked by heavy contamination by normal DNA. Manual microdissection may eliminate the gross contamination in LOH analysis [77].

ii) Poor markers. The polymorphic repeat units present near TSGs are only surrogate markers for the gene itself. Therefore, the distance between the TSG and the polymorphism will affect how tightly the polymorphism is linked to the TSG [77].

iii) Inadequate DNA. When the DNA concentration is low, one allele may be preferentially amplified over the other. When one allele has insufficient amplification for detection, this is termed *allelic dropout*. Allelic dropout can yield false-positive results for LOH.

iv) Lack of a normal comparison sample. Sometimes in tumour pathology, a concordant normal sample, such as blood or a buccal swab, from the same patient may not be available [77].

As stated above, LOH is one of the most important clinical assays that can be used to assess cancer prognosis, disease diagnosis and to offer individualized treatment options.

1.4 Observational studies: case-control study and cross-sectional study

In genetics, association studies evaluate the correlation between genetic variants and a disease phenotype in a certain population. In other words, they examine the relationship between disease and exposure. Instead of assessing the recombination events, linkage disequilibrium (LD) is investigated. LD describes a situation in which some combinations of

alleles or genetic markers occur more, or less, frequently in a population than would be expected from random formation of haplotypes based on the allele frequencies [90]. If an association is observed, a particular allele, genotype, or haplotype will be found more or less often, than expected by chance among individuals carrying the trait. Basically, such investigations compare the frequencies of variant alleles among affected individuals and unaffected individuals.

A case-control study is an observational study in which there are only two sample groups drawn from the same study population: the case group sample and a control group sample. These studies are usually used to suggest factors that may contribute to a disease outcome by comparing patients who have that disease (the case group) and closely matched subjects from the same population who do not have the disease (the control group) [91]. The comparison between cases and controls is by relevant statistical analyses which should show a statistically significant difference between the cases and the controls based on statistical power (p-value). A population-based case-control study comprises cases and matched controls from the same population. The advantage of a population-based case-control study is that, it collects large sample sizes with high statistical power to detect small or moderate effects. However, the disadvantage of this study design is that the genotypes and haplotypes frequencies may differ among different ethnic groups or geographic populations [90]. To overcome this drawback, the cases and the controls must therefore be matched for ethnicity, or geographic ancestry/origin.

A cross-sectional study is a type of observational study that analyzes and investigates the entire population at a specific point in time [91]. At one time point, a population is assessed to determine factors for disease causation and whether they were exposed to the relevant agent to develop the outcome of interest. A subgroup of the studied population may not have been exposed nor have the disease outcome of interest [91]. Cross-sectional studies are seldom conducted because of the ethical challenges of not treating subjects for the duration

of the study. Data are collected randomly at one point in time and multiple outcomes are studied. However, to be scientifically viable, a cross-sectional study should be of adequate sample size. To set up a cross-sectional study, a research question has to be formulated. Thereafter, a study population and variables of interest relevant to the research question must be identified. The researcher will then decide which variables of the study population are relevant to the research question.

This thesis is based on the above-mentioned observational study designs. Firstly, we used a case-control study with candidate gene study approach, which focused on investigating the associations between individual genetic variations within a certain population. *HLA* II of interest and cervical cancer disease states were studied according to HIV-1/HPV co-infection. The *HLA* II candidate genes were selected based on prior knowledge of their biological functions and potential impact on cervical cancer development, or because they have been implicated in cervical disease in previous studies [90]. The candidate gene approach is better suited for the detection of genetic effects for common and more complex diseases like cervical cancer [92].

Secondly, this thesis used two cross-sectional studies to investigate an entire study population for HPV genotypes, HIV-1 status, clinical immune cell markers, and frequency of LOH/MSI at chromosome 6p associated with age of patients and stage of cervical disease. Associations discovered by cross-sectional studies, may raise more questions for further studies, particularly because observed effects are typically very small and only confined to a certain population. Therefore, further investigations are required to identify the cause-effect relationship.

1.5 Overall main objective and justifications for this study

1.5.1 The main objective

To study the influence of host molecular genetic variations and specifically alterations at the *HLA* II locus, on HIV-1/HPV co-infection and cervical cancer progression in a cohort of South African women.

1.5.2 Specific objectives

i) Specific objective I

To identify genes, host molecular genetic variations and genetic alterations in cervical cancer progression that may play a role in disease progression in HIV-1/HPV co-infected women.

ii) Specific objective II

To characterize HPV genotypes within cervical tumour biopsies, and assess the relationship**s** with cervical disease stage**,** age, HIV-1 status, absolute CD4 count, and CD4 percentage, and to identify the predictive power of these variables for cervical disease stage in the cohort of South African women.

iii) Specific objective III

To determine whether host *HLA*-DRB1 and -DQB1 backgrounds were involved in progression of cervical cancer disease in HIV-1/HPV co-infected women.

iv) Specific objective IV

To investigate whether HIV-1/HPV co-infection provokes additional LOH/MSI at the *HLA* II locus to influence the rate of cervical disease progression in HIV-1-positive women.

1.5.3 Rationale

A subgroup of women who are co-infected with HIV-1 and HPV, progress rapidly to cervical disease regardless of CD4 count status. During infection, viral peptides are subjected to the host immune system. Host genetic factors associated with susceptibility or resistance to cervical disease following HPV infection are likely to involve viral immune response genes, such as the *HLA* II. However, the impact of host *HLA* II genetic variations, HIV-1/HPV co-infection, and genetic alterations at the *HLA* II locus on cervical carcinogenesis has not been established [93, 94]. Despite a high HIV-1 prevalence in the South Africa [95], there is limited data on the impact of host molecular genetic variations, HIV-1/HPV co-infection, HIV-1 associated immunosuppression, and cervical disease progression. The relationship between LOH/MSI at the *HLA* II locus on chromosome 6p and the effects of host *HLA* II variations on HIV-1/HPV co-infected cervical carcinogenesis is not known [27]. This unknown relationship between HIV-1/HPV co-infection, host molecular genetic variations and genetic alterations at the *HLA* II locus in the development of cervical cancer in a high risk population, is a matter of great concern, especially in the light of the current HIV-1 epidemic in South Africa. This emphasizes the need to conduct a study on the influence of host molecular genetic variations and HIV-1/HPV co-infection on cervical carcinogenesis [96, 97].

The impact of this study is expected to enlighten the current knowledge and understanding of the interaction between HIV-1/HPV co-infection and rapidly progressing cervical carcinogenesis with regard to host molecular genetic variations. The findings will strengthen available theories on the primary goal of genetically-targeted therapy, molecular prevention, and individualization in cervical cancer prevention and treatment. However, development of molecular genetic markers for early cervical carcinogenesis will further individualize the treatment.

1.5.4 Summary of specimen collection

The study nurse collected two tubes of whole blood (4ml each) for HIV-1 testing and DNA-based *HLA* II typing. Furthermore, the study nurse collected buccal swabs from all consented participants. By using colposcopy inspection, Gynaecologists collected punch biopsies of abnormal cervical lesions for histopathological analyses and HPV genotyping (see **Figure 1.8**).

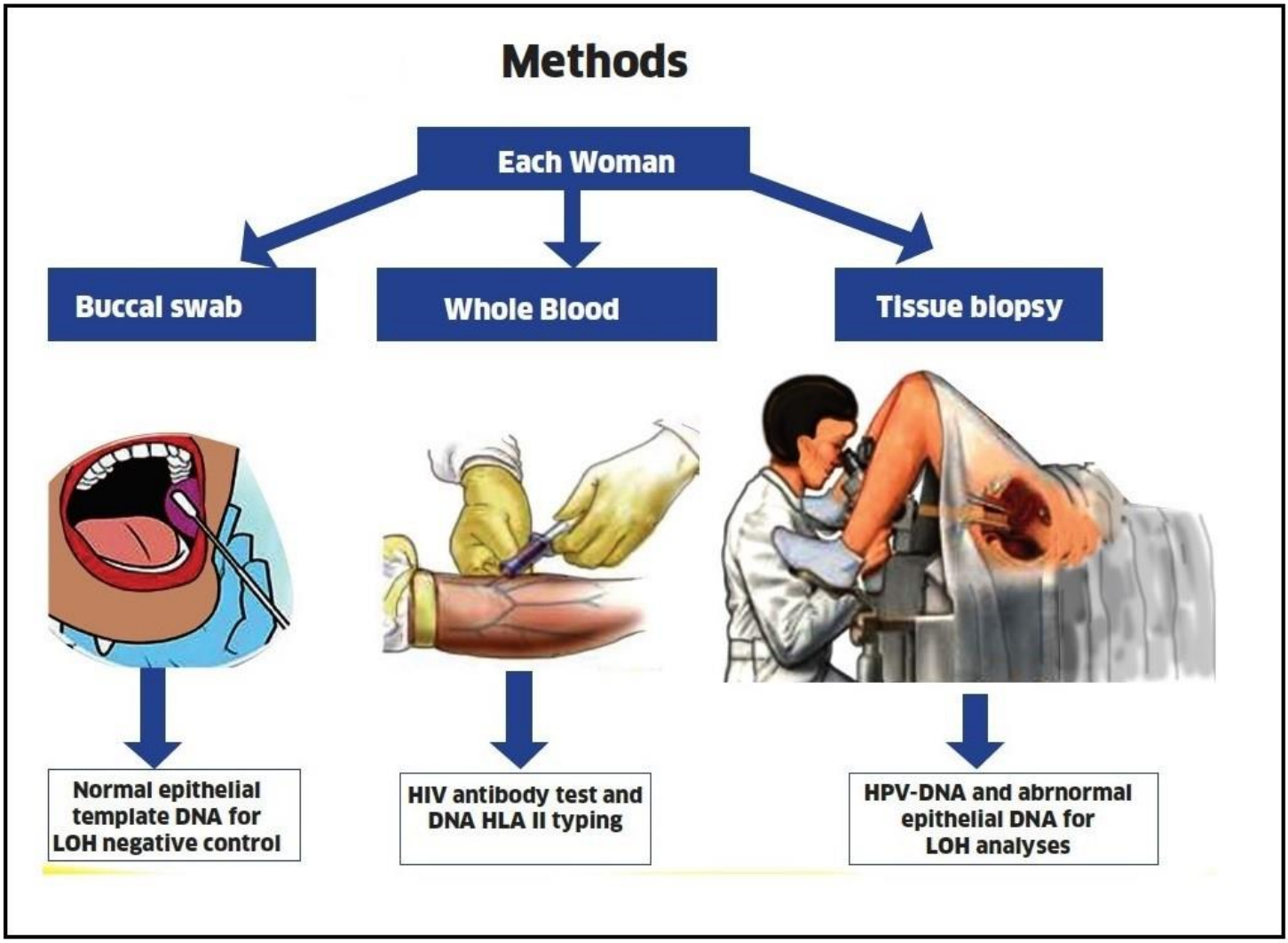

Figure 1. 8 A diagram illustrating the methods used for specimen collection.

CHAPTER 2: Impact of Host Molecular Genetic Variations and HIV-1/HPV Co-infection on Cervical Cancer Progression: A Systematic review

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ABSTRACT

Only a small subgroup of women who are co-infected with HIV-1 and persistent oncogenic HPV, progress rapidly to invasive cervical cancer by mechanisms that are currently poorly understood. The use of Highly Active Antiretroviral Therapy (HAART), with ensuing immune reconstitution of CD4 T-cells, does not appear to prevent rapidly progressing cervical carcinogenesis. Therefore, to better understand the cervical cancer pathogenesis in HIV-1/HPV co-infected women, this review focuses on identifying host molecular genetic variations and genetic alterations in cervical cancer progression that may play a role in disease progression. This is an important aspect for individualized genomic profiling and targeted molecular prevention to improve the management of the disease in this sub-population.

Keywords (Host molecular genetics, cervical cancer, HIV-1/HPV co-infection, genomic profiling and molecular targeted prevention)

2.1 Introduction

Every year, more than 530,000 women worldwide are diagnosed with invasive cancer of the uterine cervix and approximately 275,000 die from the disease [98, 99]. More than 88% of these deaths occur in developing countries especially in sub Saharan Africa [100].

Oncogenic HPV, is one of the primary causative agents for cervical cancer [101]. However, co-infection with HIV-1, may influence cervical disease progression and invasive cancer outcomes in some HIV-1-positive women [100, 102-105]. HIV-1-positive women are also more likely to have concurrent infections of single or multiple strains of oncogenic HPV, compared to HIV-1-seronegative women [101, 103, 106-109]. Furthermore, published data clearly shows that, persistent oncogenic HPV and HIV-1 co-infections contribute to rapidly progressing cervical carcinogenesis when compared to HIV-1-seronegative women with a single, multiple, or no HPV infection [26, 110-112]. Although HIV-1/HPV co-infection is common in sub-Saharan Africa, only relatively few infected patients develop cervical disease and only about one third of 'in situ' cervical carcinomas progress to invasive cancer [106, 108]. Surprisingly, the risk of developing invasive cervical cancer does not decrease following Highly Active Antiretroviral Therapy (HAART), which targets the HIV-1 infection [106, 113-116]. On the contrary, a proportion of the patients with invasive cervical cancer have no detectable HIV-1/HPV viral co-infection [55, 57].

It has been argued that, despite underlying immunodeficiency and immune reconstitution, the existing cervical carcinogenesis in HIV-1/HPV co-infected women is further influenced by host molecular genetic factors, which vary between individuals [114, 117, 118] (**Figure 2.1**). It has also been suggested that the rate of cervical disease progression and likely protection may depend on host immunogenetic variations [38, 119-122].

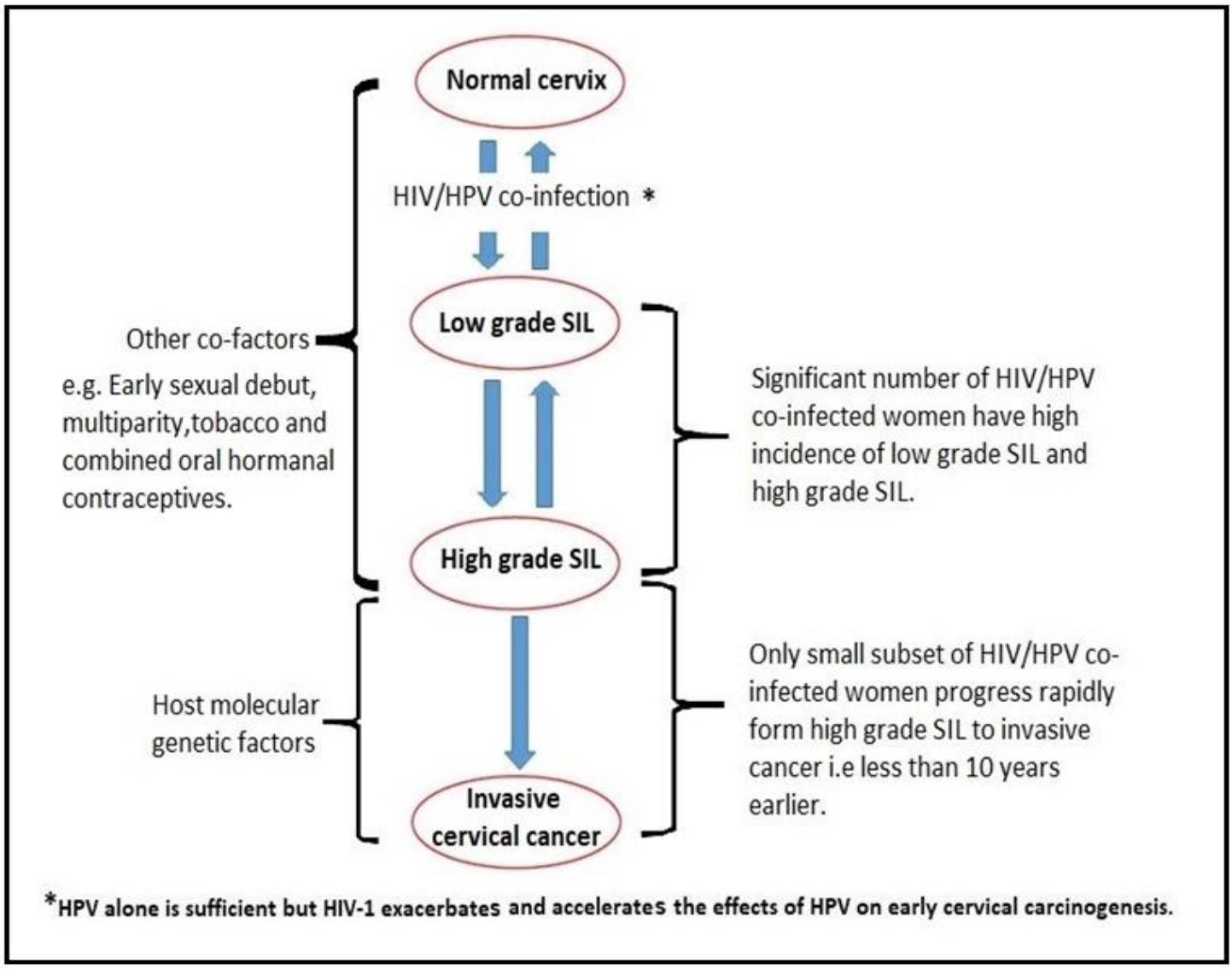

Figure 2. 1 Cervical carcinogenesis in HIV-1/HPV co-infected women

(SIL = squamous intraepithelial lesion)

(Figure from Chambuso *et al.*, (2018) [27] with reprint permission from the Journal Oncomedicine).

Specifically, the argument is that, cervical cancer progression is controlled via the microsatellite instability (MSI) pathway amongst HIV-1/HPV co-infected women and through the loss of heterozygosity (LOH) pathway amongst HPV-only infected women [114, 117, 123, 124]. While many studies have documented the association between HIV-1 infection and rapidly invasive cervical cancer development, none have shown a direct link between HIV-1/HPV co-infection and the degree of disease invasiveness and rate of progression [125-127].

These considerations have determined the focus of this review, which is to assess the current knowledge of the influence of host molecular genetic variations, genetic alterations, and HIV-1/HPV co-infection on rapidly progressing cervical carcinogenesis. We have reviewed published cohort studies and further investigations. We have also examined the evidence for possible involvement of host molecular genetic alterations and immunogenetic variations in HIV-1/HPV co-infected cervical carcinogenesis. This review stems from research conducted into existing knowledge on individualized genomic profiling and targeted molecular prevention to improve management of the disease in this subpopulation [105, 128].

2.2 Methods

This study has conducted the methodology according to similar published studies [86, 129]. A comprehensive, systematic literature search of peer-reviewed, published articles from the NCBI, PubMed, EBSCO, Medline, Elsevier Science, Springer Link and the Google Scholar bibliographic databases was carried out. The study included all original research studies, short communications, critical reviews and meta-analyses, reports on genes susceptible for cervical cancer and immune response, and factors changing risk or offering likely protection of cervical cancer development, published prior to May 31st 2018. Information on specific alleles and/or genes were noted and critically reviewed. The key words/phrases used for the search were ‘host molecular genetics’, ‘cervical cancer susceptibility’, ‘HIV-1/HPV co-infection’, ‘HIV-1 and cervical cancer’, ‘cancer genomic profiling and molecular targeted

prevention', 'cervical cancer, mutations and viruses' and 'cervical cancer genetics'. The present study excluded all studies on epigenetics of cervical cancer, as these were not deemed to be relevant to the study topic and focus. There were no specific analytical methods used for examination of articles due to the nature of the study.

2.3 Results and Discussion

In this section the study will discuss;

1. Cervical cancer pathogenesis, HAART and HIV-1/HPV co-infection.
2. Host *HLA* polymorphisms, genetic mutations and HIV-1/HPV co-infection in cervical cancer progression.
3. Molecular genetic variations, genes, chromosomes, SNPs and HPV persistence in cervical disease progression and susceptibility.
4. What is not known in host molecular genetics of cervical carcinoma?

2.3.1 Cervical cancer pathogenesis, HAART and HIV-1/HPV co-infection

The pathogenesis of cervical cancer involves three important steps; high-risk HPV viral genome integration into host genome, the oncogenic effects of HPV oncoproteins E6, E7, and the accumulation of recurrent, unrepaired genetic alterations in host chromosomal DNA [117, 130-132]. Furthermore, there is an interaction between HPV and HIV-1 co-infections, resulting in an increased risk of HPV-associated morbidity and cervical cancer mortality among HIV-1-positive women [127, 133]. In a few cases, HIV-1/HPV-associated cervical cancerous cells, non-genetically regress spontaneously to pre-cancerous cells (but not going back to normal cells). Persistent accumulation of uncorrected mutations and additional pro-oncogenic effects of HIV-1 Tat and HPV E6/E7 oncoproteins, lead to the progression of cancerous cells to invasive malignant cancer cells, regardless of the use of HAART [108, 111, 127, 134, 135] (**Figure 2.2**).

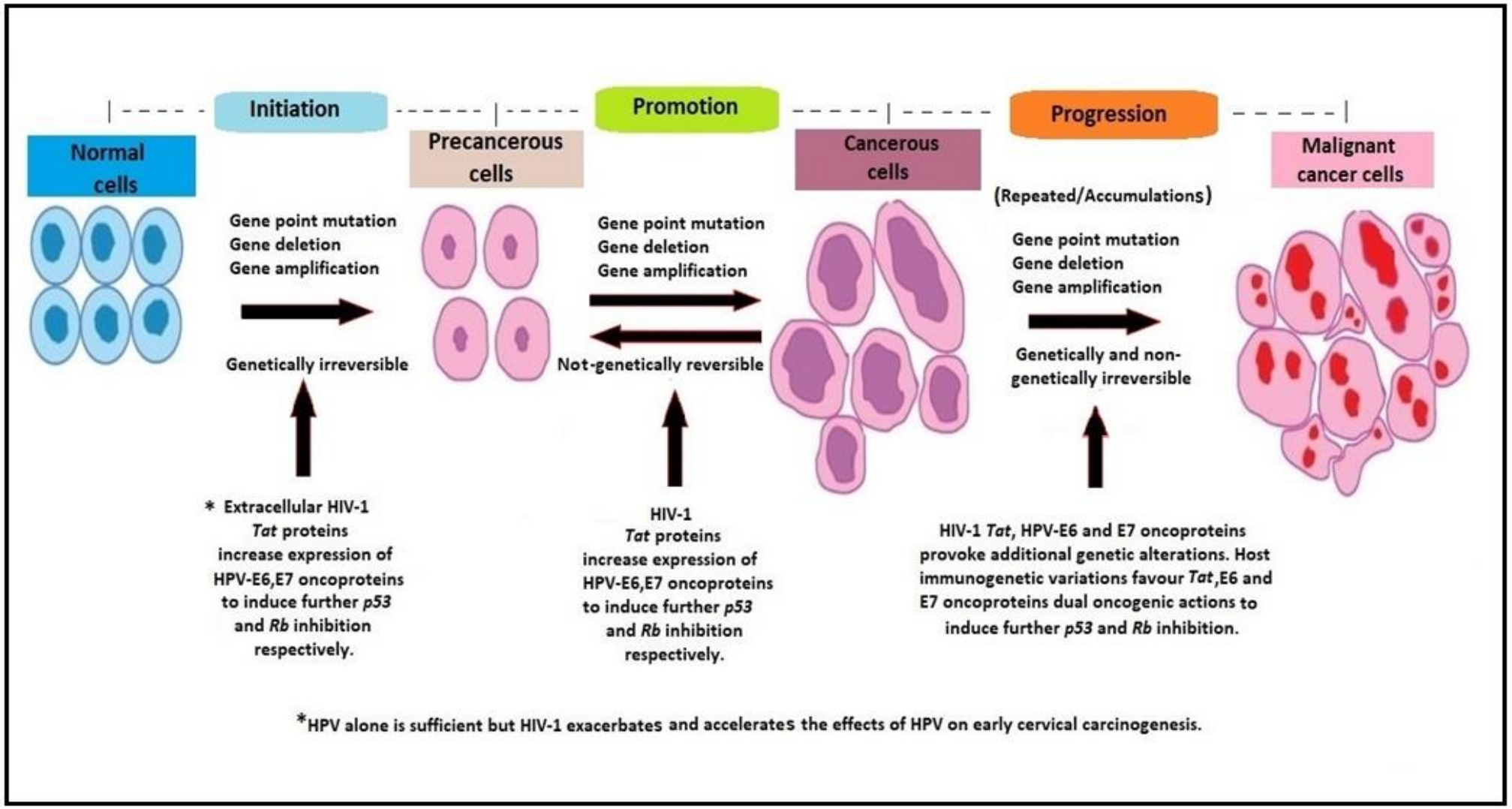
Initiation
Promotion
Progression
Normal cells
Precancerous cells
Cancerous cells
Malignant cancer cells
Gene point mutation
Gene deletion
Gene amplification
Genetically irreversible
Gene point mutation
Gene deletion
Gene amplification
Not-genetically reversible
(Repeated/Accumulations)
Gene point mutation
Gene deletion
Gene amplification
Genetically and non-genetically irreversible
* Extracellular HIV-1 Tat proteins increase expression of HPV-E6,E7 oncoproteins to induce further p53 and Rb inhibition respectively.
HIV-1 Tat proteins increase expression of HPV-E6,E7 oncoproteins to induce further p53 and Rb inhibition respectively.
HIV-1 Tat, HPV-E6 and E7 oncoproteins provoke additional genetic alterations. Host immunogenetic variations favour Tat,E6 and E7 oncoproteins dual oncogenic actions to induce further p53 and Rb inhibition.
*HPV alone is sufficient but HIV-1 exacerbates and accelerates the effects of HPV on early cervical carcinogenesis.

Figure 2. 2 Host molecular genetic variations and alterations in HIV-1/HPV co-infected cervical carcinogenesis.

(Figure from Chambuso *et al.*, (2018) [27] with reprint permission from the Journal Oncomedicine).

The immunosuppressive effects of HIV-1 infection are associated with the rapid progression of HPV-induced cervical pre-malignant lesions. This may influence the rapid onset of cervical disease and further effects on clinical outcome. However, the direct mechanism is not clear [93, 136, 137]. Additionally, the effects of duration of HAART use in cervical carcinogenesis prevention are still unknown [138, 139] .

A study by De Jong *et al.* [140], they suggested that the absence of functional HPV16-specific CD4 T-cell immune responses found in women with cervical cancer may explain further development of the cervical disease despite immune reconstitution following HAART initiation or in patients with competent CD4 cell count. Additionally, it has been already reported that not all women who progress rapidly to invasive cervical cancer are HIV-1-positive. This suggests that there are many other possible host factors apart from immunosuppression or HIV-1/HPV co-infection that may play a role in rapidly progressing cervical carcinogenesis [117, 133, 141, 142].

HIV-1 proteins can directly cause cancer growth by interfering with cellular functions [105, 133, 139, 143]. For example, HIV-1 Tat proteins directly interact with the host tumour-suppressor genes, *p53/pRb/p130/p107*, and induce increased cell proliferation, which promote the effect of HPV oncoproteins E6/E7 in the rapidly progressing cervical carcinogenesis [126, 133, 139, 143, 144]. The increased rate of HPV-associated cervical disease in HIV-1-positive women is aggravated by HIV-1/HPV molecular interactions because HIV-1 Tat proteins can modulate HPV *E2* expression, which in turn, influences HPV viral replication [145, 146]. However, Hirbod *et al*. [147], Nkwanyana *et al*. [148] and Bebell *et al*. [149] have suggested that cervical mucosal inflammation in HIV-1 infected women

may be associated with low CD4 cell counts during acute infection. Although this suggestion agrees with cancer microenvironment theories that chronic and persistent inflammation contributes to cancer development and can predispose to rapidly progressing cervical carcinogenesis, the effect has been observed only in a subgroup of HIV-1/HPV co-infected women [150-152]. It has been shown that, inflammatory mediators on the surface of the uterine cervical epithelial cells, can promote adhesion of HIV-1 infected leukocytes. This in turn facilitates HIV-1 Tat proteins uptake by cervical epithelial cells before their transformation into cancerous cells [144].

The oncogenic potential of high-risk HPV may contribute to the accumulation of mutations in proto-oncogenes and tumour suppressor genes between the G1-phase and S-phases of the cell cycle in the host [117, 153]. This process is mostly mediated by the oncoproteins E6/E7, which hijack host genomic DNA, then bind and inactivate the tumour-suppressor genes, *p53* and the retinoblastoma (*pRb*), respectively, to further inhibit apoptosis. This process is achieved through the Ubiquitin-mediated degradation pathway [110, 154-156].

It is clear therefore that, apart from immunosuppression, additional genetic and genomic alterations are necessary for pre-cancerous cells to sufficiently progress into malignant and invasive cancer cells, especially following HAART, which reconstitute the host immune competency [157].

2.3.2 Host *HLA* polymorphisms, genetic mutations and HIV-1/HPV co-infection in cervical cancer disease progression

In some populations, carcinogenesis of squamous cell carcinoma is more susceptible to genetic variations and alterations than adenocarcinoma [158, 159]. Since the immune system normally mobilizes to clear viral infections, the development of virus-associated cancers results from a failure of anti-viral immunity. Furthermore, in cervical carcinogenesis, host immune-competence gradually diminishes as cervical disease progresses to invasive cervical cancer [154].

The genes coding for the *HLA* system, which reside on the short arm of human chromosome 6, (except for the gene for β2-microglobulin) (see **Figure 2.3**) play a central role in immune recognition and the subsequent clearance of virally infected cells [39, 160-164]. Therefore, any variations or structural genomic changes within the HLA region, which influence immune evasion by viruses and cancer cells, may determine the lesions likely to progress to invasive cancer [39, 134, 139, 162, 165]. In a study of *HLA* in cervical cancer patients compared to healthy controls, expression of *HLA* I, were downregulated, suggesting that host genetic variations of *HLA* I also have a significant bearing on specifically HPV-16 related cervical carcinogenesis [166].

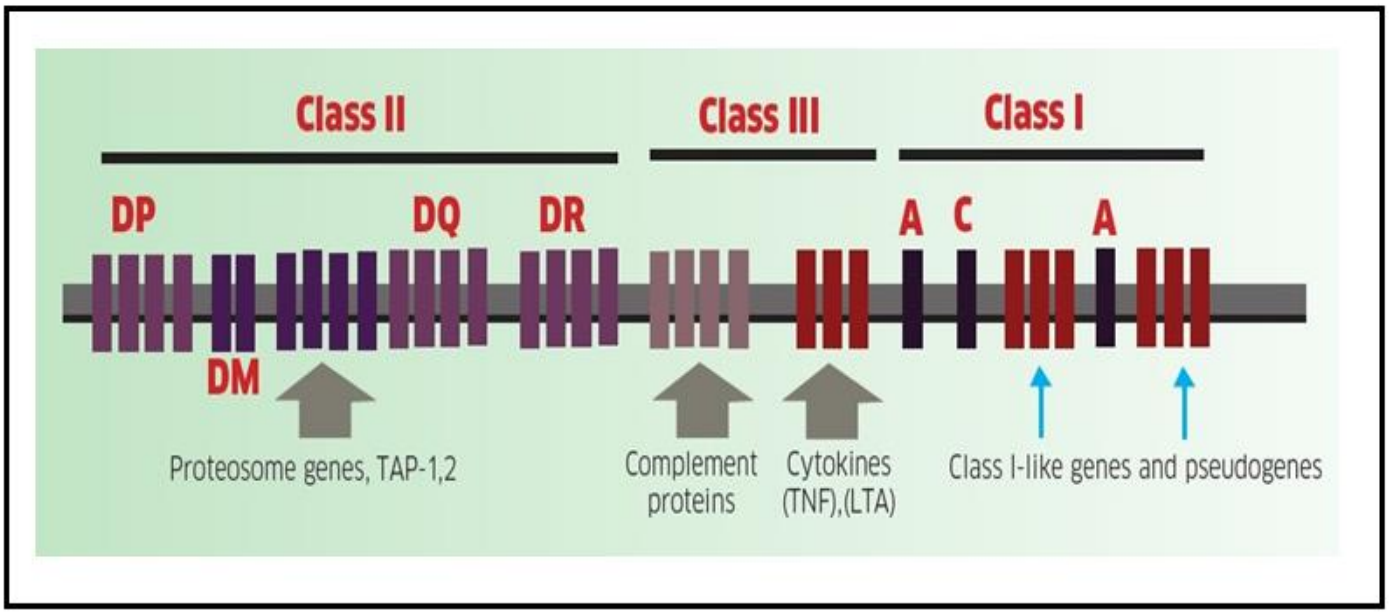

Figure 2. 3 A schematic diagram showing an overview of the Human Leukocyte Antigen (HLA) genes on chromosome 6p.

HPV-16 E5 oncoprotein and downregulation of the surface *HLA* I expression has already been reported [167]. However, genetic predisposition and variability in the HLA genes have shown considerable contradictory findings in different study populations [85, 86, 168]. Variations in *HLA* II and cervical cancer susceptibility have been investigated in different geographical populations with inconsistent findings, highlighting the complexity of the viral/host/environmental ecosystem [39, 159, 168-172]. For example, predispositions of the *HLA* system and a positive cervical cancer association with the *TNF* for apoptosis were previously reported [173-175]. Although in the mixed ethnicity South African populations, *TNF G*-308 did not show any association with cervical cancer susceptibility [129, 176].

It has been widely reported that LOH mutations and MSI at multiple genomic loci, including the *HLA* region, are the most common host genetic alterations seen in cervical cancer tissues [79, 80]. This possibly indicates that the integrity of the *HLA* region is compromised during the process of carcinogenesis [79, 81, 82]. LOH and MSI are caused by genetic alterations such as the physical deletion of a chromosomal region, a TSG, or chromosomal non-disjunction

during mitotic recombination. It has been reported that the mechanisms of LOH/MSI may be remarkably chromosome-specific [32, 77, 79, 162], with some chromosomes being completely lost while more than half of the losses are associated with the loss of only a part of the chromosome [78, 79]. Essentially, the mechanism underlying HIV-1/HPV co-infection and cervical cancer development with regard to LOH/MSI or immunogenetic variations is still poorly understood [161]. It has been hypothesized that, cervical cancer progression is influenced by the extent of LOH/MSI and *HLA* variations in immune response towards oncogenic HPV clearance and the combined pro-oncogenic effects of the HIV-1/HPV co-infection [32, 35, 83, 84].

Host genetic variations in the *HLA* that influence the primary immune response and the severity of LOH/MSI at the *HLA* genomic loci may, therefore, determine which lesions are at the highest risk for rapid progression to invasive cervical cancer in HIV-1/HPV co-infected women [85, 86].

2.3.3 Molecular genetic variations, genes, chromosomes, SNPs and HPV persistence in cervical disease progression and susceptibility.

Currently, there are no consistent data on the association between any host gene polymorphism and disease outcome for cervical cancer [177]. In studies of different chromosomes, the changes involving loss of 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q, 17q and 18q regions and gain of 1q, 3q, 5p and 8q at various stages of cervical cancer have shown a possible association with either oncogenic HPV persistence or cervical cancer disease progression [153, 178]. (**Table 2.1**). Presence of chromosomal aneuploidy, which increases genomic instability in rapidly progressing carcinogenesis, was reported in both cervical pre-cancer and cervical invasive cancer [179-181]. Furthermore, the presence of an isochromosome 5p associated with cervical cancer susceptibility has been reported in several studies [182-184].

Table 2. 1 Summary of host gene polymorphisms, SNPs and chromosomal locations found to be associated with either HPV persistence or cervical disease progression worldwide.

(Table from Chambuso *et al.*(2019) [185] with reprint permission from the Journal Oncomedicine)

HOST GENES AND SNPs	CHROMOSOME	COHORT SIZE AND NATURE OF THE STUDY	POPULATION STUDIED	HPV 16 OR 18 GENOME INTERACTION WITH HOST GENES
A) Genes involved in HPV persistence				
CTLA-4,−318 C/T	2q33.2	Case-control, 144 cases vs 378 controls	Taiwan [186]	-
STING	5q31.2	Cross-sectional, 148 patients	Thailand	*E2* downregulates *STING* [187]
***HLA*-DQB1**	6p21.32	Case-control, 1306 cases vs 288 controls	Sweden [169]	*E5*, *E7* downregulate expression of MHC molecules [188]
GTF2H4 rs2894054	6p21.33	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 controls	Costa Rica [189]	-
MICA	6p21.33	GWAS, 1075 Cases and 4014 Controls	Sweden [32]	*E5*, *E7* downregulate expression of MHC molecules [188]
SULF1, rs4737999	8q13.2-q13.3	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 controls	Costa Rica [189]	-
IFNA1	9p21.3	Cross-sectional, 148 patients	Thailand	*E2*, *E6* downregulate *IFNA1* [187]
IL2RA, rs2476491	10p15.1	Double case-control, 141 cases, 38 HSIL and 176 controls	Portugal [190]	*E6*, *E7* interferes with cytokines pathways [188, 191]
PRDX3, rs7082598	10q26.11	Cross-sectional, 68 patients	China	*E6*, *E7* downregulate *PRDX3* [192]
C1RL, rs12227050	12p13.31	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 control	Costa Rica [193]	-
OAS3, rs12302655	12q24.13	Double case-control, 469cases, 390 women with	Costa Rica [189]	-

		persistent Hr-HPV and 452 control		
DUT, rs3784621	15q21.1	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 control	Costa Rica [189]	-
TP53(p53), rs1042522	17p13.1	Cross-sectional, 577 patients	USA [194]	*E6* degrades p53 [54]
NLRP1, rs11651270 ***NLRP3***, rs10754558 and ***IL18***, rs1834481	17p13.2	Case-control, 246 cases vs 310 controls, 12 SNPs in seven genes	Brazil [195]	-
TYMS, rs2342700	18p11.32	Case-control, 65 cases vs 202 controls	Nigeria [196]	-
RPS19, rs2305809	19q13.2	Case-control, 65 cases vs 202 controls	Nigeria [196]	-
IRF3, rs7251	19q13.33	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 control	Costa Rica [197]	*E6* prevents *IFN*-α mRNA [198]
B) Genes involved in cervical disease progression				
EXO1, rs4149963	1q43	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 controls	Costa Rica [197]	-
TIPARP, rs2665390	3q25.3	Case-control, 790 cases vs 717 controls	Algeria, Morocco, India, Thailand [199]	-
PIK3CA	3q26.32	Cross-sectional, 285 cases	Mexico, Guatemala, Venezuela [200]	*E6*, *E7* increase APOBEC-mediated mutagenesis [201].
LAMP3	3q27.1		Japan [202]	-

		Double case-control, 47 cases, 15 tissues with CIN and 5 tissue controls		
RFC4	3q27.3	Case-control and Meta-analysis, 40 cases vs 20 controls	Brazil [203]	*E1, E2* ORFs disruption interferes *RFC4* [204]
POLN, rs17132382	4p16.3	Double case-control, 416 cases, 356 women with persistent Hr-HPV and 425 controls	Costa Rica [189]	E6 interacts with *POLN* [205]
MIR146A, rs2910164	5q33.3	Case-control, 447 cases vs 443 controls	China [206]	*E6* under-express miRNAs[207]
TNF	6p21.33	Descriptive, *in vitro*	USA [208]	*E6* down-regulates *TNF* [209]
URG4	7p13	Cross-sectional, 167 cc patients	China [210]	-
MYC	8q24.2	Descriptive, Case series, 1 cc patient	USA	*E7* fusion causes *MYC* overexpression [41]
CDKN2A (p16)	9p21.3	Cross-sectional, 139 cases	Japan [211]	*E7* inactivates *Rb*1 (p16 overexpression) [211]
TAP	11q12.3	Descriptive, *in vitro*	China [42]	*E7* down-regulates *TAP* [209]
IFNG , rs11177074	12q15	Double case-control, 416 cases, 356 women with persistent Hr-HPV and 425 controls	Costa Rica [189]	*E6* inhibit interferon related responses [43, 209].
MDM2	12q15	Descriptive, *in vitro*	Italy	*E2* interacts *MDM2* ubiquitin ligase [212]
RB1	13q14.2	Descriptive, *in vitro*	USA [213]	

				E7 binds and degrades *pRb* [213, 214].
CYP1A1 m2, rs1048943	15q24.1	Case-control, 100 cases vs 100 controls	India [215]	-
TELO2, rs4786772	16p13.3	Double case-control, 416 cases, 356 women with persistent Hr-HPV and 425 controls	Costa Rica [193]	-
FANCA, rs2239359	16q24.3	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 controls	Costa Rica [197]	*E6* promotes reprogramming of *FANCA* [216]
CYBA, rs7195830	16q24.3	Double case-control, 469cases, 390 women with persistent Hr-HPV and 452 controls	Costa Rica [197]	-
EVER1/EVER2, rs9893818	17q25.3	Double case-control, 416 cases, 356 women with persistent Hr-HPV and 425 controls	Costa Rica [189]	*E7* binds zinc ions to prevent *EVER1/2* and E5 binds to EVER1/2 [167]
FGFR-TK1	17q25.3	Descriptive, Case series, 3 cc patients	USA and Brazil [217]	*E6* induce expression of *FGF-BP* [217]

It has been observed that, Hr-HPV-DNA potentially integrates into more than 117 unique sites into the host genome to influence cervical carcinogenesis [218, 219]. Several other genomic regions with changes in the number of DNA copies (copy number-altered regions or CNAs), common in solid tumours, have been confirmed by comparative genomic hybridization (CGH), Florescent '*In situ'* Hybridization (FISH) and SNPs, reflecting the important role of HPV infection and specific genomic alterations in cervical carcinogenesis [220].

Individuals with a *Tp53* polymorphism in codon 72 (Arginine homozygosity) have been reported to be at a seven times higher risk for HPV-associated cervical cancer development than the heterozygous genotype [129, 194]. However, a pooled data analysis of 49 studies worldwide published in 2009, found no association between cervical cancer and the *TP53* codon 72 polymorphism [221]. The most frequently-mutated tumour-suppressor gene in cervical cancer is believed to be *Cystatin E/M*, however, these results are not consistent in different study populations [222, 223].

Previous studies in cervical cancer molecular genetics using SNPs have shown that the accumulation of cellular genomic damage such as point mutations, gene amplifications, and LOH/MSI in both pre-cancerous and cancerous lesions occurs at rs13117307 at 4q12, rs8067378 at 17q12, rs4282438 and rs9277952 at 6p21.32 [176, 219]. There were, however, no SNPs associated with cervical cancer risk in *p21* rs1801270, BRIP1 rs2048718, and rs11079454 polymorphisms [224]. Furthermore, the recurrent cellular genetic alterations in cervical cancer were observed only in primary mutational signatures, 1B and 2(APOBEC) [225, 226]. Women who are carriers of genes or alleles that may affect the expression of immune molecules capable of HPV infection recognition are at increased risk for developing cervical cancer. However, variations in *CD83*, a marker of dendritic cell maturation that may assist the T cell response to HPV infection, have shown little or no influence on cervical cancer [227, 228]. Contrarily, Yu *et al.* [229] in 2009, confirmed an association between *CD83* polymorphisms and cervical cancer susceptibility and suggested that polymorphisms in this gene and cervical disease association may depend on tumour histology.

Mutations in the cyclin dependent kinase inhibitor, *WAF1* have shown a positive association with cervical cancer susceptibility, although some studies, based on different study populations report contradictory findings [129, 230, 231]. Somatic genomic mutations, notably copy number variations, in the genes *PIK3CA, STK11, PTEN, TP53,* and *KRAS 4-7*, have been associated with cervical cancer development [128, 153]. Mutations, and/or polymorphisms in transporters associated with antigen processing genes, *TAP-1* and *TAP-2* were not associated with the development of cervical cancer [232, 233]. Although, Zoodzma *et al.* [159] reported an increased risk of cervical cancer in individuals with allele 184 at the *MICA* locus (with a recessive effect), subsequent investigations have not been able to replicate this finding [234].

Certain heritable syndromes involving defects in the DNA damage-repair system, which present susceptibility to cervical cancer have been studied. For example, Fanconi anemia syndrome (caused by mutations in the genes *FANCA, FANCC, FANCL*), is an inherited genetic disorder characterized by defects in the DNA damage-repair system. In addition, Mathew, [235] suggested an association between this syndrome and cervical cancer. However, in a subsequent study in a Swedish population, no association between Fanconi anemia and susceptibility to cervical cancer was shown [236]. Enigmatically, however, in a study using *FANCA* deficient mice, Park *et al.* [237] demonstrated susceptibility of HPV 16 *E7*-driven cervical cancer.

The *ERAP1-575* on chromosome 5, and the *TAP2-379*, and *TAP2-651* loci on chromosome 6, have been tested in Asian populations, and shown to be consistently associated with cervical cancer risk [238, 239]. The rs799917 TT genotype in the *BRCA1* has been associated with a significantly decreased risk of cervical cancer [176, 240]. Some variants in the chemokine receptor-2A (*CCR2A*), a transcribed isoform of CD192, situated on chromosome 3p21, have demonstrated a protective effect against the invasive cervical cancer development from squamous intraepithelial lesions (SIL) in Swedish, Portuguese, and South African indigenous and Mixed-Ancestry populations. It has also been described as a risk allele for high-grade squamous intraepithelial lesions and cervical cancer development in healthy individuals. In other studies, however, the *G46295A* variant in *CD192* was reported not to confer genetic susceptibility towards cervical cancer development [129, 241-243].

The activation of *Caspase 8* (*CASP8*) represents an important initiating event in the *death receptor-induced apoptosis* gene. However, the deleted allele of the *CASP8* polymorphism has been associated with decreased risk for cervical cancer in a Chinese population [244]. Studies in African populations have not found any association between *CASP8* polymorphisms and cervical cancer susceptibility [155].

Although, we have discussed numerous interactions of several genes, chromosomes, SNPs and the HPV oncoproteins E6/E7, the exact mechanisms of interactions with the HIV-1 co-infection, and the mechanisms by which the combined pro-oncogenic effects of HIV-1 Tat proteins and HPV oncoproteins E6/E7, further provoke additional genetic alterations in some women to influence the rate of cervical cancer progression is not yet known [144, 245].

2.3.4 What is not known in host molecular genetics of cervical carcinoma?

There is a lack of available research addressing some specific questions that need to be asked:

a) Why do variations at the MHC II locus increase the risk of cervical cancer? Although this has not been studied before in an HIV-1/HPV co-infected population, it does appear that the main host genetic susceptibility factors for cervical cancer may be related to the immune recognition of HPV-infected or HPV transformed cervical epithelial cells [170].

b) Do variations in the *HLA* II-DRB1 and -DQB1 influence HIV-1-positive cervical carcinogenesis [246]?

c) Are host molecular genetic polymorphisms at certain genomic loci more likely to influence cervical carcinogenesis in HIV-1/HPV co-infected women [170]? The immunogenetics of cervical carcinomas from HIV-1-positive women have not yet been studied [247, 248].

d) What are the effects of LOH/MSI at 6p in HIV-1/HPV co-infected cervical carcinomas [32]?

e) What are the effects of HAART on the incidence and severity of cervical cancer in HIV-1/HPV co-infected women with regard to molecular genetic variations?

f) How do the anti-apoptotic effects of Protease Inhibitors (PI), influence cervical carcinogenesis [135]?

g) Can the significant genomic loci, candidate tumour suppressor genes and the biological pathways of the genetic framework of susceptibility or heritability to cervical cancer, which have been indicated by the reported genome-wide association studies, be elucidated [171, 194, 234]?

h) Can further research confirm whether susceptibility loci in one population are specifically replicated in another e.g. *HLA*, 4q12, 17q12 [168, 224, 249, 250]?

i) Why is the apparent effect of the *P72R* polymorphism in the *TP53* not consistent in different ethnicities [224]? This is despite the evidence that cervical cancer susceptibility loci in each ethnic group vary considerably.

j) Does HIV-1/HPV co-infection provoke additional host genetic alterations on chromosome 6p specifically at the *HLA* II loci, DRBI and DQB1, to influence the rate of cervical disease development in HIV-1/HPV co-infected women [246]? However, this thesis has answered this question in chapter five (5) after molecular investigation in a cohort of South African women.

Overall, the evidence suggests that HPV persistent infection with oncogenic genotypes is a necessary, but not sufficient, risk factor for cervical carcinogenesis. This study hypothesizes that, HIV-1 co-infection exacerbates and increases the rate of progression to invasive cervical cancer by promoting additional genetic alterations and mutations on chromosome 6p by the cumulative oncogenic effects of the combined viruses.

2.4 Conclusions and recommendations

This is the first study to assess the HIV-1/HPV co-infection and correlation with host molecular genetics in the development of cervical cancer. Although there are limited published data on the interaction of the HIV-1 Tat proteins and HPV oncoproteins, E6/E7, and host molecular genetic susceptibility to cervical cancer progression, this review compiles the reports on the major host molecular genetic risk factors that have been shown to be associated with both rapidly progressing cervical cancer progression and susceptibility.

This review considers the gaps in knowledge for cervical cancer progression in general, and in HIV-1/HPV co-infected women, particularly. This study has provided an updated literature review, which includes a large number of genes and possible mutations for host molecular genetic susceptibility to cervical cancer development. The influence of HIV-1/HPV co-infection, HIV-1 Tat protein, HPV oncoproteins E6/E7, and host tumour suppressor-genes in the disease time course has been discussed and compared in various published studies. Proper focus on high-risk populations for cervical cancer disease (e.g. HIV-1 infected women) should decrease the number of advanced cases of invasive cervical cancer by identification of early molecular genetic changes. This can be achieved by assessing molecular genetic risk factors using predictive testing (PT) for cervical disease development in HIV-1/HPV co-infected women [251, 252]. In addition, the implementation of new genetic profiling and cervical cancer-screening programs for all women infected with HIV-1 is envisaged [252].

At this stage, it is not easy to point to one or a few genes that may affect susceptibility, severity or increase rate of progression of cervical cancer in the presence of HIV-1 and HPV co-infection as many genes are involved in different molecular pathways. Generally, the genetic variations or mutations that affect host genes for the immune response against oncogenic HPV clearance, oncogenes, tumour-suppressor genes, apoptosis-related genes, DNA damage-repair genes and cell cycle-regulatory genes are responsible for cervical cancer susceptibility and rapidly progressing disease. There is also inter-population or multi-population differences and a range of confounding host and viral factors, including environmental effects such as host behaviour and demographics. This may be why different genes have been found to be associated, or not associated, with cervical cancer development in different study populations.

CHAPTER 3: Investigation of Age, Absolute CD4 count, and CD4 Percentage in Relation to HPV infection and the Stage of Cervical Disease in HIV-1-Positive Women.

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ABSTRACT

Background

A subgroup of women who are co-infected with HIV-1and HPV, progress rapidly to cervical disease. HPV genotypes within cervical tumour biopsies were characterized and assessed for relationship with cervical disease stage, age, HIV-1 status, absolute CD4 count, and CD4 percentage, and the predictive power of these variables for cervical disease stage was identified in a cohort of South African women.

Methods

One hundred and eighty one (181) women who were histologically diagnosed with cervical disease were recruited, of whom 87 were HIV-1-positive and 94 were HIV-1-seronegative. Colposcopy-directed tumour biopsies were confirmed by histology and used for genomic DNA extraction. The Roche Linear Array® HPV genotyping test was used for HPV genotyping. Peripheral whole blood was used for HIV-1 rapid testing. Fully automated FC500 MPL/CellMek® with PanLeucogate (PLG) was used to determine absolute CD4 count, CD4 percentage and CD45 count. A chi-squared test, logistic regression model, parametric

Pearson's correlation, and ROC curves were used for statistical analyses. The Benjamini-Horchberg test was used to control for false discovery rate (FDR, q-value). All tests were significant when both p and q were <0.05.

Results

Age was a significant predictor for invasive cervical cancer (ICC) in both HIV-1-seronegative **(p<0.0001, q<0.0001**) and HIV-1-positive women (**p=0.0003, q=0.0003**). Sixty eight percent (59/87) of HIV-1-positive women with different stages of cervical disease presented with a CD4 percentage equal to or less than 28%, and a median absolute CD4 count of 400 cells/µl (IQR 300-500 cells/µl). Of the HIV-1-positive women, 75% (30/40) with ICC, possessed ≤28% CD4 cells versus 25% (10/40) who possessed >28% CD4 cells (both **p<0.001, q<0.001**). Furthermore, 70% (28/40) of women with ICC possessed CD4 count >350 compared to 30% (12/40) who possessed CD4 count ≤ 350 (both **p<0.001, q<0.001**).

Conclusions

Age is an independent predictor for ICC. In turn, development of ICC in HIV-1-positive women is independent of the host CD4 count and associates with low CD4 percentage regardless of absolute CD4 count that falls within the normal range. Thus, using CD4 percentage may add a better prognostic indicator of cervical disease than absolute CD4 count alone.

3.1 Introduction

Cervical cancer is the most common cause of cancer-related morbidity and mortality [99, 253], and the most common AIDS-related cancer in women in sub-Saharan Africa [254, 255]. Women who are infected with HIV-1, are more likely to have higher prevalence of genital oncogenic high-risk HPV infection than women who are not infected with HIV-1 [26, 100]. Persistent infection of HPV can lead to the development of cervical precancerous lesions [127]. These precancerous lesions can progress to ICC if not treated or without an effective immune response to clear the persistent oncogenic Hr-HPV infection [256].

HPV-infection screening is conducted to identify HPV genotypes causing the lesion and proof of viral replication in the cervical lesion [257, 258]. Partial genotyping is recommended when

HPV screening is used [259]. In HIV-1-positive women with cervical disease, multiple infections with different HPV genotypes are common [26]. Additionally, genotype-specific HPV burden from the primary cervical lesion may determine the progression of pre-invasive to invasive cancer [26, 260, 261]. However, there is limited data on HPV genotypes associated with cervical abnormalities in HIV-1-positive women drawn from the tumour itself in correlation with immune cell markers, such as absolute CD4 count and CD4 percentage [257, 262-264].

Within the tumour, extrachromosomal HPV viral genomes often become integrated into the host genome. This integration event is thought to drive oncogenesis by dysregulating expression of the viral oncogenes *E6* and *E7*, leading to inactivation of critical cell-cycle checkpoints and increased genomic instability in the host [130, 265]. However, many previous studies used only cytology results synonymously with cervical premalignant changes and none of them correlated with absolute CD4 count and CD4 percentage, concurrently [266-268]. Because cytology is only a screening test, it is important that the oncogenic HPV genotypes be confirmed inside the tumour itself [263]. Lack of characterization of age, CD4 count and CD4 percentage concurrently in women with cervical disease, makes the association of diagnostic clinical immune parameters in women with cervical disease unclear, particularly in HIV-1 infected women in South Africa, where HIV-1 prevalence and infection rates in women are amongst the highest in the world [95].

The aim of this study was to characterize HPV genotypes within cervical tumour biopsies and to assess the relationships of cervical disease stage with age, HIV-1 infection status, absolute CD4 count, CD4 percentage and to identify the predictive power of these variables for cervical disease stage in a cohort of South African women.

3.2 Methods

Research ethics

Ethical approval was granted from the Human Research Ethics Committee (HREC) of the University of Cape Town (HREC903/2015). All methods were performed in accordance with the relevant guidelines and regulations of the Departments of Gynaecology of all respective hospitals and the Provincial Department of Health of the Western Cape Province and the South African National Health Laboratory Service (NHLS). Subjects were recruited with informed consent: Written and signed consent forms in the language of the subject's choice were obtained in the presence of a witness. This was after detailed discussion with patients regarding the aims and nature of the study. A trained Registered Professional Nurse who was fluent in the relevant languages explained the details of the study and answered questions from the patients before their consent was requested.

Study design, subjects, HIV-1 testing and CD4 T lymphocytes enumeration

As part of a large ongoing hospital-based project, this cross-sectional study recruited 181 women with histologically confirmed cervical cancer biopsies from the Groote Schuur Hospital, Somerset Hospital and Victoria Wynberg Hospital in Cape Town, in the Western Cape Province of South Africa. All participants were above 18 years of age and the recruitment process was conducted from June 2016 to March 2017. All women were referred from peripheral health centers to these three hospitals after cervical screening with abnormal Pap smear or suspicions of cervical malignancy after gynaecological examination. Patients were recruited from the outpatient gynaecological cancer assessment clinics, colposcopy clinics and the gynaecological emergency rooms. Inclusion criteria were newly diagnosed patients with cervical disease and ability to consent. Using colposcopy inspection, Gynaecologists collected punch biopsies of abnormal cervical lesions. The biopsied tissues were stored in transport medium and sent to the Anatomical Pathology laboratory for histopathological analyses. The staging summary of cervical disease presented in this study was done as recommended by the revised FIGO staging guidelines [269].

According to the South African HIV-1 testing algorithm, peripheral whole blood (4ml) was collected in EDTA tubes (BD Vacutainer®, Johannesburg, South Africa). Approximately 20µl of the collected peripheral whole blood was used for rapid HIV-1 antibody testing (Determine, Alere, Inc., Johannesburg, South Africa) [270]. All HIV-1-positive women were on antiretroviral therapy (ART), and absolute CD4 count, CD4 percentage and CD45 white cell count were performed automatically using fully automated FC500 MPL/CellMek ™ system with a PanLeucogate (PLG) (modified gating strategy) platform as described elsewhere by Coetzee et al [271]. Generally, a CD4 percentage of 14-28% corresponds to an absolute CD4 count between 200 and 500 cells/microliters [272]. The cut-off for CD4 percent depends on the region and inter-assay or inter-laboratory variability [65, 66, 273]. For example Guiguet et al [274], used a cut-off of below or equal to 20% and above 20% in their study population in France. However, in South Africa, the laboratory CD4% reports show in a cut-off as below or equal to 28% and above 28%.

HPV DNA detection and typing

Genomic DNA was extracted from histologically confirmed cervical tumour specimens by using reagents in the Qiagen® QIAamp DNA Mini purification kit (Qiagen, Johannesburg, South Africa) according to the manufacturer's protocol. The extracted DNA was quantified using a Nanodrop® Spectrophotometer (Thermo Fisher Scientific, Johannesburg, South Africa). Due to the high concentration of genomic DNA from the tissue biopsies, the DNA was diluted using nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa) to a recommended final concentration of 0.2ng/µl. The PCR-based Roche Linear Array® HPV genotyping test (Roche Molecular Systems, Pleasanton, CA, USA) which identifies and distinguishes about 37 different HPV genotypes [HPV-6, -11, -16, -18, -26, -31, -33, -35, -39, -40, -42, -45, -51, -52, -53, -54, -55, -56, -58, -59, -61, -62, -64, -66, -67, -68, -69, -70, -71, -72, -73, -81, -82, -83, -84, -89 (HPV-CP6108) and –IS39] was used for typing HPV according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed as recommended in previous studies [275, 276]. A Chi-squared test was used with descriptive statistics to summarize the simple statistics in different stages of cervical disease according to different clinical predictor variables such as age, HPV and HIV-1 infections, age at sexual debut, parity and tobacco use. For HIV-1-positive women, in order to study immune predictor variables and age of patients, concurrently, a multinomial logistic regression model was used with cervical disease as a dependent categorical variable and CIN 1 as a reference category to show the relationship of the stage of cervical disease with age, absolute CD4 count, CD4 percentage and CD45 count for each stage of cervical disease. The correlation between absolute CD4 count and CD4 percentage was performed using parametric Pearson's correlation analyses. Receiver operating characteristic (ROC) curves were used to assess sensitivity and specificity by calculating the area under the curves (AUC) to predict disease outcome by GraphPad Prism® 8 software (www.graphpadPrism.com). Normal distribution two-proportion z-test was used to test for statistically significant differences between two proportions within the same categorical group. All *p-values* were corrected for False Discovery Rate (FDR) by the Benjamini-Hochberg's method and the adjusted *p-values* (q-values) were reported. All odds ratios (ORs), 95% confidence intervals (95% CIs) and the p-values calculated for multiple comparisons were 2-tailed, and considered significant if both p and $q<0.05$.

3.3 Results

Characteristics of the study cohort

The study cohort comprised of 181 women histologically diagnosed with cervical disease, of whom 87 were HIV-1-positive and 94 were HIV-1-seronegative (**Table 3.1**). The clinical predictor variables associated with cervical disease in this study population were: age in years (**p<0.001, q=<0.001**), HPV infection (**p=0.012, q=0.018**), HIV-1 infection (**p<0.001, q=<0.001**), parity (number of children) (**p<0.001, q<0.001**) and tobacco use (**p=0.031, q=0.0372**) **(Table 3.1),** consistent with previously published reports on factors associated with cervical disease [277, 278]. Of this study cohort: about 18.8% were documented with age of sexual debut below 16 years, while 85.5% were positive for HPV infection by DNA test, and

67.8% who were diagnosed with ICC were HIV-1-positive. There were significant associations between age above 40 (**p=0.021, q=0.063**), HIV-1 positive status (both **p** and **q <0.001**), and having more than four (4) children (both p and q=0.001), with ICC (**Table 3.1**).

Table 3. 1: Demographics and the range of variables including clinical predictors measured and the stages of cervical disease in the subjects of this study.

Predictor variable	% (n/N)	CIN 1,n= 29 (%)	CIN 2,n= 48 (%)	CIN 3, n= 45 (%)	Invasive, n=59 (%)	95%-CI	p-value	FDR q-value
Median age, y (range)		37 (28-64)	35 (20-51)	41 (26-53)	47 (26-91)			
Age in years								
<30	7.7 (14/181)	1(3.4)	9 (18.8)	2 (4.4)	2 (3.4)	Ref.		
30-40	48.6 (88/181)	22(75.9)	27(56.3)	20(44.4)	19(32.2)	1.65(0.3-16.4)	0.729	0.94
> 40	43.6 (79/181)	6 (20.7)	12 (25)	23 (51.1)	38 (64.4)	5.6(1.1-53.5)	**0.021**	0.063
Age at sexual debut (in years)								
< 16	18.8 (34/181)	9 (31)	10 (20.8)	7 (15.6)	8 (13.6)	Ref.		
16 to 18	45.3 (82/181)	13 (44.8)	17 (35.4)	21 (46.7)	31 (52.5)	1.2(0.4-3.2)	0.812	0.812
> 18	35.9 (65/181)	7 (24.1)	21 (43.8)	17 (37.8)	20 (33.9)	0.9(0.3-2.4)	0.803	0.9
HPV infection *								
Untypable (Negative)	14.5 (25/173)	7 (26.9)	8 (17.4)	5 (11.4)	5 (8.8)	Ref.		
Positive	85.5 (148/173)	19 (73.1)	38 (82.6)	39 (88.6)	52 (91.2)	2.1(0.7-7.7)	0.17	0.31
Single HPV**	42.6 (63/148)	6(33.3)	17(44.7)	23(57.5)	17(32.7)	Ref.		
Multiple HPV**	57.4 (85/148)	12(66.7)	21(55.3)	17(42.5)	35(67.3)	0.53(0.2-1.1)	0.084	0.189

HIV-1 status								
Negative	51.9 (94/181)	20 (69)	34 (70.8)	21 (46.7)	19 (32.2)	Ref.		
Positive	48.5 (87/181)	9 (31)	14 (29.2)	24 (53.3)	40 (67.8)	3.4(1.7-6.5)	**<0.001**	**<0.001**
Parity								
0 to 4	55.8 (101/181)	20 (69)	35 (72.9)	24 (53.3)	22(37.3)	Ref.		
> 4	44.2 (80/181)	9 (31)	13 (27.1)	21 (46.7)	37 (62.7)	3.1(1.6-5.9)	**<0.001**	**<0.001**
Tobacco use								
No	72.4 (131/181)	26 (89.7)	36 (75)	29 (64.4)	40 (67.8)	Ref.		
Yes	27.6 (50/181)	3 (10.3)	12 (25)	16 (35.6)	19 (32.2)	1.4(0.7-2.7)	0.38	0.57

Key;

***** 8 samples with invalid HPV results were disregarded in the HPV infection analysis; 3 samples from CIN 1, 2 samples from CIN 2, one sample from CIN 3 and two samples from ICC.

** 25 samples with untypable HPV (HPV negative) were disregarded in the analysis; 8 samples from CIN 1, 8 samples from CIN 2, 4 samples from CIN 3 and 5 samples from ICC.

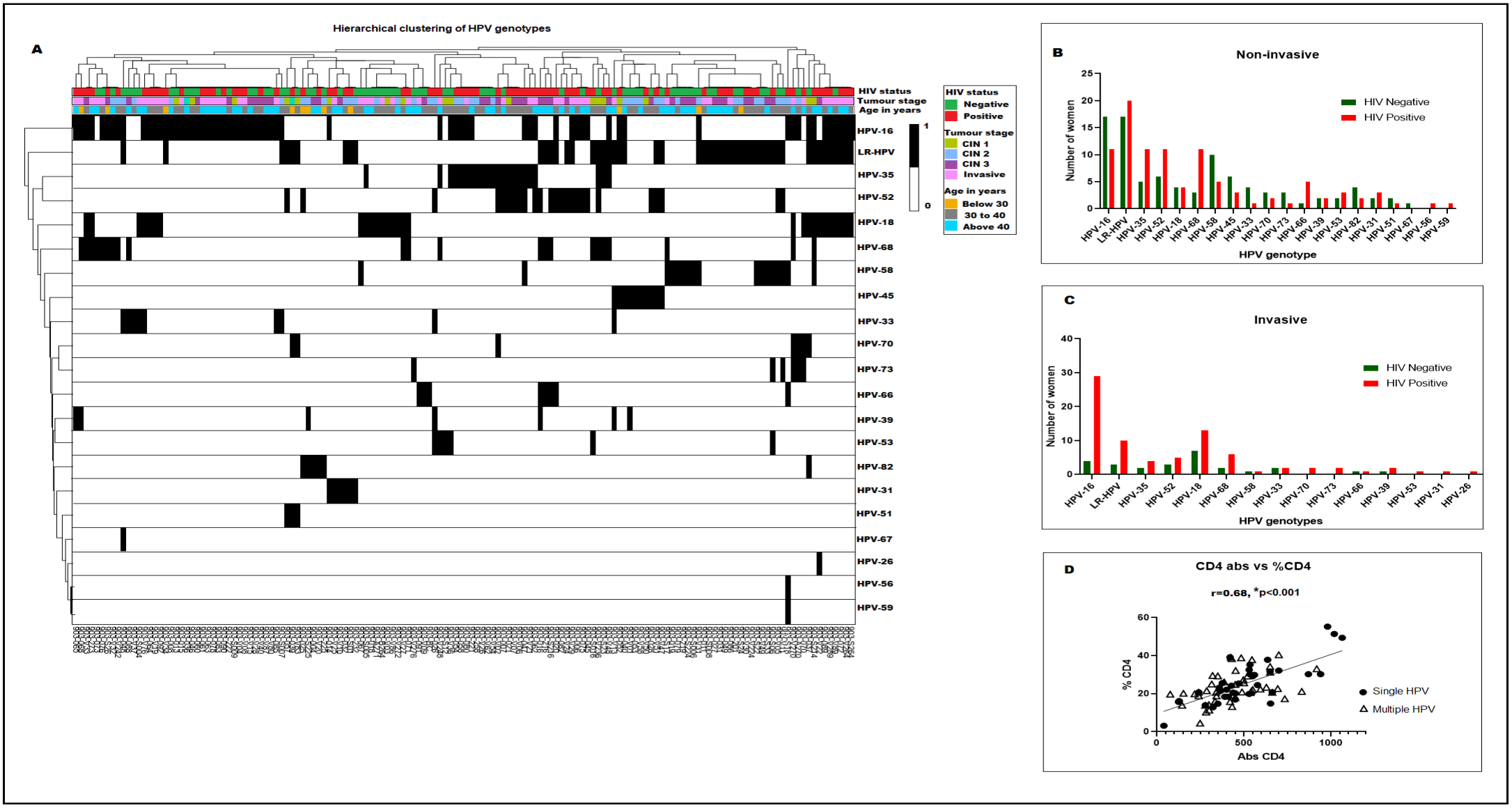
(Univariate analysis by linear regression model, cervical disease as a dependent variable, all *p-values* were adjusted for false discovery rate by the Benjamini-Hochberg test)

Whereas: n=actual number of patients, N=total sample size, CIN = Cervical intraepithelial neoplasm, 1 = Mild, 2= Moderate, 3 = Severe.

- (Chi-squared test, continuity of correlation (Yates) was not used due to the nature of the analysis).

Relationship between specific HPV genotypes, HIV-1 status, and tumour stages within different age groups.

Age, HPV genotypes, and HIV-1 status at different stages of cervical disease were investigated in the study cohort. As anticipated, HPV-16 genotype was evident most frequently, either as a single infection or in mixed infections with other HPV genotypes. Furthermore, HPV-16 genotypes were present at a significantly higher frequency in the ICC of HIV-1-positive patients (72.5%, 29/40) than in the ICC biopsies from HIV-1-seronegative women (21.1%, 4/19). There was a moderate correlation between CD4 count and CD4 percentage, irrespective of single or multiple HPV infections **($r=0.68$, $p<0.001$).** Collectively, these results show the presence of mixed HPV infections in both HIV-1-positive and HIV-1-seronegative women (**Figure 1A, 1B, 1C and 1D**).



-Some women may be counted more than once due to multiple HPV infections

Figure 3. 1. Illustration of HIV-1 status, HPV genotypes and age groups at different stages of cervical disease.

(**A**) Unsupervised hierarchical clustering showing HPV genotype distribution according to HIV-1 status (green = HIV-1-seronegative: red = HIV-1-positive), tumour stage (CIN 1-3 and invasive, and age in years <30; 30-40; >40). The X-axis represents individual biopsy samples and the Y-axis shows HPV genotypes. The presence of a specific HPV genotype is shown as solid black bars (1) and no colour (0) represents the absence of HPV genotype. The colour bars above the map indicate the HIV-1 status, tumour stage and age groups in years. (**B &C**) Histograms showing the numbers of women with specific HPV genotypes with precancerous lesions and with ICC, respectively. (**D**) Pearson correlation between absolute CD4 count and CD4 percentage in women with single HPV genotype (solid circles) and multiple HPV genotypes (open triangles).

Where;
CIN is cervical intraepithelial neoplasm, 1= mild, 2= moderate, 3= severe. Colour blocks do not represent the actual number of patients.

Hr-HPV = High risk HPV: HPV-16,-18,-31,-33,-35,-39,-45,-51,-52,-56,-59.
PHr-HPV = Probable high risk HPV: HPV-26,-53,-66,-67,-68,-70,-73 and -82.
Lr-HPV = Low risk HPV: HPV-6,-11,-40,-42,-54,-55,-61,-62,-64,-69,-71,-72,-81,-83 (HPV-CP6108) and –IS39.

Comparison between CD4 status, HPV infection and cervical disease in HIV-1-positive patients.

Consequently, the relationship between absolute CD4 count with CD4 percentage, HPV infection and cervical disease was examined in the HIV-1-positive women group in this study. Previous studies have shown that in some women, the stage of clinical immunosuppression during HIV-1 infection does not associate with stage of cervical disease (or ICC) [279]. Equally, high CD4 count and antiretroviral therapy do not appear to prevent HPV infection and cervical disease development [26, 279]. In this study, approximately 68% (59/87) of HIV-1-positive women with different stages of cervical disease presented with a CD4 percentage below or equal to 28% and a median absolute CD4 count of 400 cells/µl (Inter quartile range,

IQR, 300-500 cells/µl (**Figure 3.2A**). When the association between absolute CD4 count versus CD4 percentage was examined, there were significant moderate to strong correlations in all CIN (overall r=0.68, **p<0.001**; for CIN r=0.83, **p<0.001**, and a weak correlation in ICC (r=0.32, **p=0.043**), **Supplementary figure 3.1, -A, -B, and -C**). Cervical disease was more prevalent in women with low CD4 percentage regardless of the absolute CD4 count (**Figure 3.2, -B, and -C**). Moreover, larger proportion of women with ICC (70%, 28/40) possessed CD4 count higher than 350, compared to 30% (12/40) who possessed CD4 count ≤ 350 (**p<0.001, q<0.001, Figure 3.2D**). Furthermore, 75% (30/40) of women with ICC, possessed a CD4 percentage ≤28 versus 25% (10/40) who possessed CD4 percentage > 28% (**p<0.001, q<0.001, Figure 3.2E**). Notably, high absolute CD4 count ≥350 cells/µl was not protective against CIN 3 and ICC despite significant differences in CD4 count (All **p<0.001, q<0.001, Figure 3.2D**). Additionally, there was no significant difference between single and multiple HPV infections by immune cell status in both CIN and ICC (**Supplementary figure 3.2, -A, -B, and -C**).

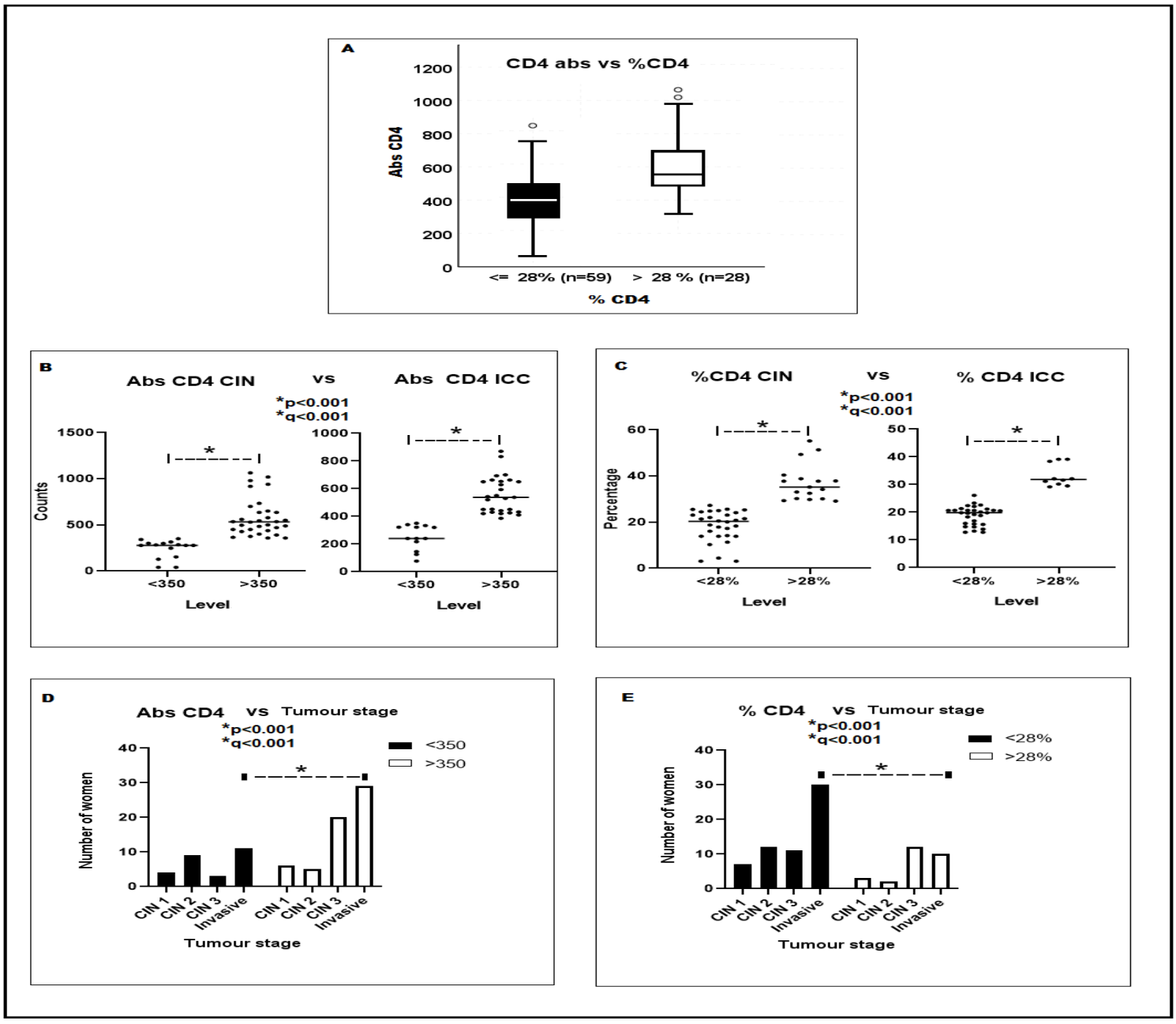
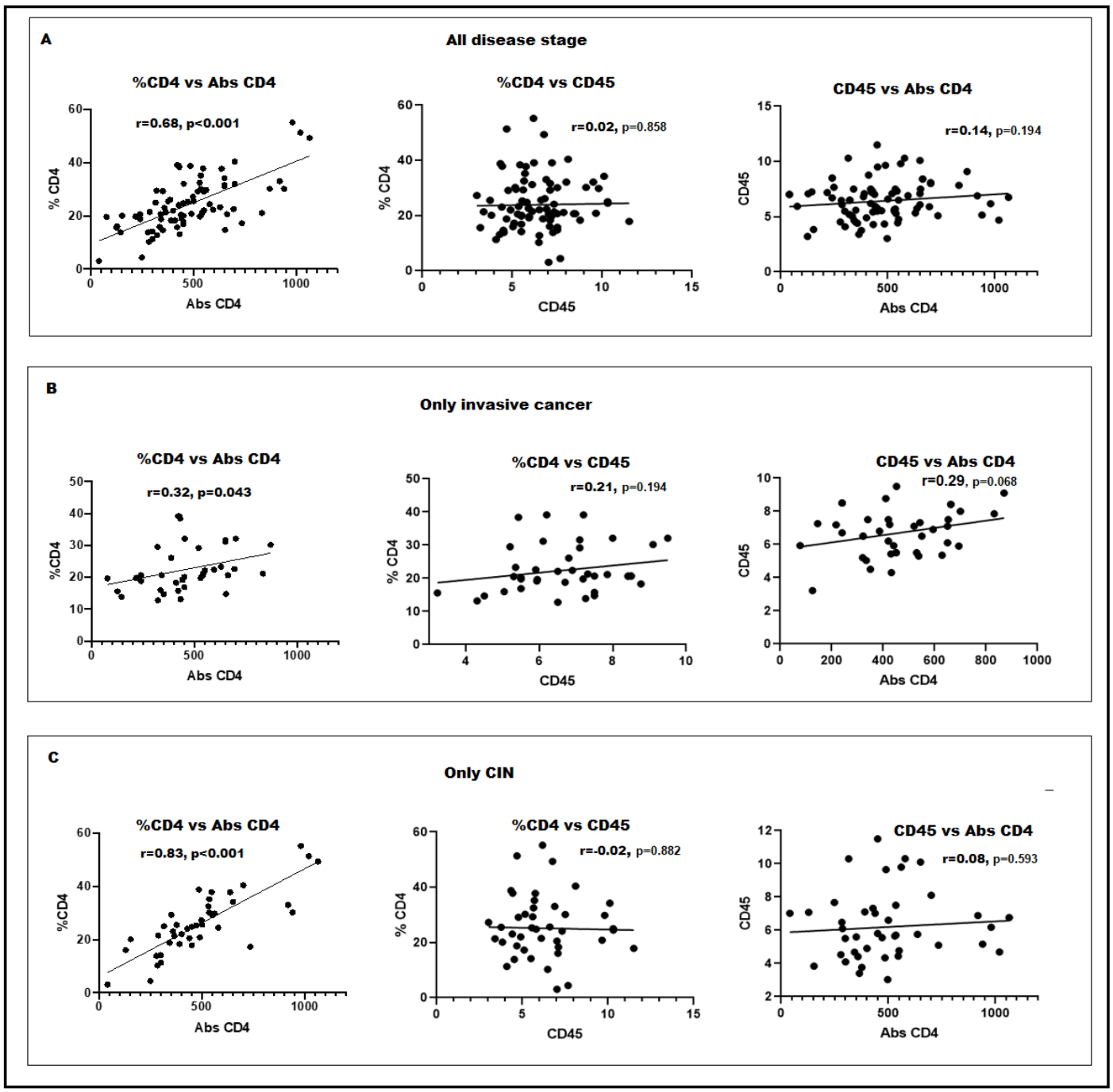


Figure 3. 2. Relationships between absolute CD4 count (Abs CD4), CD4 percentage (%CD4), HPV single or multiple infections, number of women and cervical disease stages in HIV-1-positive women.

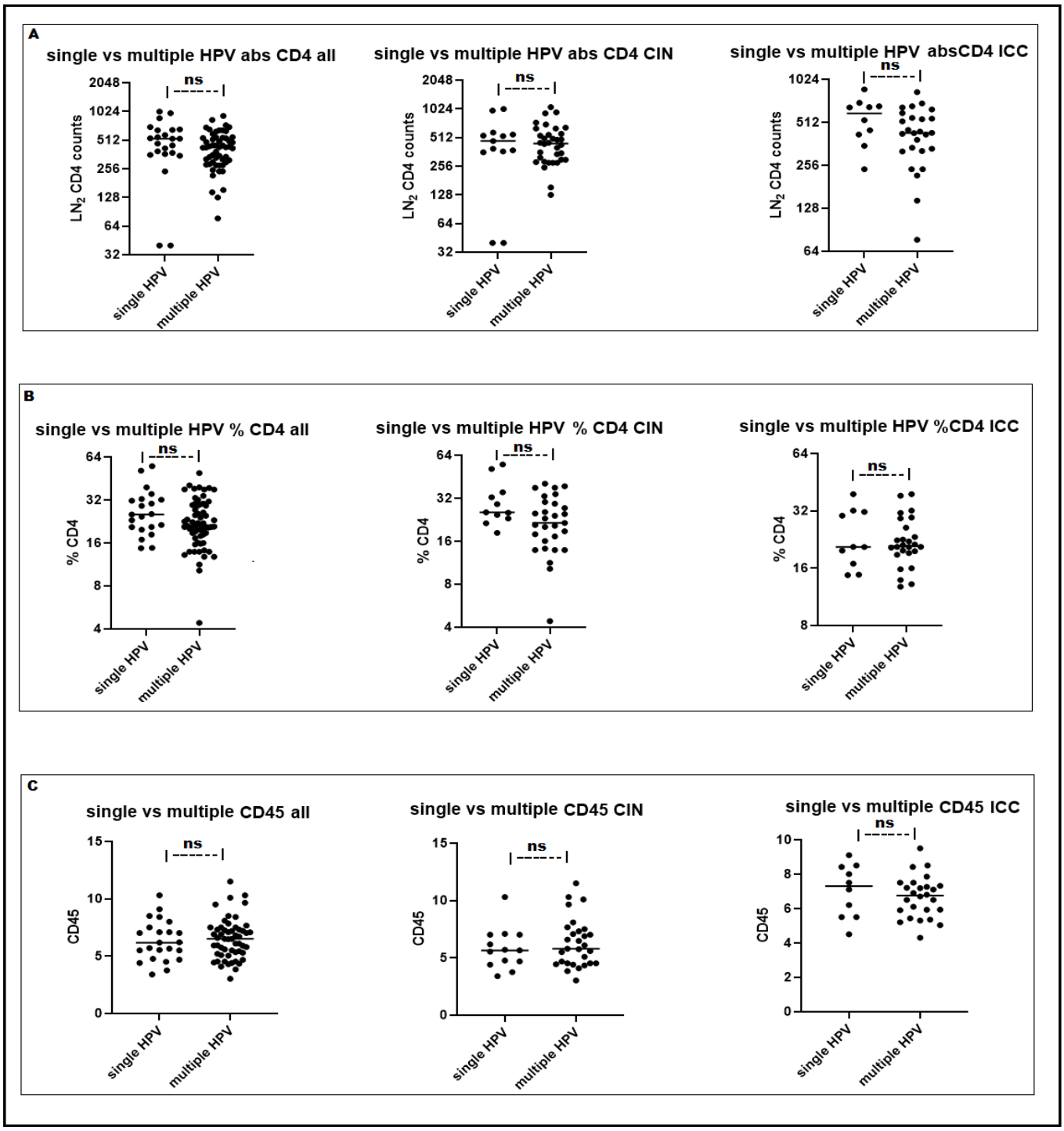
(A) Box and whisker plots showing the relationship between % CD4 and Abs CD4 with the number of patients and median CD4 count. **(B)** Scatter plots showing comparisons of levels of Abs CD4 between CIN and ICC **(C)** Scatter plots showing comparisons of % CD4 between CIN and ICC (**D**) Histograms showing the number of women and comparisons of tumour stages and Abs CD4 within cervical disease groups (**F**) Histograms showing the number of women and comparisons between tumour stage and % CD4 within cervical disease groups.

Where; R^2 = is the proportion of the variance (coefficient of determination), p = statistical power and q= Benjamini-Horchberg false discovery rate.



Supplementary Figure 3. 1. Scatter plots showing the relationships between absolute CD4 count (Abs CD4), CD4 percentage (%CD4) and CD45 count (CD45) in CIN and ICC.

(**A**) Overall correlations between abs CD4 versus % CD4 versus CD45 count in all disease stages. (**B**) Correlations between abs CD4, % CD4 and CD45 only in ICC. (**C**) Correlations between abs CD4, % CD4 and CD45 only in CIN.

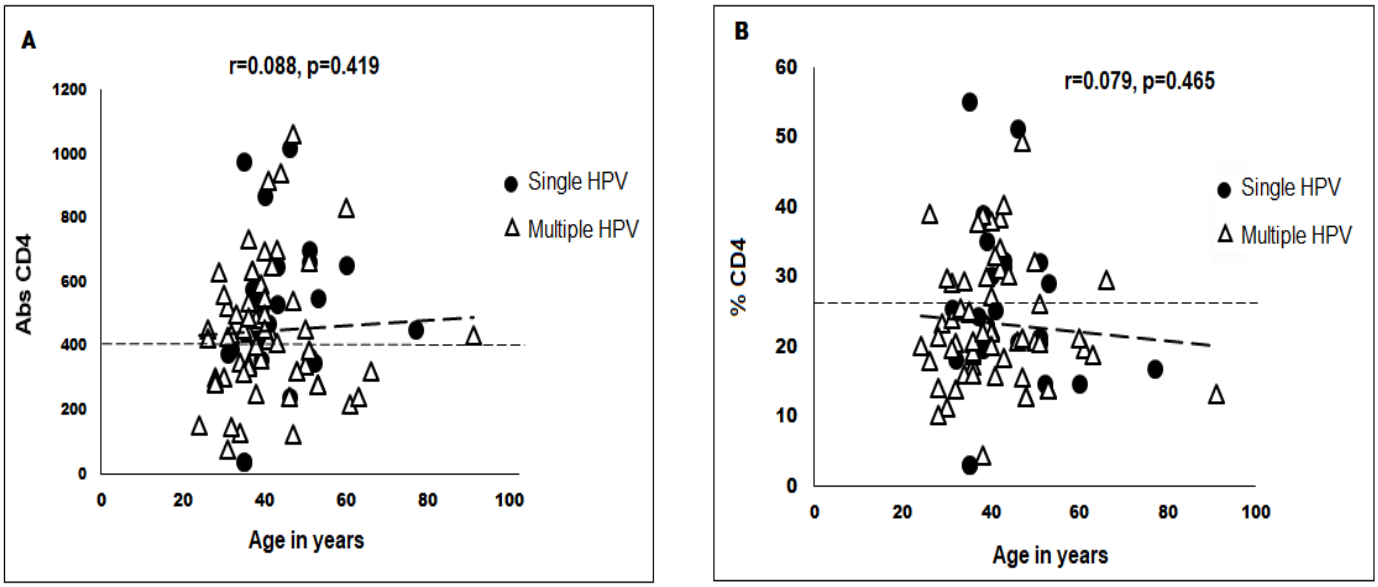


Supplementary Figure 3. 2. Scatter plots showing the relationships between single and multiple HPV infections by absolute CD4 count (Abs CD4), CD4 percentage (%CD4) and CD45 count (CD45) in CIN and ICC.

(A) Relationships between single and multiple HPV infections by Abs CD4 in CIN and ICC **(B)** Relationships between single and multiple HPV infections by % CD4 in CIN and **(C)** Relationships between single and multiple HPV infections by CD45 in CIN and ICC.

Comparison between age of patients, CD4 status and cervical disease in HIV-1-positive patients.

Aging and cancer are highly interconnected, ageing being a significant risk factor for cancer development [280]. However, in cervical cancer, it has already been reported that HIV-1-positive women develop invasive cervical cancer earlier, and at a younger age compared to HIV-1-seronegative women [99, 100, 281]. Since there is limited data on the impact of age and clinical immunological markers according to single or multiple HPV infections in cervical cancer development amongst HIV-1-positive women in Africa, this study sought to investigate the relationship between stage of cervical disease, single or multiple HPV infections, age, absolute CD4 count and CD4 percentage in HIV-1-positive women by using parametric Pearson's correlation test. Although there was no statistically significant correlation between age of patients and levels of CD4 count, as well as the age of patients and CD4 percentage, an even distribution of HPV single or multiple (genotype) infections was observed, regardless of age, absolute CD4 count or CD4 percentage. **(Supplementary figure 3.3, -A, and -B**).



Supplementary Figure 3. 3. Relationship between age of patients and levels of immune cell markers.

(A) Correlation between age and absolute CD4 count (Abs CD4). (B) Correlation between age and CD4 percentage (%CD4).

To investigate the effects of multiple immune predictor variables and age of patients at different stages of cervical disease, a statistical model of risk was created by using multinomial logistic regression that adds age, absolute CD4 count, CD4 percentage and CD45 white cell count as covariates to correct for multiple comparisons. Women older than 30 years had nearly 4 times the odds of developing ICC, however, the q value was not significant (**p = 0.043,** q= 0.172) **(Table 3.2)**. The results show that both CD4 count > 350 cells/µl and CD4 percentage > 28% did not appear likely prevent the risk of any cervical disease stage (all p>0.05) **(Table 3.2)**.

Table 3. 2: Cervical disease with different immune predictor variables and age of HIV-1-positive women

	Cervical disease stage									
	CIN 2 (n= 14)				CIN 3 (n=23)			ICC (n=40)		
Predictor variable	**%(n/N)**	**Adjusted OR(95% CI)**	**p-value**	**FDR q-value**	**Adjusted OR(95% CI)**	**p-value**	**FDR q-value**	**Adjusted OR(95% CI)**	**p-value**	**FDR q-value**
Age (years)										
≤30	9.2(8/87)	Ref.								
>30	90.8(79/87)	0.6(0.2-2.2)	0.42	0.84	1.4(0.4-5.1)	0.626	0.936	3.7(1.5-13.1)	**0.043**	0.172
CD4 count										
≤ 350	31(27/87)	Ref.								
> 350	69(60/87)	0.9(0.1-6.1)	0.911	1.215	3.6(0.5-24.6)	0.192	0.576	2.7(0.5-15.7)	0.255	0.255
CD4 percentage										
≤ 28	68(59/87)	Ref.								
> 28	32(28/87)	0.2(0.03-2.1)	0.194	0.776	0.9(0.2-5.3)	0.908	0.908	0.3(0.04-1.5)	0.131	0.175
CD45										
≤ 4 x 10 9	5.7(5/87)	Ref.								
> 4 x 10 9	94.3(82/87)	1.1 (0.1-12)	0.92	0.92	N/A	N/A	N/A	17(1-307)	0.051	0.102

CIN = Cervical intraepithelial neoplasm, 1 = Mild, 2= Moderate, 3 = Severe (Multinomial logistic regression model with cervical disease as a dependent variable, CIN 1 as reference category and all p-values were adjusted for false discovery rate (FDR) within a column of each cervical disease stage by the Benjamini-Hochberg test

This study next set out to determine whether there was any relationship between cervical tumour stage, age, CD4 cell levels, and HIV-1 status. The results show that there was no significant age difference between HIV-1-positive and HIV-1-seronegative women ($p=0.4792$, **Figure 3.3A**). However, there were weak to moderate significant correlations between age and stage of cervical disease in both HIV-1-positive and HIV-1-seronegative women ($r=0.39$, **$p=0.0002$ and $r=0.44$, $p<0.0001$**, respectively, **Figure 3.3, -B, and -C**). However, there was no correlation between tumour stage and absolute CD4 count, CD4 percentage or CD45 count (All $p>0.05$, **Figure 3.3, -D, -E, and -F**).

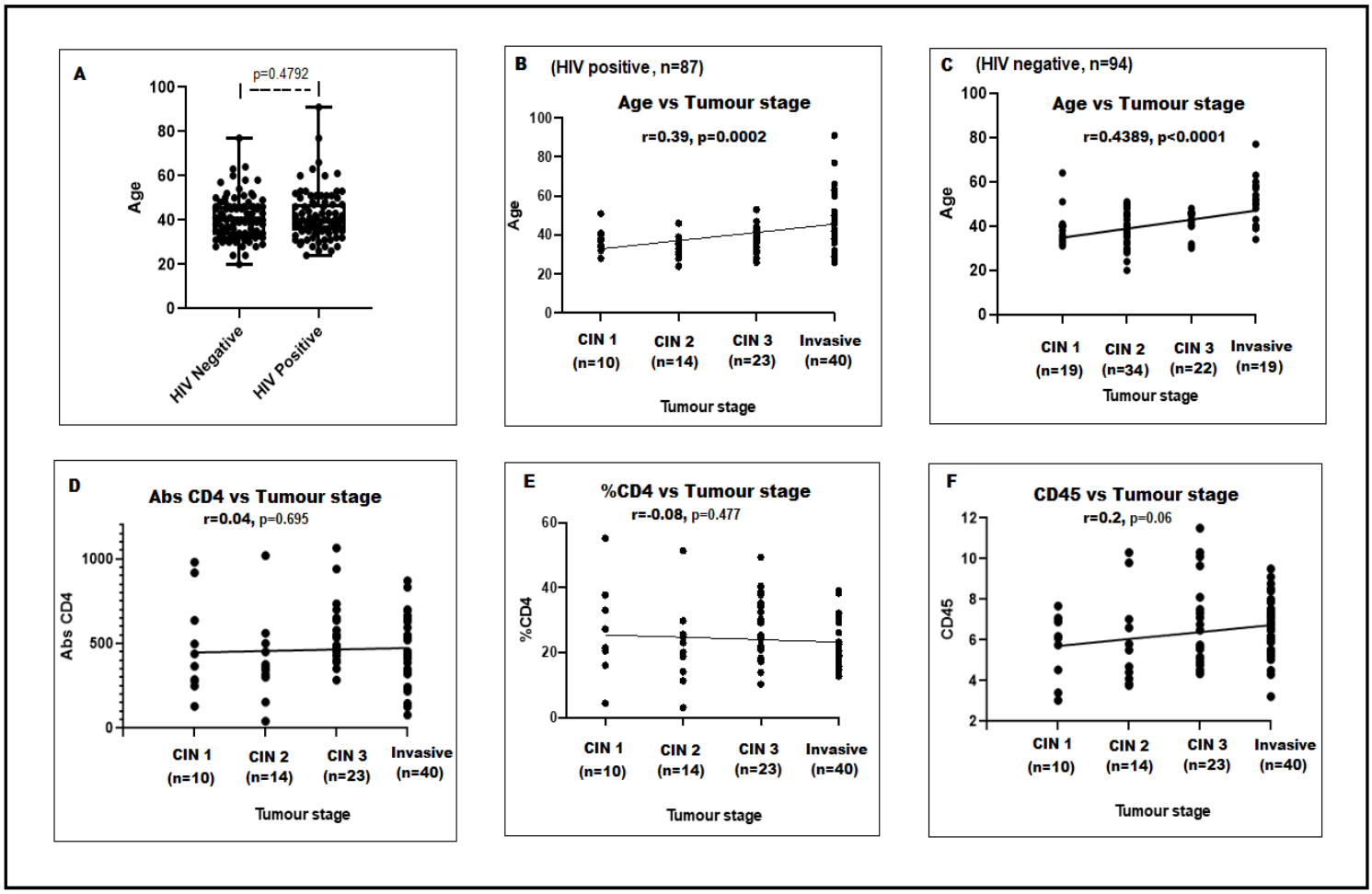


Figure 3. 3. Relationships between age, absolute CD4 count (Abs CD4), CD4 percentage (%CD4), CD45 count (CD45) and tumour stages.

(**A**) Box and whisker plots showing difference in age between HIV-1-positive and HIV-1-seronegative women with cervical disease. (**B**) Correlation between age and tumour stage in HIV-1-positive patients. (**C**) Correlation between age and tumour stage in HIV-1-seronegative patients. (D, E &F) Correlations between Abs CD4, %CD4, CD45 and tumour stage in HIV-1-positive patients.

Lastly, this study wished to determine whether age or the immune status of patients was predictive of cervical disease stage. ROC curves reflected that age was highly predictive of whether a woman was likely to be diagnosed with any of the CIN stages, including ICC, and not CD4 absolute count, CD4 percentage, nor CD45 count. Thus, only age was an independent predictor of cervical disease for both HIV-1-positive and HIV-1-seronegative patients [**p=0.0003 and <0.0001**, respectively, **Figure 3.4 and supplementary figure 3.4 (-A, -B, -C, -D, and -E)].**

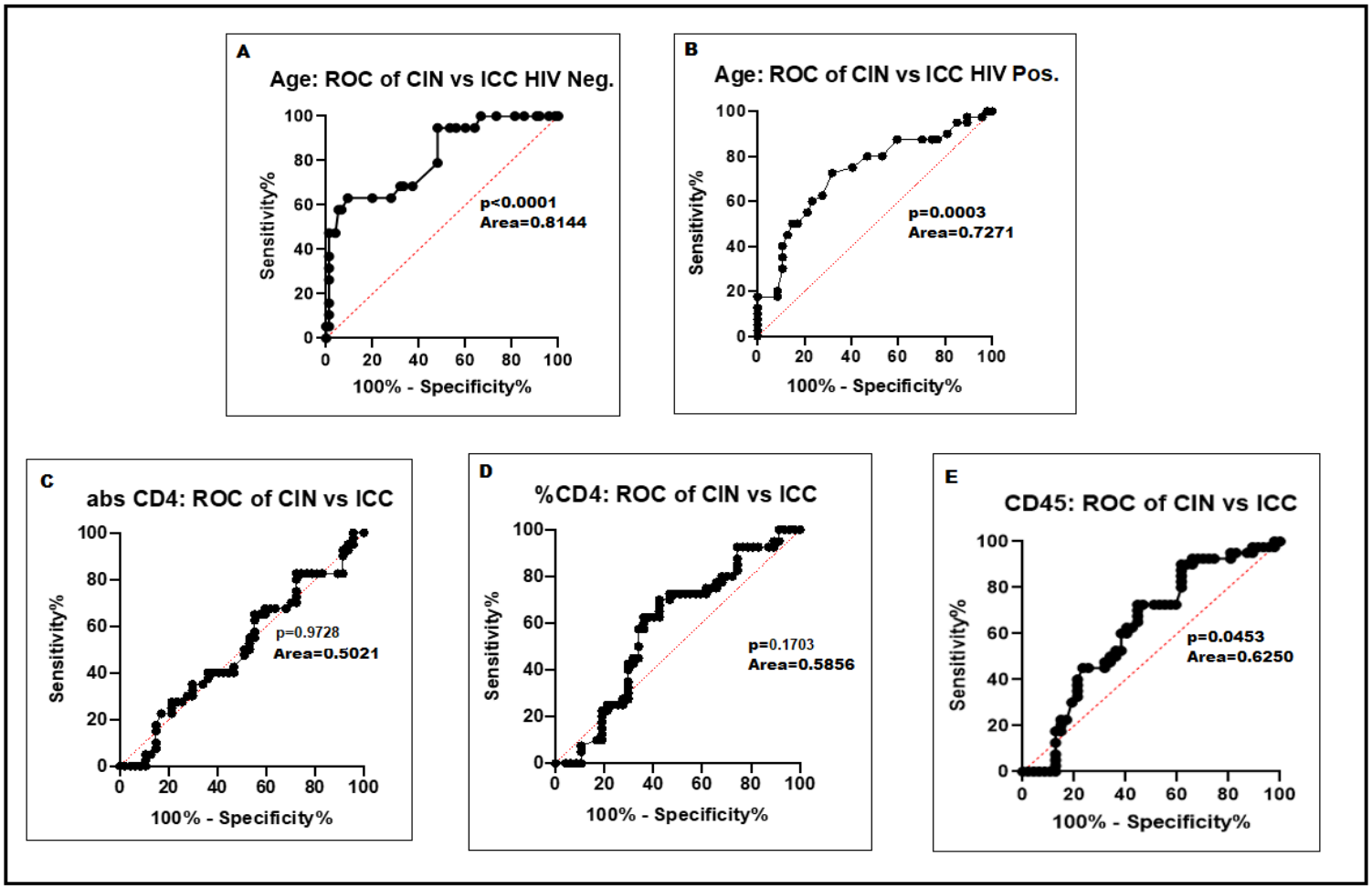
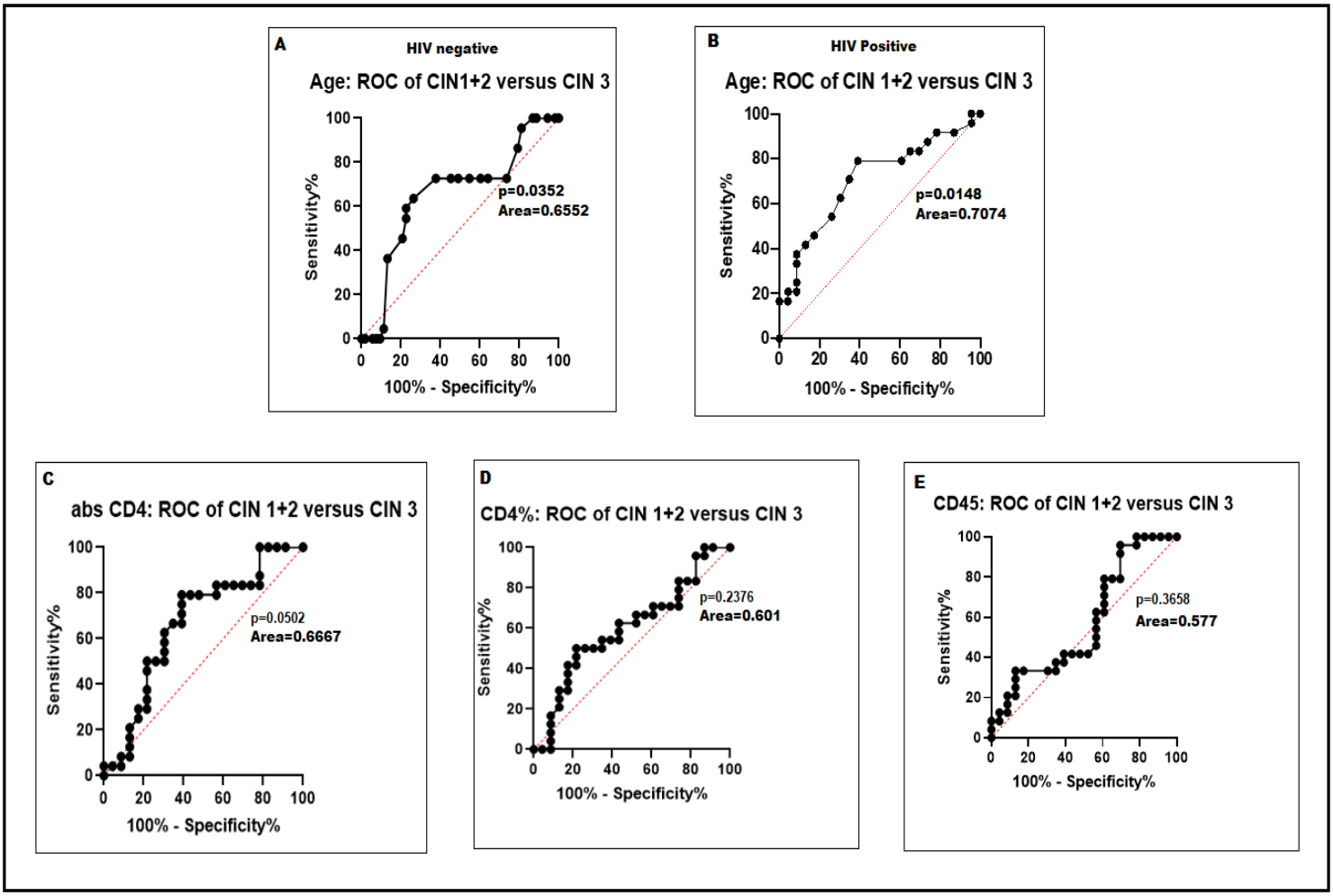


Figure 3. 4. Receiver operating characteristic (ROC) curves between age, absolute CD4 count (Abs CD4), CD4 percentage (%CD4), CD45 count (CD45) in CIN versus ICC.

(**A**) ROC curve of age between CIN versus ICC in HIV-1-seronegative women. (**B**) ROC of age between CIN versus ICC in HIV-1-positive women. (**C**) ROC curve of Abs CD4 between CIN versus ICC in HIV-1-positive women. (**D**) ROC curve of % CD4 between CIN versus ICC in HIV-1-positive women. (**E**) ROC curve of CD45 between CIN versus ICC in HIV-1-positive women.



Supplementary figure 3. 4. Receiver operating characteristic (ROC) curves between age, absolute CD4 count (Abs CD4), CD4 percentage (%CD4), CD45 count (CD45) in CIN 1 &2 versus CIN 3.

(**A**) ROC curve of age between CIN 1 &2 versus CIN 3 in HIV-1-seronegative women. (**B**) ROC of age between CIN 1 &2 versus CIN 3 in HIV-1-positive women. (**C**) ROC curve of Abs CD4 between CIN 1 &2 versus CIN 3 in HIV-1-positive women. (**D**) ROC curve of % CD4 between CIN 1 &2 versus CIN 3 in HIV-1-positive women. (**E**) ROC curve of CD45 between CIN 1 &2 versus CIN 3 in HIV-1-positive women.

4.4 Discussion

In this study, the distribution of HPV genotypes from 181 histologically-confirmed cervical tumour biopsies from HPV-unvaccinated women was investigated. An attempt was made to relate the stage of cervical disease with age, HIV-1-infection status and immune status (as measured by absolute CD4 count and CD4 percentage). A partial disconnect was observed between absolute CD4 count and CD4 percentage in HIV-1-positive women with cervical disease, where more women with invasive cervical cancer showed low CD4 percentage irrespective of the absolute CD4 count that falls close to normal range. This finding reveals some intriguing distinctions in low CD4 percentage in HIV-1-positive women diagnosed with invasive cervical cancer that requires further investigation. This is an important information because cervical disease progression differs significantly amongst HIV-1-positive women. It further suggests that other clinical immune cell markers apart from CD4 cells may be involved in the progression of cervical disease. Also the study results show that the distribution of HPV genotypes is related to stage of cervical disease in HIV-1-positive women.

This study is the first to report a partial disconnect between absolute CD4 count and CD4 percentage with cervical disease. There is also a novel association between low CD4 percentage and age (above 30 years) for HIV-1-positive women with invasive cervical cancer, where age was also significantly predictive for cervical cancer disease. In fact, only age was a significant independent predictor for stage of cervical disease in both HIV-1-seronegative and HIV-1-positive women. These results suggest that, both age and CD4 percentage are related to the development of cervical disease, but only age is a strong predictor for ICC disease outcome. Previously, Denny *et al.* [282], van Aardt *et al.*, [283] and Naucler *et al.*, [284] studied the distribution of HPV genotypes between HIV-1-positive and HIV-1-seronegative women, by using cervical tissue biopsies. However, they investigated only women with invasive cervical cancer and they did not consider the effects of age, CD4 count and CD4 percentage concurrently, while the present study does.

In the present study population, the significant positive linear correlation between absolute CD4 count and the CD4 percentage was not perfect **($r=0.69$, $r^2=0.48$)** and this was interpreted as only a partial disconnect between absolute CD4 count and CD4 percentage. In clinical HIV-1 studies, discordance between absolute CD4 count and the CD4 percentage have been reported in HIV-1-infected patients depending on the study population and the disease of interest [65, 285-287]. However, Anyimadu *et al.* [288] reported a discordance of 71.4% (20/28) in one of the sub-groups of HIV-1 infected patients. In this study, a discordance of about 68% (59/87) in HIV-1-positive women diagnosed pathologically with cervical disease was observed. The discordance between absolute CD4 count and CD4 percentage suggests that other lymphocytes apart from CD4 cells may be involved in the development of cervical disease. This is because CD4 percentage shows the relationship of CD4 cells in consideration with white blood cell count and lymphocyte differential in the body [65, 66]. Apart from CD4 cells, other immune cells could play a role in the development of cervical disease include, cytotoxic T cells (CD8+ T cells), B lymphocytes, natural killer cells, and other natural killer T cells [289].

An even distribution of single and multiple HPV infections, and cervical disease stage, regardless of CD4 count which falls within the normal range, was an observation similar to two previous reports [26, 290]. However, Denny *et al.* [291] found that having a CD4 count of more than 500/mm^3 was protective against the development of cervical disease for 36 months, once the analysis was adjusted for age, sexual activity and HIV-1 viral load. This discrepancy between the present study and that of Denny *et al.* [291], may be due to the fact that this study did not consider patient follow up and HIV-1 viral load. However, de Jong *et al.* [140], suggested that, there is an absence of functional HPV-16 specific CD4 T-cell immune response in some women. This may justify and explain the findings of ICC despite competent CD4 count observed in some women. Since there is no level of absolute CD4 count that puts HIV-1-positive women at lower risk for persistent infection with oncogenic HPV and cervical disease development, we suggest that HIV-1-positive women may be ideal for individualized HPV vaccination of adult HIV-1-positive women, as proposed by Dlamini *et al.* [292].

In HIV-1-positive women, HPV-16 genotype presented more in ICC biopsies than in non-invasive cancer biopsies (**Figure 3.1B &C**). However, we did not have enough sample size to assess the statistical significant difference. Future studies should focus on large sample size for this type of association analysis. Similar findings of high frequency of HPV-16 genotype infection as a single infection or in mixed infections with other HPV genotypes in HIV-1-positive women with cervical disease have been reported previously in different parts of the world, namely Brazil [262], Burkina Faso and South Africa in one study with samples from the two countries [293], Cameroon [294], South Africa [115, 257, 282] and the USA [295, 296]. Three studies; one from Mozambique [284], one from two countries, Kenya and South Africa [297], and one from South Africa [56], did not find the association of HIV-1 infection and HPV-16 genotype infection. However, two studies; the one from Kenya and South Africa, and the one from South Africa, used cervical swabs or brushes to collect cells/tissues around the cervix for HPV testing. Inconsistency in HPV-16 association in HIV-1-positive women using tumour biopsies and cervical swabs between the present study and other studies, could be due to sample size difference, different study populations and possibility of cross contamination with other genital HPV genotypes during biopsy processing or swab collection.

The strengths of the present study include: the use of histologically-confirmed cervical biopsies from precancerous lesions and invasive cancer, showing HPV distribution from cervical tumours from both unvaccinated HIV-1-positive and HIV-1-seronegative women, timely enumeration of CD4 cells in HIV-1-positive women. The limitations may include: being a cross-sectional study, hospital-based studies are likely to have some selection bias, limited sample size, possibilities of genital HPV contamination during biopsy collection and processing; a lack of focus on detailed information on immune status with regard to HIV-1 and HPV viral loads, duration of ART, no follow up of absolute CD4 count, and the time of acquisition of HIV-1 or HPV infection need to be taken into account. Also there was no confirmatory testing for presence of HIV-1 RNA in the studied tumour biopsies.

4.5 Conclusions

The present study suggests the presence of a partial disconnect between absolute CD4 count and CD4 percentage in a subgroup of HIV-1-positive women histologically diagnosed with cervical disease. HPV infection and cervical disease stage are irrelevant to the host CD4 immune status, but the cervical disease was more prevalent in women with low CD4 percentage regardless of the number of absolute CD4 count that falls within the normal range. However, only age is a strong independent predictor for ICC in both HIV-1-positive and HIV-1-seronegative women. Additional results of CD4 percentage should be used concurrently with absolute CD4 count to monitor cervical disease in HIV-1-positive women. Comprehensive investigation of low CD4 percentage with regard to HIV-1 and HPV viral loads in women with cervical disease is warranted in order to determine if this relationship is causal.

CHAPTER 4: Human Leukocyte Antigen (*HLA*) Class II -DRB1 and -DQB1 Alleles and the Association with Cervical Cancer in HIV-1/HPV Co-Infected Women in South Africa.

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ABSTRACT

Background

A subgroup of women who are co-infected with HIV-1 and HPV, progress rapidly to invasive cervical cancer, regardless of antiretroviral therapy (ART) or immune status. We posit that HIV-1/HPV co-infection along with specific host *HLA* II -DRB1 and -DQB1 alleles are involved in the development of cervical cancer.

Methodology

A hospital-based genetic susceptibility case-control study was conducted in Cape Town, South Africa. A total of 256 women of the same ethnicity were recruited, from which a total of 624 *HLA*-DRB1 and -DQB1 class II genotypes were determined. *HLA* II candidate genes were characterized using PCR-based, Luminex intermediate-resolution genotyping and confirmed significant associated genotypes at four-digit resolution by high-resolution gel

typing. Overall, 160 alleles were analyses from subjects with cancer, 64 alleles from subjects with precancerous lesions, and 400 alleles were analyzed from healthy control women. Whole blood was used for HIV-1 antibody testing and *HLA* II typing. Cervical tumour tissue biopsies were used for HPV genotyping. Tests were statistically significant if $p<0.05$.

Results

Women who were co-infected with HIV-1/HPV had advanced cervical disease compared to women who were HIV-1-seronegative. *HLA* II -DQB1*03:01 and -DQB1*06:02 alleles were associated with cervical cancer in HIV-1/HPV co-infected women ($p=0.001$ and $p<0.0001$, respectively) while *HLA* II-DRB1*13:01 and -DQB1*03:19 were rare or absent in women with cervical disease when compared to the control population ($p=0.012$ and 0.011, respectively).

Conclusions

Associations between *HLA* II genotypes with cervical cancer, or likely protection from cervical cancer disease in HIV-1/HPV co-infected South African women were described. Identifying mechanisms that give rise to this likely protective *HLA* association will provide insight into the development of immune-based prevention measures.

Keywords: HIV-1/HPV co-infection, *HLA* II allele association, cervical cancer disease.

4.1 Introduction

Each year, cervical cancer accounts for 528,000 new cases and 266,000 deaths worldwide [99]. Despite the recent implementation of HPV vaccine programs, cervical cancer still remains the most common cause of cancer-related mortality in women in Sub-Saharan Africa [18]. Approximately 99% percent of all cervical cancer cases are associated with persistent infection with oncogenic HPV, which is a causative organism for this disease [26]. Furthermore, women with HIV-1 infection are more likely to have a concurrent persistence of HPV infection than women who are not HIV-1-positive [26]. However, only a small

subgroup of HIV-1/HPV co-infected women will develop invasive cervical cancer [100]. This is regardless of HIV-1 suppression with antiretroviral therapy (ART) or with high CD4 cell count [282].

Although associations between HPV infection and Human Leukocyte Antigen (*HLA*) genotypes have previously been described [38], the relationship between HIV-1/HPV co-infection, *HLA* II and cervical cancer development has not been reported [27]. It is also unclear what the immune mechanism would be [104]. It is well established that T-cell recognition of virally-infected cells works through class I restricted-epitope recognition and that down-regulation of the viral peptide-*HLA* complex on the infected cell may lead to protection from cytotoxic T cells [166]. This would impair the ability of the individual to clear virus and thus allow HPV infection to persist [298]. Moreover, apart from the frequent overexpression of HPV E6 and E7 viral oncoproteins in cervical cancer, HPV-16 E5 protein has been shown to interfere with both *HLA* I and II antigen presentation [299]. This suggests that the HPV may inhibit CD4+ helper T-cells recognition through down-regulation of *HLA* II molecules and thereby evade host immunity. This effect might be exacerbated by the added effect of HIV-1 co-infection [300]. HIV-1 Tat proteins can directly interact with the *pRb/p130/p107/p53* tumour-suppressor genes and induce increased cell proliferation which may promote the effect of HPV oncoproteins E6 and E7 in accelerated cervical carcinogenesis [143, 145].

The etiology of cervical cancer has been related to certain high-risk *HLA* II genes [39]. Polymorphisms in *HLA*-DRB1 and -DQB1 genes are hypothesized to play a role in carcinogenesis of cervical cancer [38]. Despite considerable scientific interest, findings of different published studies have been inconsistent [86, 169, 298]. Several studies have reported the protective effects of *HLA*-DRB1*13:01-DQB*1**06:03 haplotypes on cervical cancer development and a positive association between *HLA* -DQB1*03:02 genes and cervical disease progression. However, this is not consistent in different population groups [111, 122, 171].

This study seeks to determine whether host *HLA*-DRB1 and -DQB1 backgrounds in HIV-1/HPV co-infected South African women, are involved in cervical cancer disease development. These results add new knowledge to the existing theories of rapid cervical cancer progression in HIV-1-positive women and aim to further inform on individualized immune-based host-directed cervical cancer prevention [86].

4.2 Methods

Research ethics

Ethics approval was obtained from the Human Research Ethics Committee of the University of Cape Town (HREC903/2015) and the National Health Laboratory Service (NHLS). Study approval was also obtained from the Western Cape Provincial Department of Health.

Sample size, study population and selection criteria

A total of 256 indigenous South African women were recruited in a hospital-based genetic susceptibility case-control study conducted at Groote Schuur Hospital, Cape Town, in the Western Cape province of South Africa. The recruitment processes were conducted from June 2016 to March 2017. The ethnicity and geographic ancestry of cases and the controls were the same in order to reduce the bias due to the diversity of *HLA* II genes within South Africa [301]. The study categorized the cases and controls as 'Black African' in terms of South Africa's five official population categories, similar to a previous study [302]. Consent was obtained from women who attended the outpatient gynaecological cancer assessment clinic, the colposcopy clinic or who were admitted to the gynaecology emergency room, to participate in the study. The control group consisted of age-matched healthy women from the same study population. The control group was compiled from historical data derived from 200 archived records in the Laboratory for Tissue Immunology (LTI) at the National Health Laboratory Service (NHLS), at Groote Schuur Hospital. The study compiled data from unrelated age-matched female donors of related bone marrow, renal and other organ

transplants, which represented the background population for the *HLA* II genes. These *HLA* typing results from the control group were counter checked by a second experienced person in the laboratory in order to confirm the allele genotypes, sex, age and ethnicity as "Indigenous African" women.

Specimen collection

Written and signed consent forms were used in the language of the subject's choice in the presence of a witness, and after detailed discussion with patients regarding the aims and nature of the study. A trained registered nurse who was fluent in the local languages explained the details of the study and answered questions from the patients before their consent was requested. Peripheral blood (8ml) was collected using two EDTA tubes (BD Vacutainer®, Johannesburg, South Africa) for genomic DNA isolation and *HLA* typing. After visual inspection of the cervix, Gynaecologists collected punch biopsies of abnormal cervical lesions. A small section of the tissue biopsy was preserved in Digene® specimen transport medium (Qiagen, Johannesburg, South Africa) and then stored at -80^0C until processed in order to preserve the genomic DNA for HPV genotyping. Another fraction of the same biopsy was stored in formalin and sent to the Anatomical Pathology Laboratory at the NHLS for histopathology analyses as described in a previous study [303]. All patients were recruited before the initiation of radiotherapy or chemotherapy in order to avoid DNA damage during cancer therapy [304].

HIV-1 antibody test

According to the South African HIV-1 testing algorithm, 20 µl of the collected peripheral whole blood was used for rapid HIV-1 antibody testing (Determine, Alere, Inc., Johannesburg, South Africa) for all cases [270]. For the control group, a retrospective follow-up was done on the NHLS Bone Marrow Registry and the organ transplant database for HIV-1 status. All women in the control group were HIV-1-seronegative.

HPV DNA genotyping and detection

Genomic DNA was extracted from cervical tumour tissue specimens using the Qiagen ® QIAamp DNA Mini purification kit (Qiagen, Johannesburg, South Africa) according to the manufacturer's protocol. The concentration of the extracted DNA was quantified with a Nanodrop® spectrophotometer (Thermo Fisher, Johannesburg, South Africa). Due to generally high genomic DNA concentration from the tissue biopsies, the DNA was diluted using nuclease- free water (Thermo Fisher, Johannesburg, South Africa) to reach a recommended final concentration of 0.2ng/µl. The HPV genotyping and detection tests were performed by using the Linear array® PCR-based HPV genotyping kit (Roche, Johannesburg, South Africa). The HPV status was only genotyped for cases, depending on the availability of tissue specimens in this study.

***HLA* Typing**

Genomic DNA was extracted from blood collected from patients using a modification of the salting out DNA extraction method for whole blood [305]. A Nanodrop® spectrophotometer (Thermo Fisher Scientific, Johannesburg, South Africa) was used to measure the concentration and purity of the extracted DNA; using the A260/280 ratio was to be in a range of 1.8 to 1.9. The DNA was further diluted using nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa) to reach a recommended final concentration of 0.3ng/µl. Intermediate resolution by commercial kits using the Luminex platform for reverse sequence-specific oligonucleotide primers (SSOP) *HLA* typing technology were used (Immucor, Sussex, England) [306]. The PCRs were performed in thermal cyclers using both PCR-SSOP and PCR-sequence-specific primers (SSP) supplied by the manufacturer in the *HLA* II typing kit. Amplification reactions were carried out according to the manufacturer's protocol. The *HLA* II alleles were analyzed using the *HLA* II data analysis software, *Match It* ® [307]. Further confirmation of the *HLA* II genotypes that resulted in significant associations were undertaken with Olerup® SSP (Immucor Inc. Johannesburg, South Africa) *HLA* II high-resolution gel typing according to the manufacturer's protocol.

Statistical analyses

A total of 624 *DRB1 *DQB1 *HLA* II alleles were analyzed. This study characterized 160 alleles from cancer patients, 64 alleles from pre-cancer patients and 400 alleles from unrelated age-matched women in the healthy control group. Allele frequencies for *HLA*-DRB1and *HLA*-DQB1 were calculated by direct counting, as described in a previous study [37]. The observed genotype frequencies in the controls were tested for Hardy-Weinberg equilibrium. The *HLA* allele frequencies for DRB1 and DQB1 alleles were compared between the cases and controls using the Fisher's exact test with 2 × 2 tables or by the $\chi 2$ test with Mantel-Haenszel correction, where appropriate. Multiple testing correction was not required due to the nature of the study [308]. The odds ratios (ORs), 95% confidence intervals (95% CIs) and the *p-values* calculated for multiple comparisons were considered significant at $p<0.05$.

4.3 Results

The results for *HLA*-DRB1 and -DQB1 allele frequencies in women, regardless of HIV-1 co-infection are summarized in Table 4.1, which shows that, without consideration of HIV-1 co-infection status, in terms of cervical cancer susceptibility, *HLA*-DQB1*03:01 (p=0.002, OR 4.66, 95% CI 1.85-11.72) and *HLA*-DQB1*06:02 (p=0.002, OR 2.68, 95% CI 1.45-4.96) were significantly associated with cervical cancer disease in cases compared to the healthy control group. Furthermore, *HLA*-DRB1*13:01 (p=0.012, OR 0.18, 95% CI 0.02-0.75) and *HLA*-DQB1*03:19 (p=0.011, OR 0.11, 95% CI 0.003-0.74) were found to be significantly low or absent for the relative risk of cervical disease development, suggestive of protective alleles.

Table 4. 1 Relative risk for cervical cancer development with specific *HLA*-DRB1and -DQB1 allele exposure without consideration of HIV-1 co-infection status.

(Table from Chambuso *et al.*, (2019) [185] with reprint permission from the Journal of Cancer)

HLA DRB1	Cancer Allele frequency (2n=83)	Pre-cancer Allele frequency (2n= 33)	Healthy controls (Allele frequency) (2n=205)	Relative Risk for pre-cancer Odds ratio (95%CI)	P-value	Relative Risk for cancer Odds ratio (95%CI)	P-value
***01:01**	0.024 (2)	0.03 (1)	0.024 (5)	1.26(0.03-11.79)	0.59	1(0.19-5.26)	>0.999
***01:02**	0.1 (8)	0.121 (4)	0.05 (11)	2.45(0.5-9.0)	0.135	1.91(0.6-5.4)	0.193
***03:01**	0.072 (6)	0.09 (3)	0.073 (15)	1.28(0.22-4.92)	0.721	1(0.31-2.86)	>0.999
***03:02**	0.072 (6)	0.15 (5)	0.093 (19)	1.8(0.47-5.43)	0.345	0.77(0.24-2.12)	0.817
***04:01**	0.036 (3)	0	0.024 (5)	N/A	N/A	1.52(0.23-8.01)	0.693
***04:04**	0	0.09 (3)	0.02 (4)	5.07(0.7-31.3)	0.057	N/A	N/A
***04:05**	0	0.03 (1)	0.005 (1)	6.42(0.08-506)	0.257	N/A	N/A
***07:01**	0.084 (7)	0.06 (2)	0.07 (14)	0.86(0.09-4.17)	>0.999	1.27(0.42-3.54)	0.62
***08:01**	0.012 (1)	0.03 (1)	0.005 (1)	6.4(0.1-506)	0.257	2.5(0.03-198.7)	0.491
***08:04**	0.012 (1)	0.06 (2)	0.04 (8)	1.6(0.3-7.9)	0.632	0.3(0.01-2.34)	0.454
***09:01**	0.036 (3)	0.03 (1)	0.01 (2)	3.2(0.1-62.6)	0.361	3.86(0.43-46.75)	0.143
***10:01**	0.06 (5)	0	0.024 (5)	N/A	N/A	2.6(0.58-11.6)	0.155
***11:01**	0.1 (8)	0.09 (3)	0.112 (23)	0.8(0.14-2.9)	>0.999	0.86(0.32-2.1)	0.835
***11:02**	0.084 (7)	0.03 (1)	0.08 (16)	0.37(0.01-2.6)	0.48	1.1(0.37-2.98)	0.813
***11:04**	0.024 (2)	0	0.005 (1)	N/A	N/A	5.1(0.26-302.5)	0.197
***12:01**	0.036 (3)	0.06 (2)	0.024 (5)	2.6(0.24-16.69)	0.249	1.52(0.23-8.01)	0.693
***13:01**	0.024 (2)	0	0.122 (25)	N/A	N/A	0.18(0.02-0.75)	**0.012**
***13:02**	0.036 (3)	0.09 (3)	0.07 (14)	1.4(0.2-5.3)	0.713	0.5(0.1-1.9)	0.411
***13:03**	0.072 (6)	0	0.03 (7)	N/A	N/A	2.2(0.6-7.9)	0.208
***14:01**	0.024 (2)	0	0.01 (2)	N/A	N/A	2.5(0.18-35.45)	0.323
***15:01**	0.05 (4)	0.03 (1)	0.024 (5)	1.26(0.03-11.79)	0.594	2.05(0.4-9.79)	0.281
***15:03**	0.05 (4)	0	0.083 (17)	N/A	N/A	0.57(0.13-1.82)	0.452
***HLA* DQB1**	**(2n=77)**	**(2n=31)**	**(2n=195)**				
***02:01**	0.078 (6)	0.065 (2)	0.09 (18)	0.67(0.07-3.06)	>0.999	0.82(0.26-2.27)	0.815
***02:02**	0.13 (10)	0.032 (1)	0.15 (30)	0.18(0.03-1.19)	0.09	0.81(0.33-1.82)	0.706
***03:01**	0.169 (13)	0.063 (2)	0.04 (8)	1.6(0.16-8.54)	0.632	4.66(1.85-11.72)	**0.002**
***03:02**	0.04 (3)	0.063 (2)	0.055 (11)	1.1(0.12-5.42)	0.696	0.67(0.12-2.6)	0.763
***03:19**	0.013 (1)	0	0.1 (20)	N/A	N/A	0.11(0.003-0.74)	**0.011**
***04:02**	0.1 (8)	0.129 (4)	0.065 (13)	2.1(0.45-7.3)	0.265	1.6(0.55-4.36)	0.322
***04:04**	0	0	0.005(1)	N/A	N/A	N/A	N/A
***05:01**	0.065 (5)	0.194 (6)	0.11 (22)	1.87(0.56-5.34)	0.24	0.54(0.15-1.54)	0.268
***05:03**	0.026 (2)	0	0.05 (10)	N/A	N/A	0.49(0.05-2.27)	0.519
***06:02**	0.324 (25)	0.29 (9)	0.145 (29)	2.3(0.85-5.81)	0.07	2.68(1.38-5.17)	**0.002**
***06:03**	0.013 (1)	0.032 (1)	0.07 (14)	0.43(0.01-3.02)	0.7	0.17(0.004-1.15)	0.075
***06:04**	0.013 (1)	0	0.075 (15)	N/A	N/A	0.16(0.004-1.05)	#**0.046**
***06:06**	0	0.097 (3)	0	N/A	N/A	N/A	N/A
***06:09**	0.026 (2)	0.032 (1)	0.02 (4)	1.58(0.03-16.6)	0.527	1.26(0.11-8.96)	>0.999

#The significant p-value was disregarded because the confidence interval crosses '1'

Table 4.2 shows consideration of HIV-1 co-infection status, whereby in HIV-1/HPV co-infected women who had cervical cancer, *HLA*-DQB1*03:01 (p=0.001, OR 5.6, 95% CI 1.9-16.9) and -DQB1*06:02 (p<0.0001, OR 4.5, 95% CI 2.2-9.0) were significantly associated with the risk of disease. However, *HLA*-DRB1*13:01, *HLA*-DQB1*03:19 showed likely protection against cervical cancer due to completely absence or rare in both HIV-1/HPV co-infected and HIV-1-seronegative cervical disease population.

Table 4. 2 Relative risk for developing cervical cancer in HIV-1/HPV co-infected women with specific *HLA*-DRB1 and -DQB1 allele exposure.

The cancers may be HIV-1-seronegative but they are not HPV-negative. (Table from Chambuso *et al.*, (2019) [185] with reprint permission from the Journal of Cancer)

HLA DRB1	HIV-1/HPV Co-infected cancer Allele frequency (2n=60)	HIV-1 Negative cancer Allele frequency (2n=23)	Healthy Controls Allele frequency (2n=205)	Relative Risk for HIV-1 Negative cancer ODDS ratio(95%CI)	P-value	Relative Risk for HIV-1/HPV Co-infected cancer ODDS ratio(95%CI)	P-value
***01:01**	0.017 (1)	0.043 (1)	0.024 (5)	1.9(0.04-17.7)	0.469	0.7(0.01-6.3)	>0.99
***01:02**	0.1 (6)	0.087 (2)	0.05 (11)	1.7(0.2-8.7)	0.623	1.9(0.57-6.17)	0.227
***03:01**	0.05 (3)	0.13 (3)	0.073 (15)	1.95(0.33-7.8)	0.399	0.7(0.12-2.5)	0.771
***03:02**	0.05 (3)	0.13 (3)	0.093 (19)	1.5(0.26-5.8)	0.464	0.5(0.1-1.9)	0.425
***04:01**	0.05 (3)	0	0.024 (5)	N/A	N/A	2.1(0.32-11.3)	0.384
***04:04**	0	0	0.02 (4)	N/A	N/A	N/A	N/A
***04:05**	0	0	0.005 (1)	N/A	N/A	N/A	N/A
***07:01**	0.083 (5)	0.087 (2)	0.068 (14)	1.33(0.14-6.5)	0.663	1.3(0.34-3.9)	0.775
***08:01**	0.017 (1)	0	0.005 (1)	N/A	N/A	3.5(0.04-275.3)	0.399
***08:04**	0.017 (1)	0	0.04 (8)	N/A	N/A	0.4(0.01-3.3)	0.689
***09:01**	0.05 (3)	0	0.01 (2)	N/A	N/A	5.4(0.6-65.6)	0.077
***10:01**	0.083 (5)	0	0.024 (5)	N/A	N/A	3.7(0.81-16.5)	**#0.049**
***11:01**	0.083 (5)	0.13 (3)	0.112 (23)	1.22(0.21-4.6)	0.728	0.7(0.21-2.1)	0.638
***11:02**	0.067 (4)	0.13 (3)	0.08 (16)	1.8(0.31-7.2)	N/A	0.9(0.2-2.8)	>0.99
***11:04**	0.033 (2)	0	0.005 (1)	N/A	N/A	7.1(0.36-421.3)	0.128
***12:01**	0.017 (1)	0.087(2)	0.024 (5)	3.9(0.35-25.54)	0.145	0.7(0.01-6.3)	>0.999
***13:01**	0	0.087(2)	0.122 (25)	0.7(0.1-3.2)	>0.99	N/A	N/A
***13:02**	0.033 (2)	0.043(1)	0.068 (14)	0.6(0.01-4.5)	>0.99	0.5(0.1-2.15)	0.537
***13:03**	0.01 (6)	0	0.034 (7)	N/A	N/A	3.1(0.83-11.4)	0.081
***14:01**	0.017 (1)	0.043 (1)	0.01 (2)	4.7(0.08-93)	0.27	1.74(0.03-33.8)	0.536
***15:01**	0.067 (4)	0	0.024 (5)	N/A	N/A	2.9(0.55-13.9)	0.118
***15:03**	0.067 (4)	0	0.083 (17)	N/A	N/A	0.8(0.19-2.6)	>0.99
HLA **DQB1**	**(2n=56)**	**(2n=21)**	**(2n=195)**				
***02:01**	0.036 (2)	0.19 (4)	0.092 (18)	2.2(0.5-7.9)	0.247	0.36(0.08-1.6)	0.263
***02:02**	0.125 (7)	0.143 (3)	0.154 (30)	0.9(0.16-3.3)	>0.99	0.78(0.27-1.9)	0.674
***03:01**	0.196 (11)	0.1 (2)	0.041 (8)	2.4(0.23-13.2)	0.259	5.6(1.9-16.9)	**0.001**
***03:02**	0.036 (2)	0.048 (1)	0.056 (11)	0.8(0.02-6.2)	>0.99	0.61(0.06-2.9)	0.739
***03:19**	0	0.048 (1)	0.103 (20)	0.4(0.01-2.9)	0.702	N/A	N/A
***04:02**	0.054 (3)	0.238 (5)	0.067 (13)	4.2(1.3-13.3)	**0.022**	0.8(0.14-3)	>0.99
***04:04**	0	0	0.005 (1)	N/A	N/A	N/A	N/A
***05:01**	0.054 (3)	0.1 (2)	0.113 (22)	0.8(0.1-3.7)	**#0.017**	0.44(0.1-1.6)	0.218
***05:03**	0.018 (1)	0.048 (1)	0.05 (10)	0.9(0.02-6.9)	>0.99	0.33(0.01-2.4)	0.465
***06:02**	0.446 (25)	0	0.149 (29)	N/A	N/A	4.5(2.2-9.0)	**<0.0001**
***06:03**	0	0.048 (1)	0.072 (14)	0.63(0.01-4.6)	>0.999	N/A	N/A
***06:04**	0.018 (1)	0	0.077 (15)	N/A	N/A	0.22(0.01-1.47)	0.131
***06:09**	0.018 (1)	0.048 (1)	0.02 (4)	2.3(0.05-24.9)	0.41	0.86(0.02-8.9)	>0.99

#The significant p-value was disregarded because the confidence interval crosses '1'.

In addition, the majority of women who were co-infected with HIV-1/HPV had advanced cervical disease compared to women who were HIV-1-seronegative. Moreover, alleles DQB1*03:01 and DQB1*06:02 were significantly associated with the HIV-1/HPV-co-infected cervical cancer population. (**Figure 4.1**).

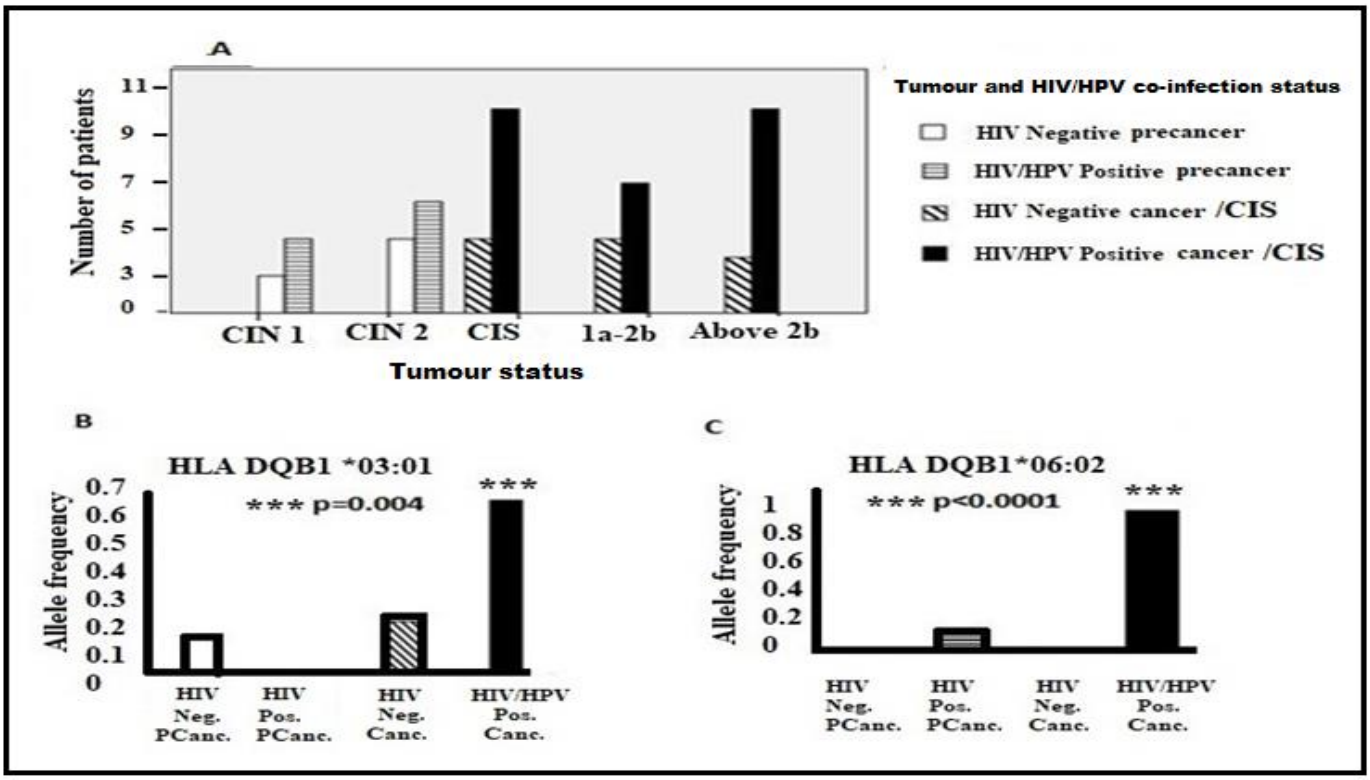

Figure 4. 1 Association of tumour status, specific *HLA* II alleles and HIV-1/HPV co-infection.

(A) HIV-1/HPV co-infection, tumour status and the number of patients. **(B)** and **(C)** show associations of *HLA*-DQB1 *03:01 and *HLA*-DQB1 *06:02 alleles, respectively, in HIV-1/HPV co-infected cervical cancer patients. (Figure from Chambuso *et al.*, (2019) [185] with reprint permission from the Journal of Cancer)

Where; FIGO = International Federation of Obstetrics and Gynaecology.

1a-2b = FIGO cervical cancer stage 1a up to stage 2b.

Above 2b = FIGO cervical cancer stage above stage 2b.

CIN = Cervical intraepithelial neoplasia, 1= Mild, 2= Moderate

CIS = Carcinoma *In situ*

Neg. Pcanc.= Negative precancer

Pos. Pcanc. = Positive precancer

Neg. Canc. = Negative cancer

Pos. Canc. = Positive cancer

The comparison between intermediate, SSP and Olerup high-resolution *HLA* II typing only for significant alleles is shown in **Table 4.3**. The study confirmed that the susceptible alleles for cervical cancer disease in HIV-1/HPV co-infected women were DQB1*03:01:01:01 and DQB1*06:02:01:01.

Table 4. 3 Association of *HLA* II alleles with cervical cancer susceptibility, likely protection and HIV-1/HPV co-infection after confirmation with high-resolution gel typing.

Cervical cancer and HLA II alleles							
Significant alleles	**Luminex intermediate *HLA* typing**	**SSP high resolution *HLA* typing**	**Olerup high resolution *HLA* typing**	**Cancer Patients #(2n=83)**	**Healthy controls #(2n=205)**	**Odds ratio (95%CI)**	**p-value**
Susceptibility	DQB1*03:01	DQB1*03:01	DQB1*03:01:01:01	0.157 (13)	0.039 (8)	4.66(1.85-11.72)	**0.002**
	DQB1*06:02	DQB1*06:02	DQB1*06:02:01:01	0.301(25)	0.141(29)	2.68(1.38-5.17)	**0.002**
Likely protection	DRB1*13:01	DRB1*13:01	DRB1*13:01:01:01	0.024 (2)	0.122(25)	0.18(0.02-0.75)	**0.012**
	DQB1*03:19	DQB1*03:19	DQB1*03:19:01	0.012(1)	0.098(20)	0.11(0.003-0.74)	**0.011**
Cervical cancer, HIV-1/HPV co-infection and HLA II alleles							
	Luminex intermediate *HLA* typing	**SSP high resolution *HLA* typing**	**Olerup high resolution *HLA* typing**	**Cancer Patients #(2n=83)**	**Healthy controls #(2n=205)**	**Odds ratio (95% CI)**	**p-value**
Susceptibility	DQB1*03:01	DQB1*03:01	DQB1*03:01:01:01	0.108 (9)	0.039(8)	4.4(1.4-13.78)	**0.001**
	DQB1*06:02	DQB1*06:02	DQB1*06:02:01:01	0.301(25)	0.141(29)	4.5(2.2-9.0)	**<0.0001**

Total number of alleles (N) from cancer patients and healthy controls do not add up because we are showing only significant alleles in HIV-1/HPV co-infected women. Where; SSP = sequence specific primers. Table from Chambuso *et al*., (2019) [185] with reprint permission from the Journal of Cancer)

4.4 Discussion

This is the first study to comprehensively investigate the association of *HLA* II -DRB1 and -DQB1 alleles, at the molecular level, with cervical cancer disease in HIV-1/HPV co-infected women. Other studies have reported cervical cancer associations with *HLA* II alleles -DQB1*03:01 [309], and -DQB1*06:02 [310], but this was in populations with unknown HIV-1 status.

In the present study, in a different population, we report a unique finding of an association of the same *HLA* II alleles -DQB1*03:01 and -DQB1*06:02 with HIV-1/HPV co-infection in women with cervical cancer. As *HLA*-II -DRB1*13:01 and -DQB1*03:19 were significantly absent or rare from women with cervical cancer, the study may infer that these are likely protective alleles for cervical cancer development in the study population. This may suggest that individuals with *HLA*-DRB1*13:01 and -DQB1*03:19 alleles, may effectively present the oncogenic HPV antigens to the immune system to provide a level of protective immunity. These findings are novel and have not previously been reported in cervical cancer disease studies in any population.

Studies done amongst different ethnic groups of patients with unknown HIV-1 status have shown the possible correlation of *HLA* II molecules with cervical cancer development [171] [129]. For example, studies done in Bolivia [311], Brazil [312], China [37] , Costa Rica [197], Mexico [308], Senegal [313], Tunisia [298] and Venezuela [314], have reported cervical cancer disease associations between different *HLA* II alleles and HPV infection but none of them have reported on HIV-1 infected patients in their study populations. In another study, however, Hu *et al.* [315] reported that *HLA* II-DQB1*06:02 was protective against cervical cancer progression in Chinese patients with unknown HIV-1 status. The inconsistency in the results of other studies to date amongst different populations, including the present study, suggests that there are other, as yet unknown, factors that influence cervical cancer disease development.

The persistence of oncogenic HPV infection frequently leads to cervical cancer development [133, 197, 298]. These results, however, add new evidence that the presence of HIV-1/HPV co-infection in addition to specific class II-DRB1 and -DQB1 alleles may aggravate or protect against cervical cancer disease development in some women. HIV-1 as a co-infection may not be a direct risk factor for cervical cancer disease, although HIV-1-positive status has been shown to influence the relative risk for cervical disease development. This means that HPV infection alone may influence cervical cancer development to a significant extent. When it is combined with a specific *HLA* II gene of risk for cervical cancer development and in the presence of HIV-1 as a co-infection, the combination may increase the relative risk for cervical cancer disease development.

The persistence of oncogenic HPV in some women with specific *HLA* II alleles nearly always leads to cervical cancer. This effect can be further exacerbated by the pro-oncogenic effects of HIV-1 co-infection, regardless of ART initiation, although the exact mechanism is not yet clear [27]. However, de Jong *et al.* [140], suggested that absence of functional HPV-16-specific CD4 T-cell immune response found in women with cervical cancer, may explain the further development of the disease despite immune reconstitution following ART initiation or in a competent CD4 count. The study acknowledges that studies on the association of *HLA* II alleles and cervical cancer progression or susceptibility show inconsistent results worldwide [248, 309, 316, 317]. However, these findings show that *HLA* II DRB1*13:01, DQB1*03:01, DQB1*03:19 and DQB1*06:02 alleles can modulate the cervical carcinogenesis in HIV-1/HPV co-infected women.

Due to small sample size, this study was underpowered to assess specific HPV types with cervical cancer risk according to *HLA* II alleles and HIV-1 status. Hospital-based studies are likely to have some selection bias because we studied only women who were referred with cervical disease, so they do not really represent HIV-1/HPV co-infected women in the general population who would have been diagnosed but not referred to the hospital in that time. Furthermore, this investigation did not study detailed information on immunocompromised status (CD4 count and CD4% of lymphocytes), HIV-1 and HPV viral loads, initiation, regime and duration of ART, and the time of acquisition of HIV-1 or HPV co-infection. Moreover, this study may have missed the effects of a window period in some of the cases because of the

use of HIV-1 antibody test, which takes at least three months for the antibodies to be detected in the blood since infection. Retrospective *HLA* data for the control group could add another bias to our study.

4.5 Conclusions

For the first time, this study provides evidence of the association of *HLA* II-DRB1, -DQB1 alleles with cervical cancer development amongst HIV-1/HPV co-infected indigenous South African women. This is a novel association from a new study population on the possible influence of variations of *HLA* II alleles and HIV-1/HPV co-infection on cervical cancer development. These findings suggest that, it is highly likely that specific *HLA* II-DQB1*03:01 and -DQB1*06:02 alleles in the presence of HIV-1/HPV co-infection influence cervical cancer disease development in the studied cohort of indigenous South African women. Furthermore, *HLA* II-DRB1*13:01 and -DQB1*03:19 were rare or absent in women with cervical disease when compared with the control population. Therefore, identifying mechanisms that give rise to this likely protective association of *HLA* II alleles will provide insight into the development of immune-based prevention measures. Additional whole genomic research for allele association studies may reveal new potential targets for individualized immune-based cervical cancer molecular prevention in HIV-1/HPV co-infected women. Furthermore, analyses of other immune-related genes and chromosomes out of the *HLA* II region may also enlighten the understanding regarding cervical cancer disease progression in HIV-1/HPV co-infected women.

CHAPTER 5: Investigation of Cervical Tumour Biopsies for Chromosomal Loss of Heterozygosity (LOH) and Microsatellite Instability (MSI) at the *HLA* II Locus in HIV-1/HPV Co-Infected Women.

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ABSTRACT

Background

A subgroup of women who are co-infected with HIV-1 and HPV progress rapidly to cervical disease regardless of the number of absolute CD4 count. LOH and MSI are early, frequent genetic alterations occurring in solid tumours. Loss of an allele or part of a chromosome can have multiple functional effects on immune response genes, oncogenes, DNA damage-repair genes and tumour-suppressor genes. To characterise the genetic alterations that may influence rapid tumour progression in some HIV-1-positive women, the extent of LOH and MSI at the *HLA* II locus on chromosome 6p in cervical tumour biopsy DNA samples with regard to HIV-1/HPV co-infection in a cohort of South African women was investigated.

Methods

A total of 164 women with cervical disease were recruited for this study, of which 74 were HIV-1-positive and 90 were HIV-1-seronegative. DNA from cervical tumours and matched buccal swabs were used for analyses. Six fluorescently-labelled oligonucleotide primer pairs in a multiplex PCR amplification were used to study LOH and MSI. Pearson chi-squared test for homogeneity of proportions using an exact *p-value*, a two-proportion z-score test, ROC curves and a logistic regression model were used for statistical analyses. All *p-values* were corrected for false discovery rate (FDR) using the Benjamini-Hochberg test and the adjusted p-values (q-values) were reported. All tests were significant when both p and $q<0.05$.

Results

Tumour DNA from HIV-1/HPV co-infected women demonstrated a higher frequency of LOH/MSI at the *HLA* II locus on chromosome 6p21.21 than tumour DNA from HIV-1-seronegative women (D6S2447, 74.2% versus 42.6%; **p=0.001, q=0.003**), D6S2881 at 6p21.31 (78.3% versus 42.9%; **p=0.002, q=0.004**), D6S1666 at 6p21.32 (79% versus 57.1%; **p=0.035, q=0.052**), and D6S2746, at 6p21.33 (64.3% versus 29.4%; **p<0.001, q<0.001**), respectively.

Conclusions

HPV infection alone can induce LOH/MSI at the *HLA* II locus in cervical tumour DNA, whereas HIV-1 co-infection exacerbates it, suggesting that this may accelerate cervical disease progression in a subgroup of HIV-1-positive women.

Keywords; Cervical cancer, loss of heterozygosity, microsatellite instability, *HLA* II locus, HIV-1/HPV co-infection, host molecular genetics, genetic alterations

5.1 Introduction

Women who are co-infected with HIV-1 and HPV are at high risk of developing cervical precancerous lesions [26]. These precancerous lesions in HIV-1/HPV co-infected women are more aggressive, but only a small subgroup progress rapidly to invasive cervical cancer. This progression is unrelated to CD4 count or antiretroviral (ARV) therapy [318, 319]. What is not clear, however, is how and why this rapid cervical carcinogenesis is only observed in some HIV-1/HPV co-infected women [27].

Both HIV-1 and HPV are classified as carcinogenic viruses [320]. On the one hand, extrachromosomal HPV viral genomes often integrate into the host genome. This integration into the host genome drives the oncogenic process due to the overexpression of HPV viral oncoproreins E6 and E7 [130], which in turn, lead to inactivation of critical host DNA-damage-repair checkpoints during the cell cycle [321]. Inactivation of the cell cycle checkpoints results in the accumulation of uncorrected mutations during normal DNA replication. These mutations increase host genomic instability and lead to further genetic alterations [2, 265]. On the other hand, intracellular HIV-1 Tat proteins can interact directly with the *Rb* and *P53* tumour-suppressor genes in the host [144, 145]. This interaction induces increased cell proliferation, which promotes the effects of HPV oncoproteins E6 and E7 in cervical carcinogenesis [143].

In two previous studies, host molecular genetic variations at the *HLA* II locus on chromosome 6p and accumulation of repeated genetic alterations were reported to influence the rate of cervical disease progression in HIV-1/HPV co-infected women [27, 185]. Furthermore, Harima *et al.* [322], reported that chromosome 6p was one of the chromosomal regions most frequently involved in the genetic alterations detected in cervical cancer. The availability of tumour biopsies in women with cervical disease can be used to interrogate the host genome for individualized tumour-specific early molecular oncogenic drivers [323]. LOH and MSI are among the most common earliest genetic alterations, and molecular oncogenic drivers, to occur in solid tumours including cervical cancer [322, 324]. Both LOH and MSI may lead to the loss of microsatellite alleles, chromosomal loci, or single nucleotide polymorphisms [325].

MSI is a locus-specific change in the length of a short tandem repeat of nucleotide sequence in tumour genomic DNA when compared to the length in the normal genomic DNA (e.g. derived from white blood cells) from the same patient [326]. MSI is caused by mutational inactivation of genes involved in DNA damage-repair [327]. LOH at chromosomal level is the loss of one copy of an allele or a chromosomal locus in a certain region of a chromosome. If both copies of a gene are inactivated, LOH can result in inactivation of functional tumour suppressor genes, oncogenes, immune-response genes and DNA damage-repair genes that occur in the region of the chromosomal loss [89, 97]. Inactivation of these important genes leads to physiologically uncontrolled cell growth and cell division in tissues where the LOH/MSI has occurred [1].

This thesis previously hypothesized that HIV-1/HPV co-infection provokes additional genetic alterations at the *HLA* II locus to influence the rate of cervical disease progression in a subgroup of HIV-1-positive women [27]. Furthermore, accumulation of repeated genetic alterations, can influence the rate of cervical disease progression in HIV-1/HPV co-infected women [27]. In the early stages of the carcinogenesis process, genetic alterations (LOH/MSI) can be identified in tumour genomic DNA by using specific DNA markers [325]. LOH/MSI can be studied in an individual's tumour genomic DNA and compared to its status in a matched control i.e. buccal mucosa cells or peripheral blood leukocyte DNA from the same patient [325].

The present study investigated the hypothesis by using host genomic DNA fragments, analyzed in a multiplex PCR using a capillary array electrophoresis platform. Six fluorescently-labelled oligonucleotide primer pairs were used; BAT 26, D6S266, D6S2447, D6S1666, D6S2746, and D6S2881 to study chromosomal LOH/MSI in cervical tumour genomic DNA. These were compared to matched control DNA derived from buccal mucosa from the same patient, with regard to HIV-1/HPV co-infection in a cohort of women histologically diagnosed with cervical disease in South Africa.

5.2 Methods

Research ethics

All procedures were performed in accordance with the guidelines of The Declaration of Helsinki. Ethical approval for the study was granted by the Human Research Ethics Committee of; the University of Cape Town (Number; HREC903/2015), all respective hospitals' gynaecology departments, the Department of Health of the Western Cape Government, and the South African National Health Laboratory Service. Consent forms were available in the language of the subject's choice and were signed in front of a witness. This was after detailed discussion with patients regarding the aims and nature of the study. A trained registered nurse who was fluent in the local languages explained the details of the study and answered all questions from the patients before their consent was requested.

Study design, subjects and samples

As part of a large ongoing hospital-based project, a total of 200 patients were recruited from three hospitals in the Western Cape, namely; the Groote Schuur Hospital, Somerset Hospital and Victoria Wynberg Hospital between June 2016 to March 2017. All patients were referred from peripheral health centers to these three hospitals after receiving abnormal Pap smear results after the routine cervical screening. Patients were recruited from; the outpatient gynaecological cancer assessment clinics, colposcopy clinics and the gynaecological emergency rooms. The only criterion for recruitment was that the patient be newly diagnosed with cervical disease. The age distribution, ranged from 24 to 91 years, with the majority of women in the age group between 30 and 40 years. Of the 200 recruited women, 164 were fully investigated in this study.

Prior to the collection of buccal swabs, patients rinsed their mouths with sterile water, and mechanically chewed both inner buccal mucosal walls for at least 30 seconds. Both sides of buccal mucosa were scraped with a 'DNA collector dry cotton swab stick' (Thermo Fisher Scientific, Johannesburg, South Africa) at least 20 times per side, as described according to the manufacturer's protocol. The buccal swabs were used to extract normal epithelial control DNA which were compared with abnormal tumour DNA from the same patients.

According to the South African HIV-1 testing algorithm, peripheral whole blood (4ml) was collected in EDTA tubes (BD Vacutainer, Johannesburg, South Africa). Approximately 20µl of the collected peripheral whole blood was used for rapid HIV-1 antibody testing (Determine, Alere, Inc., Johannesburg, South Africa) [270].

Gynaecologists used colposcopy inspection to collect punch biopsies of abnormal cervical lesions for histopathology analyses and HPV genotyping. All patients were recruited in this study before the initiation of the radiotherapy or chemotherapy treatment in order to avoid the consequences of DNA damage during cancer therapy [304].

Tumour DNA extraction and PCR amplification

Genomic DNA was extracted using Qiagen ® QIAamp DNA Mini purification kit (Qiagen, Johannesburg, South Africa) according to the manufacturer's protocol. The concentration of the extracted DNA was quantified by a Nanodrop® Spectrophotometer (Thermo Fisher Scientific, Johannesburg, South Africa). The DNA was diluted using nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa) to a recommended final concentration of 0.2ng/µl. The integrity of genomic DNA was tested by resolving DNA fragments on a 1% agarose gel by electrophoresis (Bio-Rad, Johannesburg, South Africa), migrated for 1 hour at 100 V using 0.2μl of total DNA and 2μL of orange loading dye (Thermo Fisher Scientific, Johannesburg, South Africa), followed by staining with ethidium bromide (Sigma-Aldrich, Johannesburg, South Africa) and visualization on a Ultra Violet-trans illuminator and the image was captured using a gel documentation system (Bio-Rad, Johannesburg, South Africa) [328]. Each DNA sample was graded, according to the electrophoretic migration of sample DNA compared with 100bp weight ladder (Thermo Fisher Scientific, Johannesburg, South Africa).

The extracted DNA was amplified by PCR-based assays using six fluorescently-labelled forward primers with the pair names, dyes and sequence as described in **Table 5.1**. The dream-Taq® PCR master-mix 12.5µl (1.5mmol MgCl2, 200 mmol dNTPs, 1 unit Taq DNA polymerase) (Thermo Fisher Scientific, Johannesburg, South Africa) was used for amplification according to the manufacturer's protocol. The PCR was carried out in 25µl total reaction volumes, each containing 2µl of template DNA, 1µl of each primer (Forward and reverse) and 8.5µl of nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa). The reaction mixture was heated to 95°C for 7 minutes, followed by 35 cycles, each consisting of 30 seconds denaturation at 94°C, 30 seconds annealing at 53°C, 30 seconds extension at 72°C, and a final 7-minutes extension at 72°C. The PCR amplification products (5µl) were subjected to electrophoresis (Bio-Rad, Johannesburg, South Africa) on 1% agarose gel in 1× Tris-acetate-EDTA buffer at 100 V for 1 hour and stained with ethidium bromide (Sigma-Aldrich, Johannesburg, South Africa). The images were obtained in a gel documentation system (Bio-Rad, Johannesburg, South Africa) with the expected amplicon sizes for each marker as described in **Table 5.1**.

Table 5. 1 Marker names, nucleotide sequences of primers, chromosomal positions and the fluorescent dyes used for investigation of LOH and MSI at the respective loci

Marker	Primer sequence (5'→ 3')	Chromosomal region	Amplicon size (bp)	Motif	Fluorescent dye, 5'
BAT26	F- TGACTACTTTTGACTTCAGCC	2p21	126	A(26)	FAM
	R- ACCCATTCAACATTTTTAACCC				
D6S2746	F-AGATTGTGCCACTGCACTCC	6p21.33	97-197	AAAAC	FAM
	R-ATAGTGCTGAGGTTGAGAGC				
D6S2881	F-GCTCGGGATTGAGAGGATTC	6p21.31	239-339	CA	PET
	R-AGCGGCGAGGTGAGCATGTC				
D6S266	F-GTTCCTCGGAATCATTTCCTCC	6p21.23	267-298	CTTT	FAM
	R-GGCAACAGAGTGAGGCTATCTTTG				
D6S1666	F-CTGAGTTGGGCAGCATTTG	6p21.32	131-151	GT	NED
	R-ACCCAGCATTTTGGAGTTG				
D6S2447	F-TTGAGAGGTGTGCATGTTAC	6p21.21	178-200	AC	VIC
	R-GCATTTCTCTTCCTTATCACTTC				

HPV DNA detection and genotyping

Tumour genomic DNA was diluted using nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa) to reach a recommended final concentration of 0.2ng/µl. PCR-based Roche Linear Array® HPV genotyping test (Roche Molecular Systems, Pleasanton, CA, USA) which identifies 37 different HPV genotypes (HPV-6, -11, -16, -18, -26, -31, -33, -35, -39, -40, -42, -45, -51, -52, -53, -54, -55, -56, -58, -59, -61, -62, -64, -66, -67, -68, -69, -70, -71, -72, -73, -81, -82, -83, -84, -89 (HPV-CP6108) and –IS39) was used for typing HPV according to the manufacturer's instructions. All 37 different HPV genotypes were detected accordingly.

Buccal swab DNA extraction and PCR amplification

In order to preserve the DNA, all buccal swabs were air dried for at least 20 minutes after collection and then stored frozen at -20^{o}C until processed. The genomic DNA was extracted using Qiagen QIAamp® DNA extraction kit (Qiagen, Johannesburg, South Africa) according to manufacturer's protocol [329]. Briefly, each swab was placed in a 2ml micro centrifuge tube and mixed with 20µl proteinase K and 600 µl Buffer ATL, supplied in the kit. The mixture was placed in a thermomixer and incubated at 56°C with shaking at 900rpm for at least one hour; where after, further extraction procedures followed as per the manufacturer's protocol. All samples were eluted according to the manufacturer's instructions. The integrity of genomic DNA was tested as described in the previous section.

The extracted DNA was amplified by PCR using human beta-globin gene primers –Bg1 F(5´-CAACTTCATCCACGTTCACC-3´) and Bg2 R(5´-GAAGAGCCAAGGACAGGTAC-3´), and 12.5µl Dream-Taq PCR master-mix (1.5mmol MgCl2, 200mmol dNTPs, 1 unit Taq DNA polymerase) (Thermo Fisher Scientific, Johannesburg, South Africa) according to the manufacturer's protocol. The PCR was carried out in 25µl total reaction volumes, each containing 2µl of template DNA, 0.5µl of each primer and 10.5µl of nuclease free water (Thermo Fisher Scientific, Johannesburg, South Africa). The reaction mixture was heated to 95°C for 7 minutes, followed by 35 cycles, each consisting of 30 seconds denaturation at 94°C, 30 seconds annealing at 53°C, 30 seconds extension at 72°C, and a final 7-minutes extension at 72°C. The PCR amplification products (5µl) were subjected to electrophoresis (Bio-Rad, Johannesburg, South Africa) on 1% agarose gel in 1× Tris-acetate-EDTA buffer at 100V for 1

hour and stained with ethidium bromide (Sigma-Aldrich, Johannesburg, South Africa)). The images were captured in a gel documentation system (Bio-Rad, Johannesburg, South Africa) with expected amplicon size of 268bp.

LOH/MSI analysis

LOH/MSI analysis was carried out in a 'blinded' fashion, i.e. without knowledge of HIV-1 or HPV infection status. LOH/MSI was assessed at six highly polymorphic repeat markers; BAT26, D6S2447, D6S266, D6S2666, D6S2746 and D6S2881. The markers were chosen on the basis of their high heterozygosity informative content value of 0.7 in the *HLA* II region on chromosome 6p as described in the NCBI database (https://www.ncbi.nlm.nih.gov/) with supplementary mapping information, where necessary, provided in the Genome Reference Consortium (https://www.ncbi.nlm.nih.gov/grc) and the Human Genome Database (http://morissardjerome.free.fr/infobiogen/www.gdb.org/gdb/). The forward primers were labelled with fluorescent dyes: FAM, NED, PET, or VIC [78, 330].

Five of the markers studied are located in the *HLA* II locus (Chromosome 6p21), and one marker, BAT26, was located in the vicinity of the *MSH2* mismatch repair gene on chromosome 2p21. The BAT 26 marker was used in order to compare it with other markers in the analyses due to its high percentage of LOH and its reported accuracy in predicting LOH/MSI as previously studied in colorectal tumours [331].

Multiplex PCR reactions were performed and the amplified PCR products were analysed by capillary array electrophoresis and GeneMapper® software (Applied Biosystems Inc. CA, USA). After PCR, 0.5µl of amplification product was mixed with 0.25µl GS500-LIZ Size Standard and Hi-Di™ Formamide (Applied Biosystems, CA, USA) to a final volume of 10µl. The resulting mixture was denatured for 5 minutes at 95^{o}C and then cooled on ice for at least 3 minutes. All the PCR products were genotyped on 3130x1 Genetic Analyser (Applied Biosystems Inc., Hitachi, CA, USA), according to manufacturer's instructions.

MSI was defined as the presence of novel fragment sizes in DNA from tumour which was absent in the matched normal DNA from the same patient. Furthermore, for each informative tumour DNA/normal DNA pair, the allelic-imbalance ratio (AIR) was calculated. This is the ratio of the heights of both microsatellite alleles in the normal DNA divided by the ratio of heights in the tumour DNA from the same patient [325] (**see figure 5.1**). An AIR value ≤ 0.67 or ≥ 1.35 was regarded as LOH [89]. The LOH/MSI frequency was calculated as a percentage of LOH/MSI alterations present in relation to all informative loci (Heterozygous DNA) for each marker. At least two independent experiments were required to confirm the results in each event presenting with MSI/LOH.

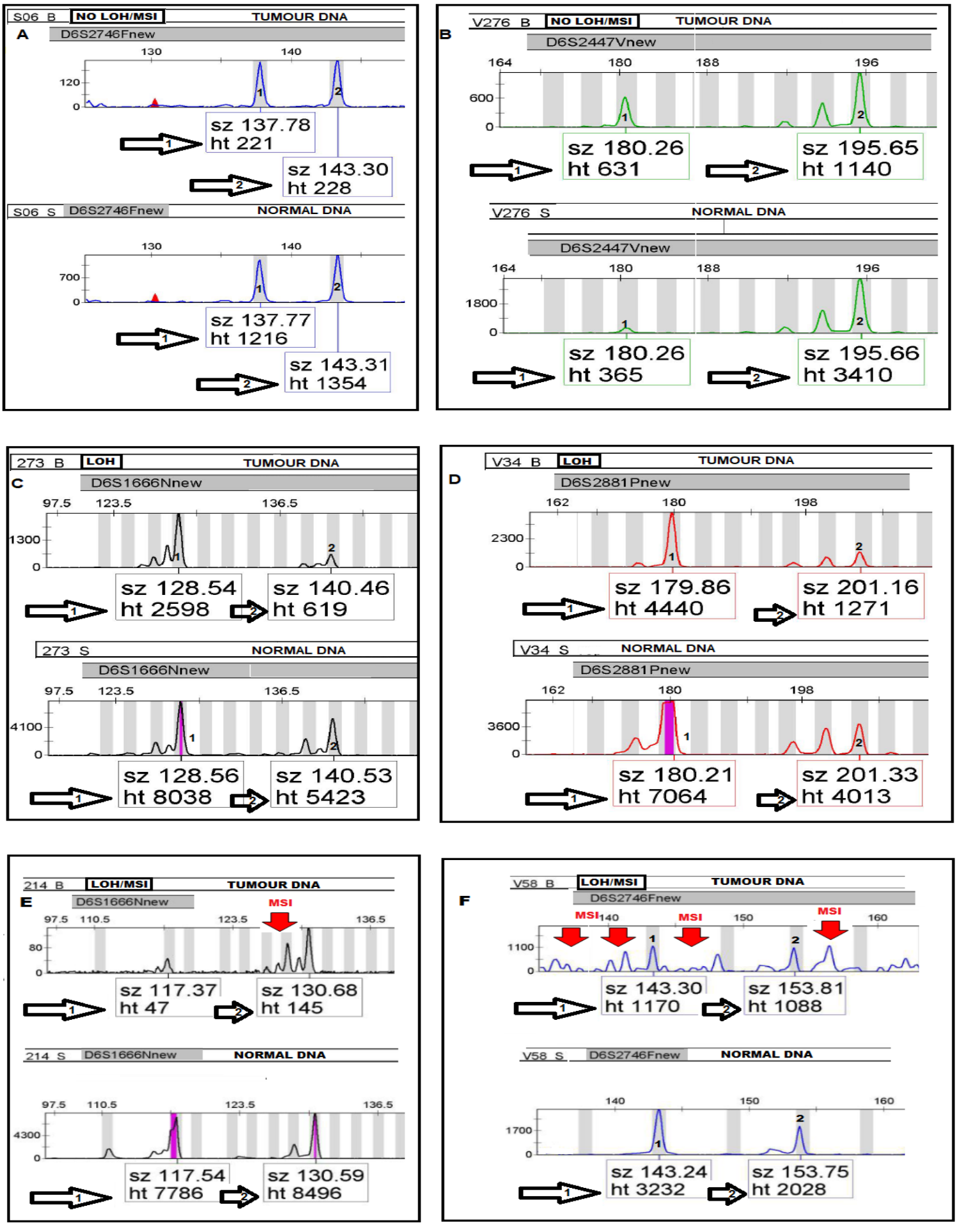

A
S06 B
NO LOH/MSI
TUMOUR DNA
D6S2746Fnew
130
140
120
0
sz 137.78
ht 221
sz 143.30
ht 228
S06 S
D6S2746Fnew
NORMAL DNA
700
sz 137.77
ht 1216
sz 143.31
ht 1354
B
V276 B
NO LOH/MSI
TUMOUR DNA
D6S2447Vnew
164
180
188
196
600
sz 180.26
ht 631
sz 195.65
ht 1140
V276 S
NORMAL DNA
1800
sz 180.26
ht 365
sz 195.66
ht 3410
C
273 B
LOH
TUMOUR DNA
D6S1666Nnew
97.5
123.5
136.5
1300
sz 128.54
ht 2598
sz 140.46
ht 619
273 S
NORMAL DNA
4100
sz 128.56
ht 8038
sz 140.53
ht 5423
D
V34 B
LOH
TUMOUR DNA
D6S2881Pnew
162
180
198
2300
sz 179.86
ht 4440
sz 201.16
ht 1271
V34 S
NORMAL DNA
3600
sz 180.21
ht 7064
sz 201.33
ht 4013
E
214 B
LOH/MSI
TUMOUR DNA
D6S1666Nnew
MSI
97.5
110.5
123.5
136.5
80
sz 117.37
ht 47
sz 130.68
ht 145
214 S
D6S1666Nnew
NORMAL DNA
4300
sz 117.54
ht 7786
sz 130.59
ht 8496
F
V58 B
LOH/MSI
TUMOUR DNA
D6S2746Fnew
MSI
140
150
160
1100
sz 143.30
ht 1170
sz 153.81
ht 1088
V58 S
D6S2746Fnew
NORMAL DNA
1700
sz 143.24
ht 3232
sz 153.75
ht 2028

Figure 5. 1 Electropherograms A, B, C, D, E and F illustrating the allelic status of tumour DNA compared to normal DNA in LOH/MSI analyses by using four fluorescently-labelled microsatellites markers.

Paired tumour and normal heterozygous allele ratios were analysed by allelic-imbalance factor by calculating the quotient of the peak ratios for each informative microsatellite alleles in the normal DNA divided by the corresponding ratio found in tumour DNA from the same patient as follows; Alleles in Normal 1:Normal 2/Tumour 1:Tumour 2.

Statistical analysis

Data analyses were based on the previously published studies [134, 275, 276]. The number of cases with LOH/MSI was divided by the total number of informative cases in that region to get the fractional locus loss. If one or more markers showed LOH/MSI, the locus was regarded as demonstrating LOH/MSI. Pearson chi-squared test for homogeneity of proportions using an exact *p-value* and multivariate logistic regression model were used for statistical analyses. LOH/MSI was used as a dependent variable for testing the significance of LOH/MSI variations between different predictor variables of interest within each marker. Normal distribution two-proportion z-score test was used to test for a statistically significant difference between two proportions within the same categorical groups. We used sensitivity, specificity and the area under the receiver operating characteristic (ROC) curves to assess if age was a predictor of invasive cervical cancer outcome in women with LOH/MSI. The *p-values* were corrected for false discovery rate (FDR) by the Benjamini-Hochberg test and the adjusted *p-values* (q-values) were reported. All odds ratios (ORs), 95% confidence intervals (95% CIs), the *p-values* and q-values calculated for multiple comparisons were 2-tailed, and considered significant if <0.05.

5.3 Results

Characteristics of the study cohort

In this study, matched paired tumour (cervical lesions) and buccal swab DNA samples were available from 164 patients of the original 200 recruited patients. The paired samples were collected from 24 patients with cervical intraepithelial neoplasm I (CIN 1), 42 patients with cervical intraepithelial neoplasm II (CIN 2), 44 patients with cervical intraepithelial neoplasm III (CIN 3/Carcinoma *in situ*) and 54 patients with ICC. Seventy-four patients were HIV-1-positive and ninety patients were HIV-1-seronegative. A total of 980 PCR reactions were performed, 490 each from matched tumour and buccal swab samples. The clinical and demographic features of the patients in the study cohort are summarised in **Table 5.2.** Six primer sets were used to analyse DNA microsatellites by a multiplex PCR; the number of paired samples examined for each marker is summarised in **Table 5.3**.

Table 5. 2 Demographic features and the range of variables including clinical predictors measured in the subjects of the study cohort.

Variable	BAT26 (N= 59)	D6S2881 (N= 81)	D6S2746 (N= 107)	D6S266 (N=37)	D6S2666 (N= 97)	D6S2447 (N=109)
Median age, y (Range)	40 (26-77)	40 (26-77)	42 (28-77)	37 (24-61)	39 (24-66)	39 (24-91)
Standard Deviation	9.7	9.7	9.6	8.4	8.7	10.6
Age group (years)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
< 30	1 (1.7)	4 (4.9)	5 (4.7)	2 (5.4)	6 (6.2)	7 (6.4)
30-40	30 (50.8)	39 (48.1)	46 (43)	21 (56.8)	54 (55.7)	60 (54.5)
> 40	28 (47.5)	38 (46.9)	56 (52.3)	14 (37.8)	37 (38.1)	42 (38.2)
HIV-1 status						
HIV-1-positive	36 (61)	46 (56.8)	56 (52.3)	19 (51.4)	62 (63.9)	62 (56.9)
HIV-1-seronegative	23 (39)	35 (43.2)	51 (47.7)	18 (48.6)	35 (36.1)	47 (43.1)
***HPV infection**						
Single HPV infection	19 (32.2)	24 (29.6)	36 (33.6)	13 (35.1)	33 (34)	36 (33)
Multiple HPV infection	33 (55.9)	39 (48.1)	50 (46.7)	18 (48.6)	48 (49.5)	54 (49.5)
High risk HPV	46 (78)	59 (72.8)	81 (75.7)	27 (73)	77 (79.4)	86 (78.9)
Low risk HPV	6 (10.2)	4 (4.9)	5 (4.7)	4 (10.8)	4 (4.1)	4 (3.7)
Tumour stage						
CIN 1 & 2	20 (33.9)	31 (38.3)	38 (35.5)	17 (45.9)	44 (45.4)	49 (45)
CIN 3	16 (27.1)	17 (21)	25 (23.4)	9 (24.3)	25 (25.8)	29 (26.6)
Invasive	23 (39)	33 (40.7)	44 (41.1)	11 (29.7)	28 (28.9)	31 (28.4)
Histopathology						
Mild dysplasia	9 (15.3)	16 (19.8)	19 (17.8)	2 (5.4)	16 (16.5)	18 (16.5)
Moderate dysplasia	11(18.6)	15 (18.5)	19 (17.8)	15 (40.5)	28 (28.9)	31 (28.4)
Carcinoma *In situ*	16 (27.1)	17 (21)	25 (23.4)	9 (24.3)	26 (26.8)	29 (26.6)
Squamous cell carcinoma	20 (33.9)	26 (32.1)	35 (32.7)	8 (21.6)	23 (23.7)	24 (22)
Adenocarcinoma	3 (5.1)	7 (8.6)	9 (8.4)	3 (8.1)	4 (4.1)	7 (6.4)

* 14 Invalid HPV results were omitted in the analyses

Table 5. 3 Number of paired samples for which each microsatellite marker was successfully resolved.

	Marker	Samples
i)	BAT26	59 paired samples
ii)	D6S2881	81 paired samples
iii)	D6S2746	107 paired samples
iv)	D6S266	37 paired samples
v)	D6S2666	97 paired samples
vi)	D6S2447	109 paired samples

Comparisons of LOH/MSI frequency:

(i) between tumour DNA from precancerous lesions and ICC

To investigate whether LOH/MSI was different between patients with precancerous lesions and ICC, the frequency of LOH/MSI in tumour DNA from precancerous lesions and ICC for each marker was compared separately. ICC tumour DNA showed more LOH/MSI only at 6p21.31 (D6S2881) than precancerous lesions tumour DNA, i.e. 78.8% versus 52.1 % respectively, **p=0.019**. However, the false discovery rate (FDR) q-value was not statistically significant (q=0.114; **Table 5.4A**).

(II**) between tumour DNA from HIV-1-positive and HIV-1-seronegative women**

To investigate if LOH/MSI was different between tumour biopsies, depending on HIV-1 infection status, the frequency of LOH/MSI for all tumour biopsy DNA by examining each marker was compared separately in HIV-1-positive and HIV-1-seronegative women. The results show that tumour DNA from HIV-1-positive women demonstrated a higher frequency of LOH/MSI than tumour DNA from HIV-1-seronegative women at 6p21.21 (D6S2447, 74.2% versus 42.6%; **p=0.001, q=0.003**), 6p21.31 (D6S2881, 78.3% versus 42.9%; **p=0.002, q=0.004**), 6p21.32 (D6S2666, 79% versus 57.1%; **p=0.035,** q=0.052), and 6p21.33 (D6S2746, 64.3% versus 29.4%; **p<0.001, q<0.001**), respectively (**Table 5.4B**).

Table 5. 4 Frequency of LOH/MSI according to cervical disease and HIV-1 status

(**A**) Frequency of LOH/MSI between precancerous lesions and ICC. (**B**) Frequency of LOH/MSI between HIV-1-seronegative and HIV-1-positive women. (Pearson chi-square test for homogeneity of proportions by using an exact *p*-value)

A

MARKER	PRECANCEROUS Cases studied	PRECANCEROUS LOH/MSI (%)	INVASIVE CANCER Cases studied	INVASIVE CANCER LOH/MSI (%)	p-value	FDR q-value
BAT 26	36/59	6/36 (16.7)	23/59	6/23 (26.1)	0.51	1.02
D6S266	26/37	12/26 (46.2)	11/37	4/11 (36.4)	0.723	1.085
D6S2666	69/97	49/69 (71)	28/97	20/28 (71.4)	>0.99	0.99
D6S2881	48/81	25/48 (52.1)	33/81	26/33 (78.8)	**0.019**	0.114
D6S2746	63/107	33/63 (52.4)	44/107	18/44 (40.9)	0.325	0.975
D6S2447	78/109	48/78 (61.5)	31/109	18/31 (58.1)	0.829	0.99

B

MARKER	HIV-1-seronegative Cases studied	HIV-1-seronegative LOH/MSI (%)	HIV-1-positive Cases studied	HIV-1-positive LOH/MSI (%)	p-value	FDR q-value
BAT 26	23/59	3/23 (13)	36/59	9/36 (25)	0.334	0.401
D6S266	18/37	9/18 (50)	19/37	7/19 (36.8)	0.515	0.515
D6S2666	35/97	20/35 (57.1)	62/97	49/62 (79)	**0.035**	0.052
D6S2881	35/81	15/35 (42.9)	46/81	36/46 (78.3)	**0.002**	**0.004**
D6S2746	51/107	15/51 (29.4)	56/107	36/56 (64.3)	**<0.001**	**<0.001**
D6S2447	47/109	20/47(42.6)	62/109	46/62 (74.2)	**0.001**	**0.003**

The present study also compared the odds of having LOH/MSI (LOH/MSI-positive versus LOH/MSI-negative cases) according to HIV-1 status for each marker. The results show that tumour biopsy DNA from HIV-1-positive women had a higher relative risk of having LOH/MSI than tumour biopsy DNA from HIV-1-seronegative women for markers; D6S2881 (**OR** 4.8, **CI** 1.8-12.7, **p= 0.002, q=0.004**), D6S2746 (**OR** 4.3, **CI** 1.9-9.7, **p< 0.001, q= <0.001**), D6S2666 (**OR** 2.8, **CI** 1.1-7.7, **p= 0.035,** q= 0.052) and D6S2447 (**OR** 3.88, **CI** 1.6-9.5, **p= 0.001, q= 0.003**) (**Table 5.5**).

Table 5. 5 The relationship between the odds of having LOH/MSI and HIV-1 status for each marker

	Outcome					
	BAT 26 (n= 59)					
Exposure	**No LOH/MSI (n= 47)**	**LOH/MSI (n= 12)**	**OR**	**CI**	**P-value**	**FDR q-value**
HIV-1-seronegative (n=23)	20 (42.6%)	3 (25%)	Ref.			
HIV-1-positive (n= 36)	27 (57.4%)	9 (75%)	2.2	0.5-14	0.334	0.401
	D6S2881 (n= 81)					
	No LOH/MSI (n= 30)	**LOH/MSI (n= 51)**				
HIV-1-seronegative (n= 35)	20 (66.7%)	15 (29.4%)	Ref.			
HIV-1-positive (n= 46)	10 (33.3%)	36 (70.6%)	4.8	1.8-12.7	**0.002**	**0.004**
	D6S2746 (n= 107)					
	No LOH/MSI (n= 56)	**LOH/MSI (n= 51)**				
HIV-1-seronegative (n= 51)	36 (64.3%)	15 (29.4%)	Ref.			
HIV-1-positive (n= 56)	20 (35.7%)	36 (70.6%)	4.3	1.9-9.7	**<0.001**	**<0.001**
	D6S266 (n= 37)					
	No LOH/MSI (n= 21)	LOH/MSI (n= 16)				
HIV-1-seronegative (n= 18)	9 (42.9%)	9 (56.2%)	Ref.			
HIV-1-positive (n= 19)	12(57.1%)	7 (43.8%)	0.58	0.1-2.6	0.515	0.515
	D6S2666 (n= 97)					
	No LOH/MSI (n= 28)	**LOH/MSI (n= 69)**				
HIV-1-seronegative (n= 35)	15 (53.6%)	20 (29%)	Ref.			
HIV-1-positive (n= 62)	13 (46.4%)	49(71%)	2.8	1.1-7.7	**0.035**	0.052
	D6S2447 (n= 109)					
	No LOH/MSI (n= 43)	**LOH/MSI (n= 66)**				
HIV-1-seronegative (n= 47)	27 (62.8%)	20 (30.3%)	Ref.			
HIV-1-positive (n= 62)	16 (37.2%)	46 (69.7%)	3.88	1.6-9.5	**0.001**	**0.003**

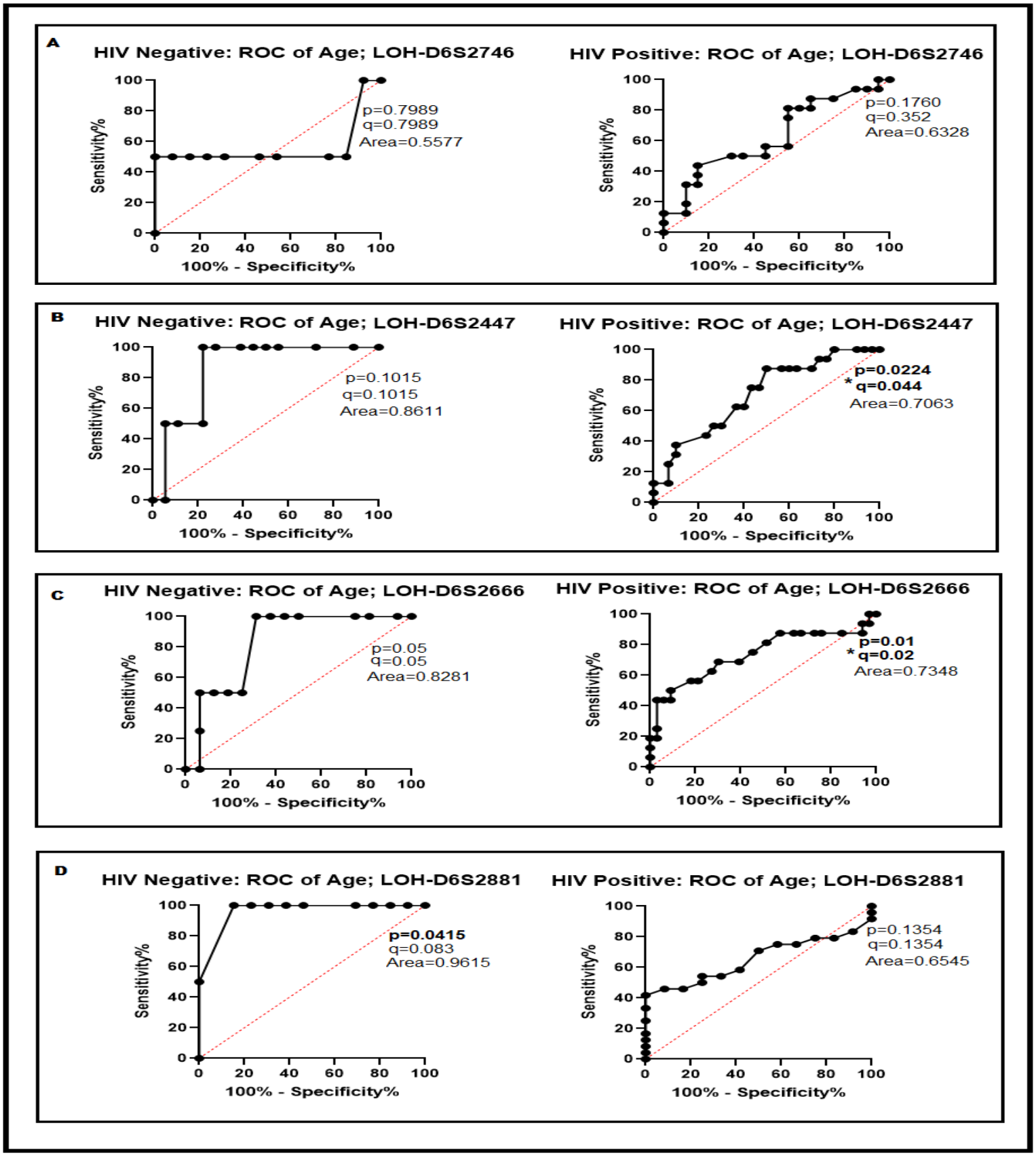
Comparison between LOH/MSI status and clinical variables

In order to control for the effects of other variables that may influence the outcome, a multivariate logistic regression model was used to study the association of each variable by considering LOH/MSI as a dependent variable for four markers that showed significantly different frequency among the studied groups. In this analysis, only HIV-1 status was significantly associated with LOH/MSI in DNA marker; D6S2746 (**p < 0.0001, q<0.001)**, D6S2881 (**p = 0.025**, q=0.15), D6S2447 (**p = 0.002, q=0.012)** and D6S2666 (**p= 0.021,** q=0.063**)** while tumour stage and histopathology were significantly associated with LOH/MSI in DNA marker D6S2666 for tumour stage (**p=0.027,** q=0.054**)** and for histopathology (p=**0.015,** q=0.09**)** (**Table 5.6**). Furthermore, aging and cancer are highly interconnected, older age being a significant risk factor for cancer development [280].

However, in cervical cancer, it has already been reported that HIV-1-positive women develop invasive cervical cancer earlier and at a younger age compared to HIV-1-seronegative women [99, 100, 281]. Since there is limited data on the impact of age on LOH/MSI in cervical cancer development amongst HIV-1-positive women, this investigation sought to study whether age could predict invasive cervical cancer outcome in HIV-1-positive women with LOH/MSI in the study cohort by plotting specificity against sensitivity for each marker by using receiver operating characteristic (ROC) curves to calculate the area under the curves (AUC), with *p* values. This study found that age could predict invasive cervical cancer outcome in HIV-1-positive women with LOH/MSI for two DNA markers, D6S2447 (**p=0.0224, q=0.044**) and D6S2666 (**p=0.01, q=0.02**) (**Supplementary figure 5.1**)

Table 5. 6 Multivariate analysis in logistic regression for each marker by using LOH/MSI as a dependent variable.

	D6S2746 (N=107)	p value	FDR q value	D6S2881 (N= 81)	p value	FDR q value	D6S2447 (N=109)	p value	FDR q value	D6S2666 (N= 97)	p value	FDR q value
Variables												
Age	n (%)	0.256	0.384	n (%)	0.687	1.031	n (%)	0.16	0.48	n (%)	0.7	0.84
< 30	5 (4.7)			4 (4.9)			7 (6.4)			6 (6.2)		
30-40	46 (43)			39 (48.1)			60 (55)			54 (55.7)		
>40	56 (52.3)			38 (46.9)			42 (38.5)			35 (36.1)		
HIV-1 status		**<0.0001**	**<0.001**		**0.025**	0.15		**0.002**	**0.012**		**0.021**	0.063
HIV-1-seronegative	51 (47.7)			35 (43.2)			62 (56.9)			35 (36.1)		
HIV-1-positive	56 (52.3)			46 (56.8)			47 (43.1)			62 (63.9)		
HPV risk		0.174	0.522		0.837	1.004		0.997	0.997		0.919	0.919
Low risk HPV	5 (4.7)			4 (4.9)			4 (3.7)			4 (4.1)		
High risk HPV	81 (75.7)			59 (72.8)			86 (78.9)			77 (79.4)		
HPV single or multiple		0.215	0.43		0.935	0.935		0.353	0.706		0.142	0.213
Single HPV	36 (33.6)			24 (29.6)			36 (33)			33 (34)		
Multiple HPV	50 (46.7)			39 (48.1)			54 (49.5)			48 (49.5)		
Tumour stage		1.171	1.171		0.632	1.264		0.818	0.981		**0.027**	0.054
CIN 1 & 2	38 (35.5)			31 (38.3)			49 (45)			44 (45.4)		
CIN 3	25 (23.4)			17 (21)			29 (26.6)			25 (25.8)		
Invasive	44 (41.1)			33 (40.7)			31 (28.4)			28 (28.9)		
Histopathology		0.646	0.775		0.267	0.801		0.536	0.804		**0.015**	0.09
Mild dysplasia	19 (17.8)			16 (19.8)			18 (16.5)			16 (16.5)		
Moderate dysplasia	19 (17.8)			15 (18.5)			31 (28.4)			28 (28.9)		
Carcinoma In Situ	25 (23.4)			17 (21)			29 (26.6)			26 (26.8)		
SCC	35 (32.7)			26 (32.1)			24 (22)			23 (23.7)		
ADC	9 (2.8)			7 (8.6)			7 (6.4)			4 (4.1)		



Supplementary figure 5.1. Receiver operating characteristic curves by using age of patients

to predict ICC between HIV-1-positive and HIV-1-seronegative women with LOH/MSI for four significant DNA markers.(**A**) ROC curves for DNA marker D6S2746, showing prediction for ICC with LOH/MSI in both HIV-1-positive and HIV-1-seronegative women by using age. (B) ROC curves for DNA marker D6S2447, showing prediction for ICC with LOH/MSI in both HIV-1-positive and HIV-1-seronegative women by using age. (C) ROC curves for DNA marker D6S2666, showing prediction for ICC with LOH/MSI in both HIV-1-positive and HIV-1-seronegative women by using age. (D) ROC curves for DNA marker D6S2881, showing prediction for ICC with LOH/MSI in both HIV-1-positive and HIV-1-seronegative women by using age.

Comparison of LOH/MSI status between tumour DNA from precancerous lesions and ICC according to HIV-1 infection

In order to further examine the effects of HIV-1 infection on the status of LOH/MSI, a comparison was made of LOH/MSI between tumour DNA from precancerous lesions and ICC according to HIV-1 infection for four markers; In marker D6S2746, tumour DNA from HIV-1-positive women showed significantly more LOH/MSI than tumour DNA from HIV-1-seronegative women with precancer (**p=0.011, q=0.011**). For marker D6S2447, tumour DNA from HIV-1-positive women showed more LOH/MSI than tumour DNA from HIV-1-seronegative women in invasive cancer, although the *p-value* was not applicable (N/A) due to a very low number of HIV-1-seronegative women with LOH/MSI. For marker D6S2666, tumour DNA from HIV-1-positive women showed significantly more LOH/MSI than tumour DNA from HIV-1-seronegative women with precancer (**p=0.001, q=0.002)**, the *p-value* was not applicable for invasive cancer due to a very low number of HIV-1-seronegative women with LOH/MSI. Finally, for marker D6S2881, tumour DNA from HIV-1-positive women showed more LOH/MSI than tumour DNA from HIV-1-seronegative women with ICC, although the *p-value* was not applicable due to very low number of HIV-1-seronegative women with LOH/MSI (**p=0.011, q=0.022) (Figure 5.2).**

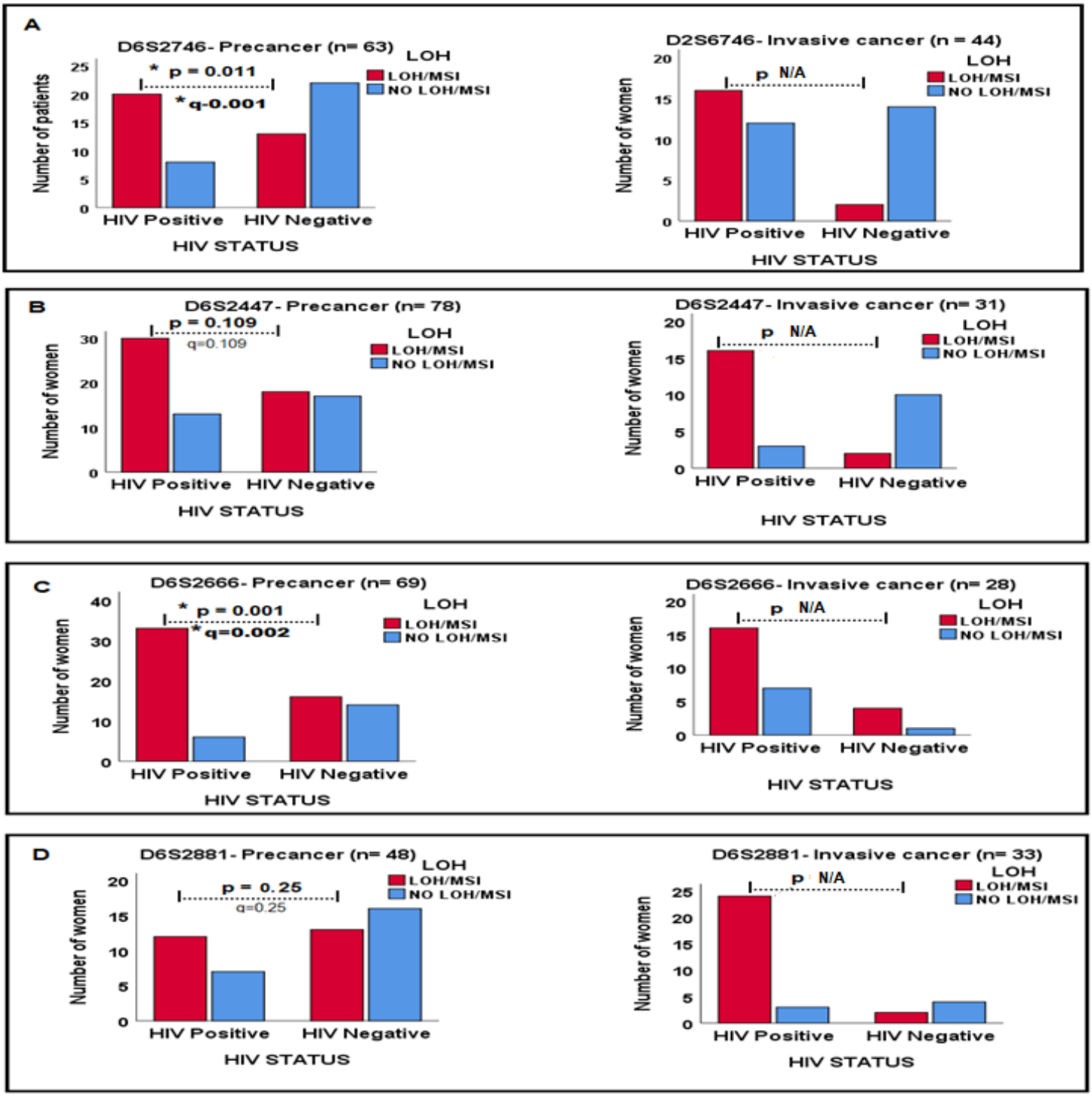


Figure 5. 2 Relationships between LOH/MSI status and HIV-1 status between precancerous lesions and ICC tumour biopsies for four DNA markers in A, B, C &D.

Comparison of LOH/MSI status between HIV-1-positive and HIV-1-seronegative women with Hr-HPV infection.

Because Hr-HPV infection is a known risk factor for cervical disease development, and HIV-1 infection increases prevalence, persistence and reduces clearance of HPV-infection [26], this study examined whether LOH/MSI between tumour (cervical lesion) biopsy DNA from HIV-1-positive women and HIV-1-seronegative women differ according to Hr-HPV infection in four markers which showed significant results. Tumour biopsy DNA from HIV-1-positive women with Hr-HPV infection showed more LOH/MSI than tumour biopsy DNA from HIV-1-seronegative women with Hr-HPV infection in marker D6S2746 (**p= 0.003**, **q=0.006**) (**Figure 5.3A**), D6S2447 (**p= 0.001, q=0.004) (Figure 5.3B)** and D6S2881 (**p= 0.005, q=0.007) (Figure 5.3D)**. However, with marker D6S2666, the difference was not statistically significant p=0.172, q=0.172 **(Figure 5.3C).**

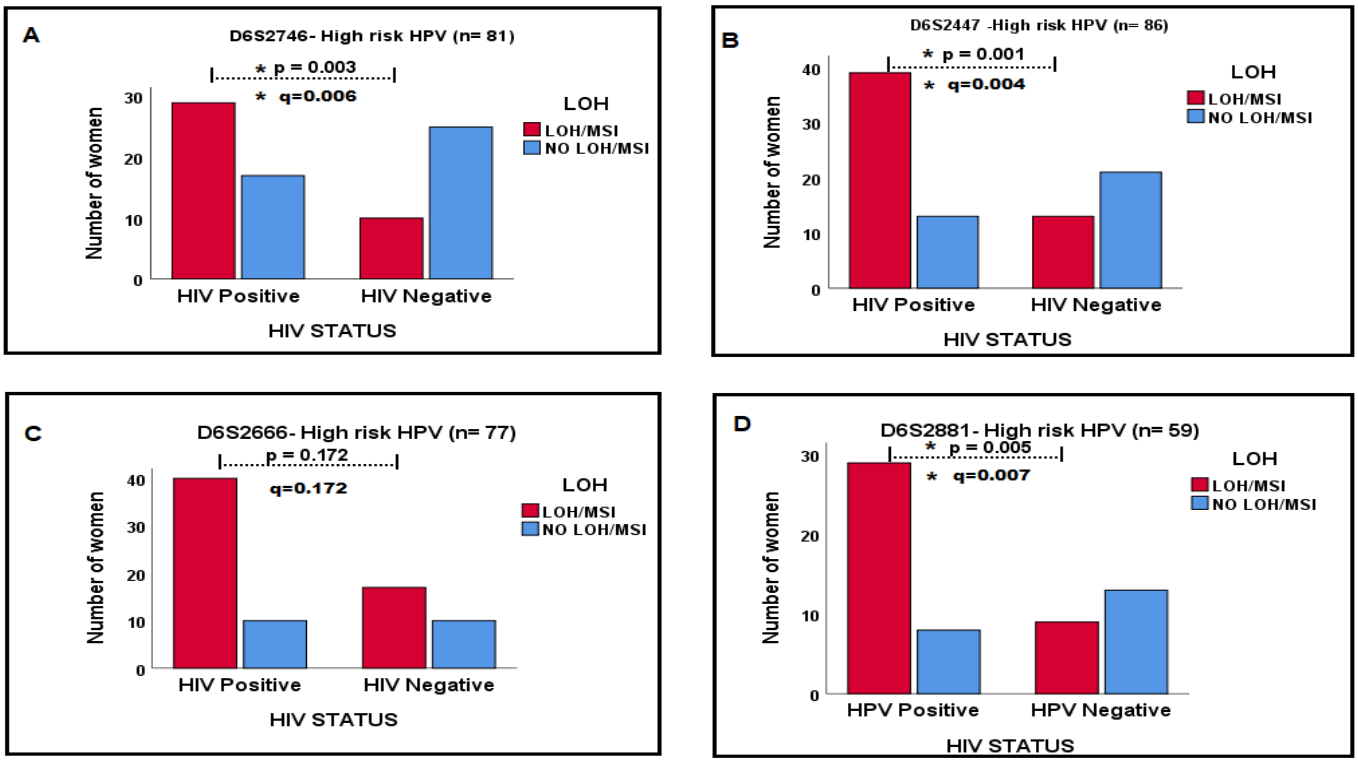


Figure 5. 3 The relationships in LOH/MSI status between HIV-1-positive and HIV-1-seronegative women with Hr-HPV infection in four markers in A, B, C&D.

5.4 Discussion

The present study represents the first in-depth analysis of cancerous changes, viral infection, and host LOH/MSI. The study performed comprehensive allelo-typing of isolated genomic DNA from buccal swab samples (control DNA), and from cervical precancerous lesions and ICC samples. All these samples were from HIV-1-positive and HIV-1-seronegative South African women, none of them were HPV vaccinated previously. More importantly, this study is the first to offer an opportunity to examine the effects of HIV-1/HPV co-infection in cervical cancer by using a host molecular genetics approach. The findings revealed a significantly higher frequency of chromosomal LOH/MSI in tumour biopsy DNA from HIV-1-positive women than from HIV-1-seronegative women at the *HLA* II locus on chromosome 6p, as summarised in **Supplementary Table 5.1**. The investigation also contributes some important information to the existing theories of host molecular genetic alterations and cervical carcinogenesis in HIV-1-positive women that requires further investigation.

Supplementary Table 5. 1 Summary of the results

	LOH/MSI, n/N (%)	LOH/MSI, n/N (%)	*p-value*	*q-value*
	Cervical disease stage			
DNA marker	**Pre-invasive lesions**	**Invasive cancer**		
BAT 26	6/36 (16.7)	6/23 (26.1)	0.51	1.02
D6S266	12/26 (46.2)	4/11 (36.4)	0.723	1.085
D6S2666	49/69 (71)	20/28 (71.4)	>0.99	0.99
D6S2881	25/48 (52.1)	26/33 (78.8)	**0.019**	0.114
D6S2746	33/63 (52.4)	18/44 (40.9)	0.325	0.975
D6S2447	48/78 (61.5)	18/31 (58.1)	0.829	0.99
	HIV-1 status			
DNA marker	HIV-1-seronegative	HIV-1-positive		
BAT 26	3/23 (13)	9/36 (25)	0.334	0.401
D6S266	9/18 (50)	7/19 (36.8)	0.515	0.515
D6S2666	20/35 (57.1)	49/62 (79)	**0.035**	0.052
D6S2881	15/35 (42.9)	36/46 (78.3)	**0.002**	**0.004**
D6S2746	15/51 (29.4)	36/56 (64.3)	**<0.001**	**<0.001**
D6S2447	20/47(42.6)	46/62 (74.2)	**0.001**	0.003
	High risk HPV			
DNA marker	HIV-1-seronegative	HIV-1-positive		
D6S2666	18/77 (23.4)	40/77 (51.9)	0.172	0.172
D6S2881	10/59 (16.9)	30/59 (50.8)	0.005	0.007
D6S2746	10/81 (12.3)	30/81 (37)	**0.003**	**0.006**
D6S2447	11/86 (12.8)	40/86 (46.5)	**0.001**	**0.004**
Multivariate logistic regression analyses				
	HIV-1 status			
DNA marker	HIV-1-seronegative	HIV-1-positive		
D6S2666	35/97 (36.1)	62/97 (63.9)	**0.021**	0.063
D6S2881	35/81 (43.2)	46/81 (56.8)	**0.025**	0.15
D6S2746	51/107 (47.7)	56/107 (52.3)	**<0.0001**	<0.001
D6S2447	62/109 (56.9)	47/109 (43.1)	**0.002**	**0.012**
Tumour stage (D6S2666)				
CIN 1 & 2	44/ 97(45.4)		**0.027**	0.054
CIN 3	25/97 (25.8)			
Invasive	28/97 (28.9)			

Histopathology (D6S2666)			
Mild dysplasia	16/97 (16.5)	**0.015**	0.09
Moderate dysplasia	28/97 (28.9)		
Carcinoma *In situ*	26/97 (26.8)		
Squamous cell carcinoma	23/97 (23.7)		
Adeno-squamous cell carcinoma	4/97 (4.1)		

ROC curves analyses for age

	LOH/MSI			
DNA marker	**HIV-1-seronegative**		**HIV-1-positive**	
D6S2666	AUC=0.8281	p=0.005, q=0.005	AUC=0.7348	**p=0.01, q=0.02**
D6S2881	AUC=0.9615	**p=0.042**, q=0.083	AUC=0.6545	p=0.1354, q=0.1354
D6S2746	AUC=0.5577	p=0.7989, q=0.7989	AUC=0.6328	p=0.1760, q=0.352
D6S2447	AUC=0.8611	p=0.1015, q=0.1015	AUC=0.7063	**p=0.0224**, **q=0.044**

Where; AUC=Area under the curve.

Although the duration of oncogenic Hr-HPV infection is recognised as an etiologic factor for the development of cervical disease, other host factors, and an understanding of the mechanisms involved in the cervical carcinogenesis pathway in the host, remain unknown [231]. In view of the association of certain *HLA* II genes on chromosome 6p with cervical cancer, this study has considered the molecular genetic basis of this association in the host.

The present study reports LOH/MSI from tumour DNA in precancerous lesions and cancerous lesions even from HIV-1-seronegative women. These results suggesting that, in HPV infected women with cervical disease, LOH/MSI is an early genetic event in the development of cervical cancer, including the pre-invasive lesions. HIV-1 infection is an additional factor that appears to change the genetic makeup of the host cell, which in turn causes the overall genomic instability of cervical tumour DNA during the carcinogenic process. Although HPV infection alone can induce LOH/MSI at the *HLA* II locus in cervical tumour DNA, HIV-1 co-infection exacerbates it, potentially accelerating cervical disease progression in a subgroup of HIV-1-positive women.

Since LOH/MSI play an important role in cervical carcinogenesis [89], five locus-specific DNA markers were used to study LOH/MSI at the *HLA* II locus on chromosome 6p. This region contains the *HLA* II, specifically, -DRB1 and -DQB1 which are responsible for viral infection recognition and antigen presentation to the immune system [32]. Loss of an allele, or loss of a part of a chromosome at the *HLA* II locus, may lead to haplo-insufficiency, and can have multiple functional effects on viral immune response genes. Furthermore, if one allele of a tumour-suppressor gene is inactivated earlier at a specific locus by somatic mutations, then deletion of a second allele, as detected by LOH, may result in loss of function of a tumour-suppressor gene or an immune-response gene [332]. These results strongly support the argument that the *HLA* II locus on chromosome 6p is critical in the pathogenesis of cervical cancer. The present study has demonstrated that several genetic alterations associated with different cervical disease stages regardless of HIV-1 infection status. Findings similar to these have been reported by Harima *et al.* in Japan [322], by Mazurenko *et al.* in Russia [333, 334] and by Pulido *et al.* in the USA [134]. However, the specific tumour-suppressor gene(s) at this locus remain unknown in different study populations.

A previous study [185], described an association, or likely protection from cervical cancer in HIV-1/HPV co-infected South African women, with certain *HLA* II genotypes. Furthermore, a study carried out by Meys *et al.* in 2016 [38] reported that specific *HLA* immunogenotypes can determine the persistence of HPV infection in HIV-1 infected patients even during antiretroviral treatment. The presence of HIV-1/HPV co-infection in combination with specific *HLA* II genotypes or a haplotype may increase or decrease the risk of cervical disease development. Therefore according to the present study, in the case of likely protective *HLA* II (*HLA* II alleles associated with decreased cancer risk), presence of LOH/MSI at the *HLA* II locus may further affect the protective function of these genes on HPV infection clearance [36]. Additionally, genomic changes and genetic alterations in the cervical pre-cancerous lesions and ICC may be induced differently by different types of HPV, and particular HPV-specific *HLA* II acting in combination [219, 335, 336]. Since cervical cancer is a complex genetic disease, this thesis acknowledges that the influence of epigenetics and other genomic changes on cervical cancer progression [128, 337], may also play a part in this process.

The presence of MSI is phenotypic evidence that DNA mismatch repair is not functioning normally [338]. Therefore, structural genomic changes within the *HLA* II locus, may determine cervical lesions that are likely to progress to invasive cancer due to inactivation of immune-response genes and the persistence of oncogenic Hr-HPV infection. HIV-1 Tat proteins can also interact directly with functional tumour-suppressor genes in the host (*Rb* and *p53*) [144, 145], which induces increased cell proliferation and increases the effect of Hr-HPV oncoproteins E6 and E7 in cervical carcinogenesis [27, 143].

Genomic instability is an early event in carcinogenesis and arises as a consequence of the disruption of critical cell-cycle checkpoints and failure of the DNA damage-repair system as observed in premalignant tumour DNA (**Table 5.4** and **Figure 5.2**). These findings are similar to those of Migdalska-Sek *et al.* [89] who reported LOH/MSI as early events in precancerous lesions according to HPV infection status, however, the HIV-1 status of their patients was unknown. Presence of LOH/MSI in premalignant tumours allows cells to acquire the additional mutations, required for malignant transformation [339]. This study further suggests that genetic instabilities are early trigger genetic events which may facilitate the subsequent establishment of all other hallmarks of cancer.

Other studies performed on LOH/MSI in head and neck cancers, colon cancer, ovarian cancer and in cervical cancer in different populations with unknown HIV-1 status, reported frequent LOH/MSI in many chromosomes, with LOH/MSI frequencies that varied from 17% to 90% [89, 134, 265, 325, 326, 340]. However, in this study, the LOH/MSI frequencies vary from 13% to 79% (**Table 5.5**). The discrepancy between the present study and other published works could be due to;

(a) The quality and concentration of genomic DNA.

(b) Differences in the specific DNA markers used.

(c) Intrinsic genetic differences in genomic DNA composition among different populations.

(d) The specific cancer disease of interest, whether it is MSI-High, MSI-Low, microsatellite stable or unstable [341].

(e) The particular chromosomes examined.

(f) The specific PCR optimization conditions used [342].

The presence of dual-oncogenic HPV and HIV-1 infections has remarkable effects on genomic instability in our study population. This was demonstrated by the observation that Hr-HPV infection influenced the frequency of LOH/MSI at the *HLA* II locus more in cervical tumour DNA from HIV-1-positive women than cervical tumour DNA from HIV-1-seronegative women, except in DNA marker D6S2666 (p=0.172) (**Figure 5.3C**). However, when this study examined the odds of having LOH/MSI with other predictor variables in a multivariate logistic regression analysis, LOH/MSI was significantly associated only with HIV-1 status, in all four markers (**Table 5.6**). This may be due to the effects of including many predictor variables at once, which can dilute the true association, scientific plausibility, and clinical meaningfulness of any individual result [276].

However, the present study obtained interesting results by using the receiver operating characteristic (ROC) curves (**Supplementary figure 5.1**) that age was a significant predictor of invasive cervical cancer outcome in HIV-1-positive women with LOH/MSI in DNA markers D6S2447 and D6S2666. In combination with **Table 5.5** and **Table 5.6**, these results suggest that, age above 30 years is a strong poor prognostic factor for ICC in HIV-1-positive women with LOH/MSI by using these two markers. Further studies are warranted on the differential effects of specific HPV genotypes and HIV-1 infection on the overall genomic instability of cervical tumour DNA during carcinogenesis.

The unique strengths of this study include the comparative molecular investigation of the frequency of LOH/MSI between tumours from HIV-1-positive and HIV-1-seronegative women. The examination of abnormal cervical tumour epithelial DNA and matched normal buccal mucosa epithelial DNA, as control DNA. Previously published studies have focused almost exclusively on control DNA from whole blood, which may not provide a reasonable comparison with a matched abnormal epithelial tumour DNA. Previously published studies examining tumour biopsies, have not reported on cervical tumours from HIV-1-positive women. The present analysis has focused on chromosome 6p21 only, with five different locus-specific DNA markers to amplify fractions of the *HLA* II locus. Mononucleotide repeats were disregarded due to difficult markers analyses and difficulty in distinguishing heterozygotes from homozygotes if the allele sizes were very similar, because of 'stutters'. The capillary electrophoresis analyses were repeated by using ethanol precipitation to clean the PCR products, wash out excess salts and unincorporated primer leftover after PCR for samples which showed poor amplification. PCR-based LOH assays include the ability to detect small deletions and the ability to enrich for tumour cells through microdissection [77]. This investigation has been able to answer the research question by demonstrating that HIV-1/HPV co-infection does provoke additional LOH/MSI in cervical tumour DNA during progression, which may influence the rate of cervical disease progression in a subgroup of HIV-1-positive women.

The study limitations include the possibility of contaminated tumour samples since histologic sections of tumours usually contain a mixture of tumour cells, inflammatory cells, stromal cells, and other cellular contaminants. Limited molecular data from the study population on the presence of polymorphisms at the primer binding sites, therefore null alleles could not be excluded. Relative fluorescent units (RFUs) were very high for some swab samples and very low or absent for others which reflects unequal amplification between swab samples. In case of inadequate DNA concentration or low DNA concentration, one allele may be preferentially amplified over the other, and where one allele has insufficient amplification, termed *allelic dropout*. Off-scale peaks could not be sized accurately and it was not possible

to determine the peak heights when the camera was saturated above 8000 RFUs. Finally, we did not make any patient follow up in our study.

Future research opportunities may include in vivo studies to demonstrate the mechanisms of HIV-1/HPV co-infection carcinogenesis with specific *HLA* II alleles or haplotypes combinations, and genome-wide assays including massively parallel DNA sequencing and single-nucleotide polymorphism arrays to investigate further in-depth mechanisms and the presence of cause-effect relationships of our findings.

5.5 Conclusions

This study has demonstrated a unique relationship between LOH/MSI in cervical tumour DNA and HIV-1/HPV co-infection in a cohort of South African women. Tumour DNA from HIV-1/HPV co-infected women demonstrated a higher frequency of LOH/MSI than tumour DNA from HIV-1-seronegative women at chromosome 6p. Loss of an allele or part of a chromosome can have multiple functional effects on immune-response genes, DNA damage-repair genes and tumour-suppressor genes. The results suggest that HPV infection alone can induce LOH/MSI at the *HLA* II locus in cervical tumour DNA, whereas HIV-1 co-infection exacerbates it, possibly accelerating cervical disease progression in a subgroup of HIV-1-positive women. This work adds to the existing theories of host molecular genetic alterations and cervical carcinogenesis.

CHAPTER 6: General Discussion, Conclusions with the Strengths and Weaknesses of This Study, and the Future Perspectives.

6.1 Summary of the main findings

The main aim of this research was to study the influence of host molecular genetic variations and alterations at the *HLA* II locus, and HIV-1/HPV co-infection on progression of cervical cancer in a cohort of South African women. The molecular analysis performed as part of this research was limited to *HLA* II (DRB1 and DQB1) on chromosome 6p mainly because the genetic polymorphisms and alterations at these loci have been reported most likely to influence cervical carcinogenesis by allowing HPV persistence. *HLA* II alleles are involved in directing CD4 T-cell responses. It is well established that T-cell recognition of virally-infected cells works through restricted epitope recognition [166]. Down-regulation of the viral peptide-*HLA* II complex on the infected cells may lead to protection from cytotoxic T-cells [343]. Therefore, HPV infection may inhibit CD4 helper T-cell recognition through down-regulation of *HLA* II molecules and thereby evade host immunity [344]. Our results strongly support the assumption that the *HLA* II locus on chromosome 6p is critical in the pathogenesis of cervical cancer.

The first specific objective was to review the published literature in order to identify genes, host molecular genetic variations and genetic alterations in cervical cancer progression that may play a role in disease progression in HIV-1/HPV co-infected women. The second specific objective was to characterize HPV genotypes within cervical tumour biopsies and assess the relationships with cervical disease stage, age, HIV-1 status, absolute CD4 count, and CD4 percentage, and to identify the predictive power of these variables for cervical disease stage in the cohort of South African women. The third specific objective was to determine whether host *HLA*-DRB1 and -DQB1 backgrounds in HIV-1/HPV co-infected women were involved in cervical cancer disease progression. The fourth specific objective was to investigate whether HIV-1/HPV co-infection provokes additional LOH/MSI at the *HLA* II locus to influence the rate of cervical disease progression as observed in a subgroup of HIV-1-positive women.

Carcinogenesis in HIV-1-positive cervical cancer has three major steps: (i) HPV integration into the host genome; (ii) dual pro-oncogenic effects of HPV oncoproteins E6/E7, and HIV-1 oncoproteins Tat within the host genome, and (iii) the accumulation of repeated, unrepaired genetic alterations in the host chromosomal DNA. Compared to HIV-1-seronegative cervical cancer, HIV-1-positive cervical carcinogenesis has additional oncogenic effects from HIV-1 Tat oncoproteins. Both HIV-1 and HPV are classified as carcinogenic viruses. Intracellular HIV-1 Tat proteins interact directly with the host *Rb* and *p53* tumour-suppressor genes in the host. This interaction induces increased cell proliferation, which promotes the effects of HPV oncoproteins E6/E7 in cervical carcinogenesis. These additional oncogenic effects may influence rapid cervical carcinogenesis observed in some HIV-1-positive women.

Rapid cervical carcinogenesis could manifest as a younger age of disease onset, or by a rapid progression from non-invasive cervical disease to invasive cervical cancer. HPV infection alone can induce LOH/MSI in cervical tumour DNA, whereas HIV-1 co-infection exacerbates it, this may influence the rate of cervical disease progression in a subgroup of HIV-1-positive women. However, the question remains as to why only few HIV-1-positive women develop rapid cervical carcinogenesis? This may be the result of one or more of the other inter-population or multi-population genetic differences and a wide range of confounding viral and host factors, which include:

(i) The type of antiretroviral drugs administered

(ii) HIV-1 and HPV viral loads

(iii) Duration of HIV-1 infection

(iv) Specific oncogenic HPV genotype infection

(v) *HLA* genotype variations

(vi) Severity of genetic alterations or genetic instabilities

(vii) Environmental effects such as host behaviour and demographics that can influence the rate of cervical disease development in HIV-1/HPV co-infected women.

(viii) The action of many other different host genes that have been found to be positively, or negatively associated, with cervical cancer development in different study populations.

This study has identified likely protective *HLA* associations with *HLA* II alleles,-DRB1*13:01 and -DQB1*03:19 which were rare or absent in women with cervical disease when compared to the age-matched control population. Conversely, *HLA* II-DQB1*03:01 and -DQB1*06:02 appear to be associated with high risk of cervical cancer development. These findings will provide insight into individualization in treatment and may direct the development of immune-based prevention measures. The presence of LOH/MSI at the *HLA* II locus may affect the likely protective function of specific *HLA* II alleles on HPV infection clearance. HIV-1 as a co-infection may not be a direct risk factor for cervical cancer disease, although HIV-1-positive status has been shown to influence the relative risk for cervical disease development. Furthermore, although cervical disease was more prevalent in women with low CD4 percentage, the crude measurement of CD4 percentage or absolute CD4 count in HIV-1/HPV co-infected women is not directly reflective of cervical disease risk, except for those above 40 years of age. However, even in those above the age 40 years, this seems to depend on the host *HLA* II background, the extent of LOH/MSI in the *HLA* II locus, and the HPV genotype infection.

This means that HPV infection alone may influence cervical disease development to a significant extent when it is combined with the apparently high-risk host *HLA* II alleles, -DQB1*03:01 and -DQB1*06:02. In the presence of HIV-1 as a co-infection, the overall combination increases the relative risk of cervical cancer development and the rate of cervical disease progression. Since there was no level of the absolute CD4 count that puts HIV-1-positive women at lower risk for oncogenic Hr-HPV infection and cervical disease

development, these women may be ideal for individualized HPV vaccination of adult HIV-1-positive women, at any absolute CD4 count level.

6.2 Strengths and Weaknesses of This Study, and the Future Perspectives

The work reported here has a number of features that could be improved. These include the quality, selection bias of the study cohort and reliability of the analyses performed. The strengths of this research include:

At the technical level: The high stringency of the DNA purification included gel visualization of DNA in order to establish DNA quality and standardized dilution of all DNA samples to 0.2ng/µl or 0.3ng/µl, if necessary. All DNA markers used were optimized to amplify in two multiplexes in order to standardize the results of amplification. Only microsatellite markers with the best PCR amplification profiles were selected. The analysis was narrowed to focus only on chromosome 6p21, with five different locus-specific DNA markers to amplify regions within the *HLA* II locus. Markers were selected with both high and low polymorphism information content (PIC), to accommodate the unknown genetic diversity of the study population.

At the clinical molecular level: In order to improve the reliability of the comparative molecular investigations of the frequencies of LOH/MSI in tumour DNA between HIV-1-positive and HIV-1-seronegative women, the patient-matched DNA samples were taken from the same tissue types, abnormal epithelial DNA and matched normal epithelial DNA. In addition, all cervical biopsies from precancerous lesions and ICC used in this study were histologically confirmed.

In the statistical analysis and research methods: Effort was made to calculate the adequate sample size required and multiple statistical tests were used with different analyses. The correction for false discovery rate (FDR) and the use of different study designs for different research objectives add more strength to the study findings.

Most importantly, the research question has been answered that HIV-1/HPV co-infection provokes additional LOH/MSI in cervical tumour DNA which may influence the rate of cervical disease progression in a subgroup of HIV-1-positive women.

Future research suggested by this study:

To address some of the shortcomings of this work, it would have been better to have more information and larger sample numbers in order to study the effects of LOH and MSI separately for each marker at the *HLA* II locus. This was not possible because of the limited study duration and budgetary constraints. Increasing the cohort size would make it possible to resolve the effects of each oncogenic HPV genotype separately. It would be useful to have patient follow up via a longitudinal study to assess cervical disease progression, prognosis and survival analysis in relation to the severity of LOH/MSI in HIV-1-positive women (amongst other parameters). Further analysis could involve additional information regarding: the duration of HIV-1 or HPV infection, detailed information on immunocompromised status with nadir CD4 count, HIV-1 and HPV viral loads, HIV-1 sub-types, and ARV drug type, and duration of ARV.

From a laboratory, and technical viewpoint, it would be ideal to do a confirmatory test for HIV-1 RNA in the tumour biopsies used for the analysis and to emphasize the considerations for genital HPV contamination during the cervical tumour biopsy processing in order to improve technical sampling issues. One of the drawbacks of using tumour biopsy samples is the lack of the potential to address tumour genetic heterogeneity. It would be useful to extend

this research to include some consideration of tumour heterogeneity, particularly by using liquid biopsy or peripheral blood to study circulating tumour DNA.

A future study should be carried out to assess specific HPV genotypes with cervical cancer risk according to age, *HLA* II alleles, LOH/MSI, CD4 count, CD4 percentage and HIV-1 infection, by using more sensitive methods such as high diversity single nucleotide polymorphism (SNP) arrays in a blinded randomized manner. This could investigate in-depth mechanisms and the presence of cause-effect relationships of our findings. *In vivo* studies to demonstrate the mechanisms of HIV-1/HPV co-infection carcinogenesis with specific *HLA* II alleles or haplotype combinations are a logical extension of this work and are warranted.

The advances in Next Generation Sequencing (NGS) and the potential outcomes of NGS analysis make it possible to include genome-wide assays, including massively parallel DNA sequencing and SNP arrays, to make an SNP-based comparison between HIV-1-positive women with persistent and clearing Hr-HPV infection and their *HLA* immunotypes. Whole genome or exome sequencing can yield information on additional rare mutations and novel tumour-suppressor genes (TSG) in HIV-1/HPV co-infected women according to specific HPV genotypes. It could also enable variant analysis of HPV-16 diversity amongst HIV-1-positive women with cervical disease in South Africa.

This thesis provides novel insights, new information, and adds new knowledge to the existing theories in the field of cervical cancer molecular genetics. It shows the influence of HIV-1/HPV co-infection on cervical disease progression with regard to: age, absolute CD4 count, CD4 percentage, host molecular genetic variations, and genetic alterations at the *HLA* II locus on chromosome 6p. This is the first published research in cervical cancer to analyze the relationships between specific host *HLA* II alleles, CD4 immune status, and the LOH/MSI on chromosome 6p in HIV-1/HPV co-infected women worldwide.

This work recognizes the urgent need to move away from the "one size fits all" generalization and focus on individualization in precision genomic medicine for prevention and treatment of cervical cancer in HIV-1/HPV co-infected women. It is, however, acknowledged that there may be other host molecular genetic factors that have not been considered in this thesis or are as yet unknown, which may influence the rate of cervical disease progression in HIV-1/HPV co-infected women.

However, identification of early molecular genetic changes and genetic risk factors for cervical disease development is very crucial. The benefit of implementing a molecular genetic profiling in cervical cancer-screening programs in all women infected with HIV-1 should be envisaged. Although this project proposed to interrogate only the *HLA* II locus on chromosome 6p, it should be obvious that other regions of the *HLA* system, other immune response genes or whole genomes that may be subject to mutations/rearrangements during cervical carcinogenesis should be investigated.

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APPENDICES

APPENDIX A

CONSENT FORM FOR PARTICIPATION IN THE RESEARCH PROJECT

Name of Researchers: Dr.Ramadhani Chambuso and Prof. Raj Ramasar

Informed Consent:

HIV/HPV co-infection, host genetics and cervical cancer study

Principal Investigator: Dr Ramadhani Chambuso, Division of Medical Virology/Human Genetics, Institute of Infectious Disease and Molecular Medicine, University of Cape Town.

Co-investigator: Assoc. Professor Jo-Ann Passmore/Prof. Raj Ramesar, Department of Pathology, Faculty of Health Science, University of Cape Town

Dear Participant,

Thank you for the interest in our study and considering being a participant in the research. You will be approached for cervical biopsy, dried blood sample and oral swab for research purposes. The decision to join our study is completely your own. If you decide not to participate this will not affect your care here at the hospital or anywhere else, now or in future. This consent form tells you about the study that the Ocean Road Cancer Institute and the Department of Medical Virology, University of Cape Town is carrying out. We do require you to read this form or have it read to you. Please take your time deciding if you wish to join this study. Do not hesitate to ask questions about anything you do not understand. If you decide to join the study, we will ask you to sign this form. Signing this form means that you have read the form (or had it explained to you), understand it and agree to join the study. Please note that the procedures involved in this study are not part of the routine care for patients and these will be over and above the standard of care provided. Women with additional HPV related conditions such as warts will be offered treatment and/or surgical removal of warts at department.

About the HIV/HPV co-infection, host genetics and cervical cancer study:
We are conducting this study in order to learn more about the influence of HIV/HPV co-infection and host genetics in the early development of invasive cervical cancer with regard to immunity. Furthermore, we want to investigate why HIV/HPV co-infected women progress faster to invasive cervical cancer in early age and others do not regardless of the immunity and to get a better understanding of what happens to host genetic changes and the immune responses towards invasive cervical cancer development, when you are immunocompromised by a virus like HIV. If you agree to take part in our study, you will be asked to be enrolled and get updates of the ongoing study results.

Number of visits:
You will be asked for contact information including your physical address and cell phone numbers, and the best way to stay in contact with you.

What samples we will need to take for this study:
We will ask for the following samples from you, if you agree to participate in this study:

- Finger pin prick of blood once from your hand.
- During your vaginal/cervical examination, a very small (2mm) piece of cervical tissue biopsy will be taken (with local anaesthetic).
- Finally, a buccal mucosal swab will be taken from your mouth. This will not be painful.

All the samples collected will be used to measure the changes of immune cells, types of viral infection (HPV and HIV) and further genetic changes if occurred. The results may be used for further publications relating to the study. The tests that we are doing will not replace the kind of care you get from the department.

Your rights and responsibilities:
As a participant in this study, we respect your rights. You may request to view the World Medical Association's Declaration of Helsinki and/or the Department of Health's booklet, entitled 'What you should know when deciding to take part in a clinical trial as a research participant. These documents will inform you of your rights and responsibilities as a study participant in our study.

Important to remember:

- Your participation in this study is voluntary. If you decide not to participate in this study, this will not affect the quality of care you receive from the department.
- Information obtained is entirely confidential and will be used for research purposes only. Please note that, Human Research Ethics Committee of the UCT have the right to inspect the patients' records.
- Participants will be given R100 for the participation as incentive. However, your involvement may help improve the care of women with invasive cervical cancer in future.
- The Human Research Ethics Committee of the University of Cape Town, has approved this study. For further information, please contact the Human Research Ethics Committee on 021-4066626, +255718716975.
- If you have any questions regarding our study, please feel free to phone Dr Ramadhani Chambuso at telephone number 0682-387836 or 021-6507963.

We would appreciate your involvement in this study.
What we learn from this study may improve the health of Tanzanian women.

STATEMENT OF CONSENT

Study title: The HIV/HPV co-infection, host genetics and cervical cancer study

I, ……(name of study participant), have agreed to participate in this study.

- I understand that I will have the following tests done:
  1. Blood samples to be taken from my hand
  2. Cervical biopsy sample to be taken from the mouth of the womb
  3. Buccal mucosal swab from my mouth

- I have had a chance to ask questions about the study.
- My questions have been answered and I understand the answers.
- I have been given all the detailed information regarding the 3 visits.
- I understand that my decision whether or not to take part in the study is voluntary.
- I understand that if I decide to continue with the study I may withdraw at any time.
- By signing this consent form I do not give up any rights that I have as a research participant.

SIGNATURES

Participant Name (Print)	Participant Signature	Date
Study Staff Conducting Consent Discussion (Print)	Study Staff Signature	Date
Witness Name (Print)	Witness Signature	Date

Clause – Insurance in Consent Form

In industry-sponsored clinical research, the consent form must include a simply-worded statement that research-related injuries will be compensated according to the Association of the British Pharmaceutical Industry (ABPI) Clinical Trial Compensation Guidelines.
In investigator-initiated research which relies on UCT's No Fault Insurance policy, the consent form must include the following language:
What if Something Goes Wrong?

APPENDIX B

Buffers used in this study

10X TBE buffer
108 g Tris, 55 g boric acid and 9.3 g EDTA.
Dissolved and make up to 1 liter. Autoclave

0.5X TBE buffer
45mM Tris-borate (v/v)
1mM EDTA (w/v)
pH was adjusted to 8.3

TE buffer
10 mM Tris-HCl,
1 mM EDTA (w/v)
pH 8.0

Lysis buffer
50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl

Ethidium Bromide (EtBr)
1x (0.2 ml/ml glycerol and 5 mg/ml bromophenol). To dissolved shake it.

Loading buffer
0.25% (w/v) bromophenicol blue and 0.25% (w/v) xylene cyanol in 30% (v/v) glycerol in sterile distilled water.

10X TE
100mM Tris-Cl pH 7.5

10mM EDTA

Dissolve 12.11 g Tris and 3.72 g of EDTA in 700 ml distilled water. pH was adjusted to 7.5 with concentrated HCL. The volume was filled up to 1 L before autoclaving