The Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Dysplasia in Women Living With HIV

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Declaration

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Summary

Title
The Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Cytological Abnormalities in Women Living With HIV

Background
Human Papillomavirus (HPV) infection causes cervical cancer. The prevalence of HPV-related dysplastic lesions is significantly higher in patients co-infected with the HI virus and thought to be linked to possible more persistent HPV infection. There is, however, conflicting evidence as to whether treatment of Human Immunodeficiency Virus (HIV) infection with antiretroviral agents may influence cervical HPV infection and the behaviour of Squamous Intraepithelial Lesions (SIL).

Aims
To examine the effect of the initiation of combination antiretroviral therapy (cART) on: 1) the persistence of cervical Low-grade SIL (LSIL); 2) The progression of cervical LSIL to High-Grade SIL (HSIL); 3) The effectiveness of excision treatment of HSIL 4) HPV genotypes detected, in HIV-infected and uninfected women at the Infectious Diseases Clinic and the Colposcopy Clinic, Tygerberg Teaching Hospital, Cape Town, South Africa.

Design and Methods
We conducted a retrospective cohort analysis of 1720 women with LSIL of the survival of progression-free-time or time-to-clearance. Time to progression or persistence was compared according to HIV status, antiretroviral treatment and CD4 count. In another retrospective cohort analysis, we investigated the effectiveness of excision treatment in 1848 women who underwent LLETZ or CKC biopsy was used. Logistic regression and survival analysis were used to compare excision treatment failure and recurrence-free time between groups according to HIV status, antiretroviral therapy and CD4 count.

To investigate the effect of antiretroviral therapy on the cervical HPV infection, 300 HIV-infected women were prospectively enrolled and followed at 6-monthly interval. Cytological testing and cervical HPV sampling were done at each visit. Biopsy of suspicious lesions and excision treatment were done at colposcopy clinic according to standard a protocol. The Roche Linear array HPV genotyping test was used for HPV detection. Generalized Estimating Equation (GEE) multivariate analysis was applied to investigate the effect of cART on the detection of HPV infection, while adjusting for time-dependent covariates such as CD4 count, sexual activity and excision treatment. The effect on each HPV type was then also compared to the effect on HPV16.

Results
Overall, we found that there was no difference between the progression of LSIL to HSIL by HIV status. However, among HIV-infected patients, those who started ART before first LSIL had a significantly lower risk for progression (HR 0.66, 95% CI 0.54-0.81). CD4 count did not have an impact on the risk for progression. We also found lower persistence of SIL in the HIV uninfected group (HR 0.69, 95% CI 0.57-0.85) and that cART was independently associated with decreased persistence of LSIL. On the other hand, a higher CD4 count at the time of first LSIL was not associated with lower persistence of the lesion.

HIV infected women with HSIL experienced much higher excision treatment failure than uninfected women (53.8% vs. 26.9%, p<0.001). Factors that improved outcome were higher CD4 count and complete excision.

cART reduced the risk of detection of any HPV type by 47% (OR 0.53, 95% 0.49-0.58, p<001). When adjusted for covariates, time of exposure to cART and CD4 had a stronger effect. Every month of cART exposure reduced the risk detection of any HPV type with 7%. The effect was also significant on HPV16 alone (OR 0.93, 95% CI 0.90-0.95). All non-oncogenic subtypes were influenced similarly or more strongly than HPV16, as well as oncogenic HPV52. Only one oncogenic subtype HPV subtype, HPV39, was influenced marginally less (ratio of OR 0.95, CI 0.90-0.99, p=0.04).

There was an increased risk for any HPV detection at CD4 count<200 (OR 1.63, 95% CI:1.50-1.77), but when adjusted, the time of cART exposure again remained the strongest predictor of risk (OR 0.94, 95% CI:0.93-0.95).

Conclusion

cART impact the outcome of cervical HPV infection by increasing clearance, decreasing progression of LSIL and recurrence after excision treatment. This effect is time dependent and also associated with CD4 count. Specifically, HPV16 detection risk is also reduced by cART, and all HPV types are influenced at least as much as HPV16, except possibly HPV39. It seems that increased cervical HIV-proviral load is associated with HPV detection risk, and both are lowered by cART time.

Opsomming

Titel
Die Effek van Kombinasie Antiretrovirale Terapie op Menslike Papilloomvirusinfeksie en Servikale Sitologiese Abnormaliteite in Menslike Immuniteitsgebrekvirus-geïnfekteerde Vroue

Agtergrond
Menslike Papilloomvirusinfeksie (MPV) veroorsaak servikale kanker. Die prevalensie van MPV-verwante displastiese letsels is betekenisvol hoër in pasiënte wie ook met Menslike Immuniteitsgebrekvirus (MIV) geïnfekteer is en dit word gereken dat dit te wyte is aan meer persisterende MPV infeksie. Daar is egter teenstrydige bewyse oor of die behandeling van MIV infeksie met antiretrovirale (ART) middels die infeksie met MPV en die gedrag van Plaveisel Intraepiletiële letsels (PIL) kan beïnvloed.

Doelwitte
Om die effek van die inisiasie van kombinasie ART op: 1) die persistering van Laegraadse PIL (LPIL); 2) die progressie van servikale LPIL na hoëgraadse PIL (HPIL) 3) die sukses van eksisiebehandeling van HPIL; 4) MPV genotypies waarneembaar, in MIV-geïnfekteerde vroue by die Infeksiesiektekliniek en die Kolposkopiekliniek,Tygerberghospitaal, Kaapstad, Suid-Afrika, te ondersoek.

Studie-ontwerp en Metodes
`n Retrospektiewe kohort-analise op 1720 vroue met LPIL van die oorlewing van progressive-vrye tyd en tyd tot opklaring van PIL is gedoen. Tyd tot progressie of opklaring is vergelyk na aanleiding van die pasiënt se MIV status, behandeling met antiretrovirale terapie en CD4-telling. In nog `n retrospektiewe kohort-analise is die effektiwiteit van eksisiebehandeling in 1848 vroue wie LLETZ or Kouemeskonus eksisie ondergaan het, ondersoek. Logistiese regressie en oorlewingsanalise is toegepas om die voorkoms van onsuksesvolle uitkoms en tyd sonder herhaling van letsels tussen groepe te vergelyk na aanleiding van MIV status, ART en CD4-telling.

Om die effek van antiretroviral therapie op servikale MPV infeksie te ondersoek, is 300 MIV-geïnfekteerde vroue opgeneem in `n prospektiewe studie en sesmaandeliks opgevolg. Sitologiese en MPV servikale smere is met elke besoek geneem. Biopsies van verdagte letsels en eksisiebehandeling is by die Kolposkopiekliniek gedoen volgens die standaardpraktyk. Die *Roche Linear Array HPV Genotyping* toets is gebruik vir MPV deteksie. Algemeen-beraamde vergelyking (*GEE*) meerveranderlike analise is toegepas om die effek van die anti-MIV terapie op die teenwoordigheid van MPV op die serviks te ondersoek. Die aangepaste effek is ook getoets deur die CD4-telling, die seksuele aktiwiteits- en eksisiebehandelingstatus by elke besoek in ag te neem. Die effek op elke MPV genotipe is laastens dan ook vergelyk met die effek op ‘n spesifieke basislyn genotype; in hierdie geval was MPV16 gekies.

Resultate
Daar was geen statisties beduidende verskil tussen die progressie van LPIL na HPIL na aanleding van HIV status nie, maar pasiënte wie met ART begin het voordat hulle vir die eerste keer met LPIL gediagnoseer was, het ‘n laer risiko gehad vir progressie (HR 0.66, 95% VI 0.54-0.81). Daar is ook gevind dat dit onafhanklik van die CD4 telling was. Die persistering van PIL was laer in die MIV negatiewe groep (HR 0.69, 95% VI 0.57-0.85), maar ook hier was antiretrovirale behandeling geassosieer met verminderde persistering. Weer eens was daar nie ‘n verband met die CD4 telling nie.

MIV-geinfekteerde vroue met HPILwas baie meer geneig tot gefaalde eksisiebehandeling (53.8% teenoor 26.9%, p<0.001). Verbeterde uitkoms was geassosieer met ‘n hoër CD4-telling en ‘n eksisie wat as volledig beskryf was. ART wat reeds voor die eksisiebehandeling begin was, het nie die risiko vir onsuskesvolle uitkoms statisties beduidend verminder nie, maar het egter die risiko vir herhaling van letsels na die eksisie sterk verlaag.

ART het die kans dat enige MPV tipe waargeneem sou word, met 47% verlaag (OR 0.53, 95% VI 0.49-0.58, p<001). Wanneer aangepas vir ander faktore, was die tyd wat verloop het sedert ART begin was, sowel as vir die CD4 telling, sterker. Vir elke maand sedert ART begin was, het die kans dat enige MPV tipe waargeneem word, met 7% verminder. `n Soortgelyke effek is op HPV16 alleen gevind (OR 0.93, 95%, VI 0.90-0.95). Die effek was net so sterk of sterker op alle subtipes. Slegs een onkogeniese subtipe, MPV39, was gering minder beïnvloed (ratio van OR 0.95, VI 0.90-0.99, p=0.04).

Die kans vir waarneming van enige MPV subtype is hoër wanneer die CD4 telling laer as 200 selle/ųl is (OR 1.63, 95% VI: 1.50-1.77), maar wanneer aangepas, was die tyd van ART weer eens die sterkste voorspeller van MPV infeksie (OR 0.94, 95% VI:0.93-0.95).

Gevolgtrekkings

ART verbeter die uitkoms van servikale infeksie met MPV deur progressie en persistering van LPIL en herhaling van PIL na eksisie te verminder. Die effek is tydsafhanklik en word ook deur die CD4 telling beïnvloed. Die kanse dat MPV16 spesifiek waargeneem word, word ook deur ART verminder, en all MPV tipes ondervind dieselfde of groter verlaging van waarnemingsrisiko as MPV16, behalwe miskien MPV39. Ons kon aandui dat verhoogde teenwoordigheid van servikale MIV verband hou met die risiko vir die waarneming van MPV infeksie, en beide word verminer deur die tyd waarmee die pasiënt met ARV terapie behandel is.

Publications

Parts of this thesis have been published as follows:

1. Zeier, MD, Botha, MH, Van der Merwe, FH, Eshun-Wilson, I, Van Schalkwyk, M, La Grange, M, Mason, D, Louw, M, Nachega, JB. Progression and Persistence of Low-Grade Squamous Intraepithelial Lesions in Women Living with HIV Infection. Journal of Lower Genital Tract Disease 16:3. 2012.
2. Zeier MD, Nachega JB, Van der Merwe, FH, Eshun-Wilson, I, Van Schalkwyk, M, La Grange, M, Mason, D, Louw, M, Botha, MH. Impact of Timing of ART Initiation on Survival of Cervical Squamous Intraepithelial Lesions. A Cohort Analysis from South Africa. International Journal of STD and AIDS, 2012. 23:890-896.

Parts of this thesis have been presented at Local and International Scientific Meetings:

1. Zeier MD, Botha MH, Atzl CA, Eshun-Wilson I, Prozesky HW, Taljaard JJ. Cervical Cancer in HIV-positive women: the Next Epidemic? Poster presentation and overall winner at Annual Academic Year Day, University of Stellenbosch, 2007. Poster presentation and overall winner at annual academic year day University of Cape Town and Stellenbosch combined academic Year day for Internal Medicine 2007.
2. Zeier MD, Botha MH, MH, Van der Merwe FH, Eshun-Wilson I, Van Schalkwyk,M, La Grange M, Mason D, Louw M, Nachega JB. Progression and Persistence of Low-Grade Squamous Intraepithelial Lesions in Women Living with HIV Infection. Oral presentation, Annual Academic Year Day, University of Stellenbosch 2011.
3. Zeier MD, Nachega JB, Van der Merwe FH, Mason D, Van Schalkwyk M, La Grange M, Eshun-Wilson I, Louw M, Botha MH, Engelbrecht S. High Incident Detection of HPV subtypes in Women Living with HIV Infection. Poster Presentation. International Papillomavirus Conference, Sep 2011 in Berlin, Germany.
4. Zeier MD, Nachega JB, Van der Merwe FH, Eshun-Wilson I, Van Schalkwyk M, La Grange M, Mason D, Louw M, Botha MH. Increased Excision Treatment Failure of Intraepithelial Squamous Lesions in Women Living with HIV infection. Winner of Best Presentation, Maternal and Women's Health, Annual Academic Year Day, University of Stellenbosch 2011.
5. Zeier MD, Nachega JB, Engelbrecht S, Van der Merwe FH, Van Schalkwyk M, Mason D, Machekano R, Botha MH. Cervical Human Papilloma Virus Infection in HIV Infected Women: The Effect of Combination Antiretroviral Therapy. Oral Presentation. Maternal and Women's Health, Annual Academic Year Day, University of Stellenbosch 2013.

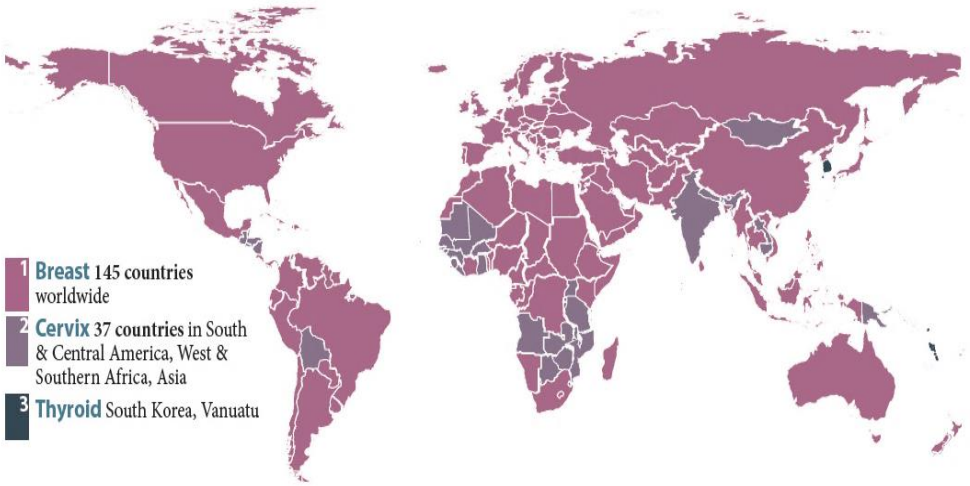
List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cells
(c) ART	(combination) Antiretroviral Treatment
ARV	Antiretroviral
ASC-H	Atypical Squamous Cells of Unknown Significance– high grade cannot be excluded
ASC-L	Atypical Squamous Cells of Unknown Significance – low-grade
ASCUS	Atypical Squamous Cells of Unknown Significance
cART	Combination Antiretroviral Treatment
CC	Colposcopy Clinic
CD4	CD4+ or helper cells
CIN	Cervical Intraepithelial Neoplasia
CKC	Cold Knife Cone
DNA	Deoxyribonucleic Acid
EB	Excision Biopsy
EC	Endocervical Cells
ELISA	Enzyme-linked Immunosorbent assay
GAVI	Global Alliance Vaccine Initiative
GEE	Generalized Estimating Equation
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HR	Hazard Ratio
HR-HPV	High-Risk Human Papillomavirus
HSIL	High-grade Squamous Intraepithelial Lesion
ICC	Invasive Cervical Carcinoma
IDC	Infectious Diseases Clinic
IFN	Interferon
LEEP	Loop Electrosurgical Excision Procedure
LLETZ	Large Loop Excision of the Transformation Zone
LR-HPV	Low-Risk Human Papillomavirus
LSIL	Low-grade Squamous Intraepithelial Lesion
mRNA	Messenger Ribonucleic Acid
MSM	Men who have sex with men
NK	Natural Killer
NNRTI	Non-nucleoside Analogue Reverse Transcriptase Inhibitor
NRTI	Nucleoside Analogue Reverse Transcriptase Inhibitor
OR	Odds Ratio
Pap	Papanicolaou
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
pVL	Proviral Load
RNA	Ribonucleic Acid
TB	Tuberculosis
TBH	Tygerberg Hospital
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VAIN	Vaginal Intraepithelial Neoplasm
VIN	Vulvar Intraepithelial Neoplasm

Chapter 1: Introduction

Cancer of the Cervix in South Africa and World-Wide

Figure 1 Most prevalent cancer by country- females



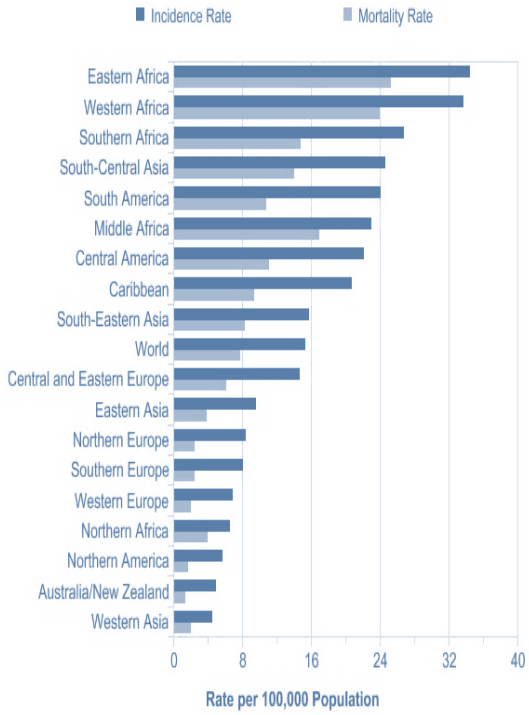
http://www.cancerresearchuk.org/cancer-info/cancerstats/world/

At an incidence rate of 26.8 per 100,000 population, cancer of the cervix is the second most common cancer in Southern African women (1). It is also the biggest cause of cancer death in South African women (1, 2). The region has the third highest cervix cancer mortality rate in the world, after Eastern and Western Africa, (1) (Figure 1). The lifetime risk of cervical cancer in South Africa has been reported as 1 in 26 (3) (Figure 2).

Figure 2 Cervical Cancer Estimates 2008

World Age-Standardised Incidence and Mortality Rates per 100,000 Population, Females, World Regions
(*http://www.cancerresearchuk.org/cancer-info/cancerstats/world/*)

World Region	Incidence Rate	Mortality Rate
Eastern Africa	34.5	25.3
Western Africa	33.7	24
Southern Africa	26.8	14.8
South-Central Asia	24.6	14.1
South America	24.1	10.8
Middle Africa	23	17
Central America	22.2	11.1
Caribbean	20.8	9.4
South-Eastern Asia	15.8	8.3
World	15.3	7.8
Central and Eastern Europe	14.7	6.2
Eastern Asia	9.6	3.9
Northern Europe	8.4	2.5
Southern Europe	8.1	2.5
Western Europe	6.9	2
Northern Africa	6.6	4
Northern America	5.7	1.7
Australia/New Zealand	5	1.4
Western Asia	4.5	2.1



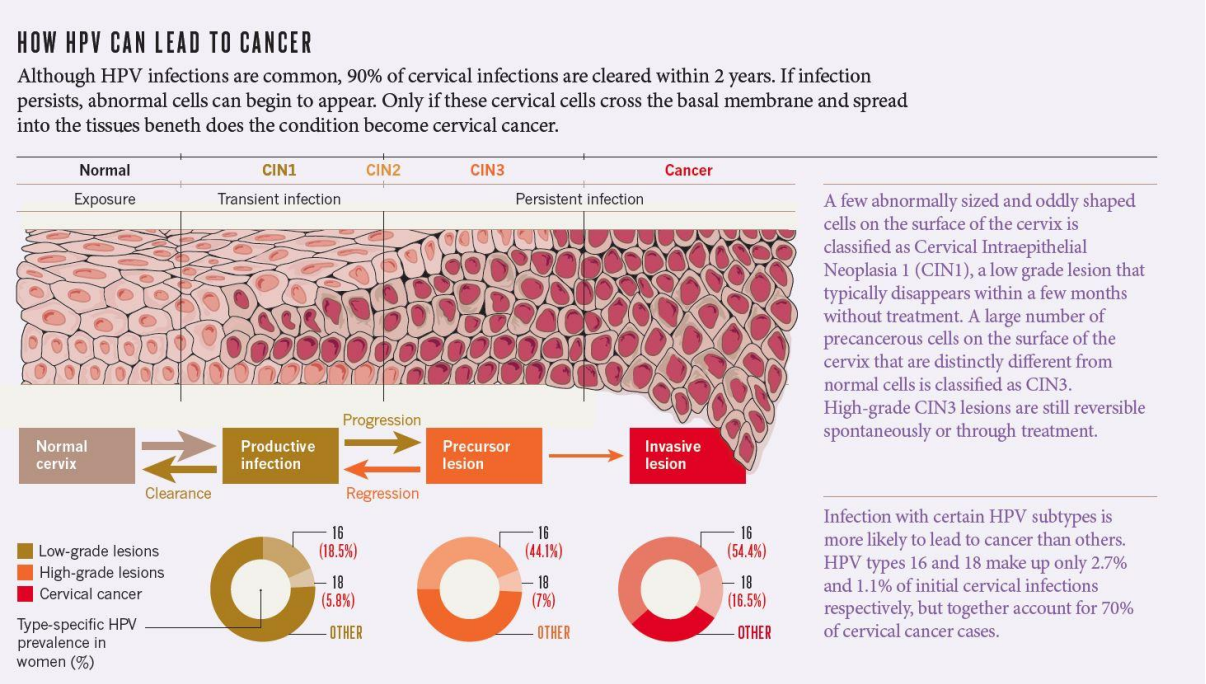
Prevention and Detection of Cancer of the Cervix

Cancer of the Cervix is a largely preventable disease. Detection and treatment of dysplastic pre-cancer lesions through screening programs is implemented internationally. Screening is done, and has been for many years, by performing a Cytological evaluation, or Papanicolaou (Pap) smear, at regular intervals. This procedure is easily performed by Health Care workers and can be performed after minimal training under even basic health service levels. When a low-grade abnormality (low-grade squamous intraepithelial lesion or LSIL) is detected on the Pap smear, it may safely be followed with a repeat smear after two years in order to exclude progression to a more severe high-grade lesion (high-grade squamous intraepithelial lesion or HSIL). If a high-grade lesion is detected, however, referral for colposcopy evaluation by a trained physician is necessary. A biopsy of abnormal-looking areas is done and if the high-grade dysplasia is confirmed, the area needs to be excised to prevent further progression to cervical cancer. Regression of high-grade pre-cancer lesions occurs seldom. Figure 3 represents a diagrammatic summary of the corresponding cytological and histological lesions

associated with the pathogenesis of cervical cancer. Sadly, access to screening facilities is most limited in the most underdeveloped regions in the world, which is reflected by the higher prevalence rates of cervical cancer in the same areas.

Figure 3 Progression of HPV infection in the cells of the Cervix

(Reproduced with permission from (4))



Cervical Cancer Screening Programme in South Africa

For HIV-uninfected or of unknown HIV status, the current National Guideline for Cervical Cancer Screening recommends three Pap smears for every woman – one every ten years, commencing after the age of 30 years (5). For women known with HIV disease and not yet eligible for Antiretroviral Treatment (ART), however, The South African Antiretroviral Treatment Guidelines recommend yearly Pap screening (6).

The management of *abnormal Pap smears* according the National Guideline for Cervical Cancer Screening is identical regardless of HIV status. HSIL is always referred to the nearest Colposcopy clinic for evaluation. For LSIL and Atypical squamous cells of undetermined

significance (ASCUS) results, the smear is repeated after one year. If it persists, colposcopy evaluation is then recommended.

Tygerberg Hospital still has a lower threshold for HIV positive women who have LSIL and recommends colposcopy examination after the first abnormal smear. This is based on evidence that cervical cytology may not be sufficient in HIV infected women as diagnostic tool: they were found to be at high risk for underlying CIN II/III on colposcopy examination when diagnosed with a single cytological diagnosis (7). This is however not widely implemented outside our institution, and this approach still deserves validation.

Human Papilloma Virus Infection and Cervical Cancer

Cervical cancer is caused by oncogenic Human Papilloma Virus (HPV) infection (8). HPV is a small double stranded DNA virus that infects epithelial cells and can cause hyperproliferation of these cells. Cervical HPV infection is asymptomatic and easily transmitted. It is the most frequent viral sexually transmitted disease. The virus can exist in most of the anogenital region and it is likely that transmission during sexual intercourse is not prevented by the use of male condoms (9-15).

Before technological advances enabled researchers to identify the aetiology of cervical cancer, the focus of research was on environmental and socioeconomic factors as causative agents. The progression from HPV infection to dysplasia and eventually invasive cancer is a slow process. In the HIV negative patient, mild dysplasia as well as moderate dysplasia almost always regresses spontaneously.

HPV Vaccine

There is currently no registered vaccine for the prevention of HIV infection. On the other hand, the development of HPV vaccines has been a modern success story. There are currently two non-infectious recombinant prophylactic HPV vaccine options with proven efficacy against some HPV strains. Gardasil ® (by Merck) targets subtypes 6, 11, 16 and 18 (16) and Cervarix ® (by GlaxoSmithKline) subtypes 16 and 18 (17). Both vaccines protect against the two subtypes that are responsible for over 70% of cervical cancer cases and 50% of high grade cervical lesions, and Gardasil ® protects against 93.5% to 96% of persistent HPV

infection, HSIL, vulvar intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VAIN) and genital warts (18). The impact of the nine valent vaccine currently in development will have an even larger impact, and is expected to prevent 90% of all invasive cervical cancer (19). Vaccination is aimed at the age group 9-12 years old, to occur before sexual debut.

Although cost factors currently restrict HPV vaccination programmes world-wide, the recent announcement of drastic price reductions of both registered vaccines will see some African countries implement national rollout programmes by 2014. By 2020, the Global Alliance for Vaccines and Immunisation (GAVI) hopes to protect 30 million girls in 40 countries (20) (figure 4). Unfortunately, due to logistic factors, no catch-up vaccination effort can be expected, and the full cancer preventative effect may not be visible in South Africa for the next 20 years.

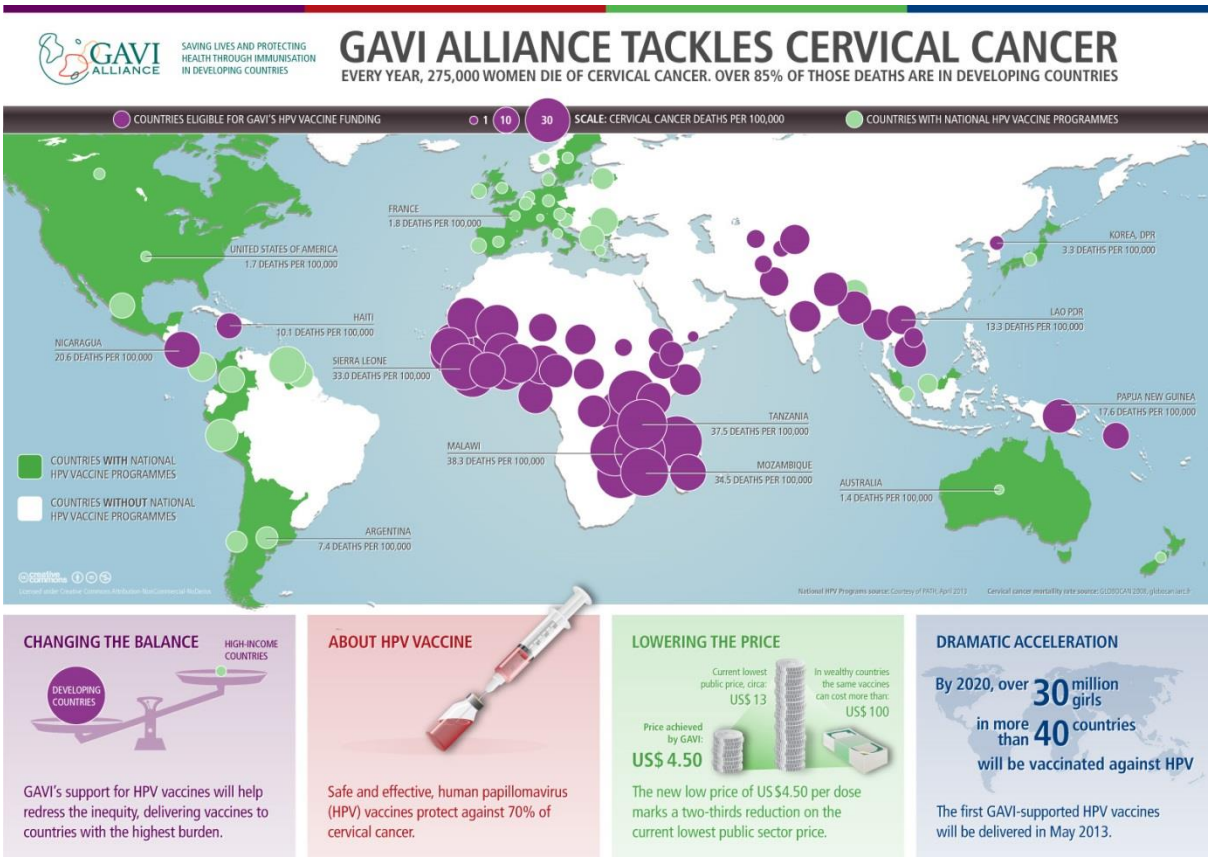


Figure 4 Poster announcing global GAVI expansion of HPV vaccine access

http://www.gavialliance.org/library/news/press-releases/2013/hpv-price-announcement

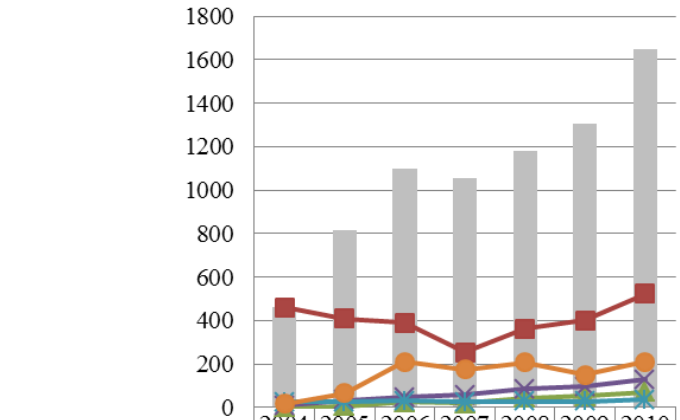
HPV Infection in HIV infected women

The National HIV prevalence amongst women in South Africa according to the 2006 antenatal survey stands at 29.1 % (21). HIV infection causes gradual weakening of cellular immune function and leaves women vulnerable to co-infection with diseases such as Tuberculosis (TB), and certain cancers, including vulval and cervical cancer (22). Even though cervical cancer has been reported as occurring at a younger age in HIV infected women, the burden of untreated HIV disease and high Acquired Immune Deficiency Syndrome (AIDS) mortality when antiretroviral therapy was not accessible, even led a reduction in the prevalence of cervical cancer reported in some areas (23).

Now, in the era of improved survival of women living with HIV infection due to the better access to combination antiretroviral therapy (cART), an expected re-emergence of cervical cancer cases has not yet been documented. Furthermore, women on ART programmes for some years have been reported to have fewer abnormal smears than those recently initiated. Could this be due to cART? And if cART provides protection (clearance and/or decrease likelihood of progression to HSIL), is this due to or mediated by the reconstitution of the immune status, as can be measured by the CD4 T-cell count?

Investigations at Tygerberg Hospital

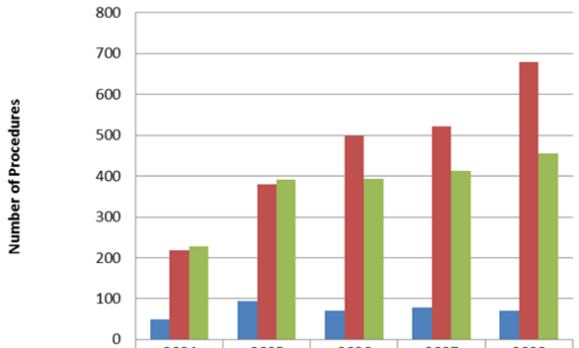
The Infectious Diseases Clinic (IDC) was established in 1993 under the Department of Internal Medicine. It is located on the eighth floor of Tygerberg Hospital (TBH) and has approximately fifty associated staff members, either on a full-time or part-time basis. Initially it treated mostly patients with sexually transmitted diseases and Tuberculosis, but as the HIV epidemic grew in the Western Cape, by 1996 most patients seen were referred for management of their HIV infection. From 1997 to 2011, it has also served as a site for Industry-driven pharmaceutical studies, in order to provide antiretroviral therapy to affected patients. On the 6 January 2004 it became the first site to provide this cART as part of the Government Rollout Programme of Antiretroviral Treatment in the Western Cape. Since then, the IDC has initiated more than 3000 patients on cART (figure 5).



	2004	2005	2006	2007	2008	2009	2010
On Rollout in year	460	818	1098	1054	1178	1304	1647
Initiated at TBH	460	408	389	254	362	400	524
Transferred In	0	5	25	20	43	53	72
LTF	8	34	47	58	85	97	129
Mortalities	25	25	30	24	29	29	37
TFO	18	66	209	175	207	150	209

Figure 5 Numbers of Patients attending the IDC clinic, Tygerberg Hospital, for ART.

The Colposcopy Clinic is located on the third floor of Tygerberg Hospital and falls under the Gynaecological Oncology Unit of the Department of Obstetrics and Oncology. The number of procedures performed at the clinic (biopsies) or referred from the clinic (hysterectomies) is depicted in Figure 1.6. There has been an increase in the number of biopsies since 2004, due to a combination of factors, which include the population growth in referral areas, but certainly, the number of HIV infected women who were referred for colposcopy evaluation also increased dramatically.



	2004	2005	2006	2007	2008
Hysterectomy	49	93	70	79	71
Excision Biopsy	218	380	499	521	679
Biopsy	229	392	393	413	455

Figure 6 Number of procedures performed annually at the Colposcopy Clinic, TBH.

We investigated the prevalence of cervical dysplasia in HIV positive women at Tygerberg Hospital in a pilot cross-sectional study in July 2007 and analysed the cytology smear and histopathology records of 648 HIV-positive females on the ARV programme at the IDC. Only 295 (45.6%) were found to have cytology or pathology results on record at the hospital. This was ascribed to the recommendation at the time that all women, regardless of HIV status, undergo the first Pap screening at the age of 30 years. The results of those women who had undergone screening were compared to those of prevalence survey done in Tygerberg Hospital Antenatal Clinic in 1996 which analysed 4762 cytology results done over a six month period (**Study1**) (24), as well as to a multicentre South African Prevalence study from 2003 (**Study2**) (25). Of the 295 TBH ARV women, 80 evaluated patients *(27.1%)* were found to have LSIL lesions (ASCUS or CIN I), 87 *(29.5%)* HSIL lesions (CIN II or CIN II) and 9 *(3.1%)* had carcinomas. These figures are compared to **Study1** and **Study2** (table 1).

Table 1 Comparison: Cervical Smear Results

Comparison: Cervical Smear Results: Percentage of Patients (Median Age)			
	Study 1: 1996	**Study 2: 2002**	**HIV ARV Group: 2007**
Normal	83.99%	Not reported	38,2% *(36,5)*
LSIL	2.66% *(25,6)*	2.42% *(33,1)*	27.1 % *(34.1)*
HSIL	0.84% *(30,8)*	1.8 % *(38,0)*	29.5 % *(35,7)*
Invasive CA	0.02 % *(not reported)*	0.47 % *(51,3)*	3.1 % *(42.8)*

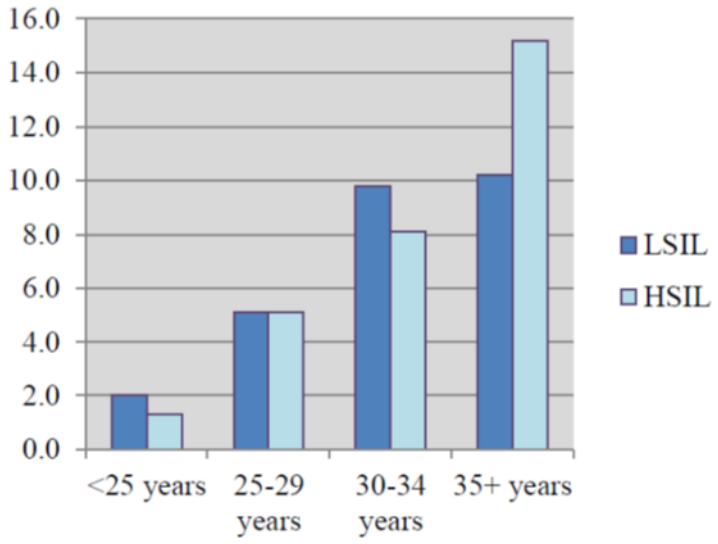


Figure 7. Comparative age distribution (percentage of total TBH HIV ARV women) with history of LSIL and HSIL on cytology report

The cross-sectional design of our investigation which relied on retrospective data routinely collected through medical records with substantial missing data, as well as the tertiary nature of our Tygerberg Hospital (tertiary referral hospital), increase the likelihood of a selection bias towards overrepresentation of LSIL and HSIL and could account for the differences with Study 1 and 2. In support of a possible selection bias, a large number of women treated for HIV infection at Tygerberg Hospital (56.6%) had a recent history of LSIL and HSIL, and these numbers appeared to be much higher than the largely HIV uninfected comparative group from 1996. Despite the above limitation leading to possible overestimation of the true prevalence rates, these results could also suggest the possibility of additional factors that could account for the high prevalence rates of LSIL, HSIL and cervical cancer observed such as failure of cervical cancer screening programmes.

Indeed, improving Pap smear screening initially met with logistical problems. Health Care systems in South Africa are fragmented and services within institutions are generally not duplicated. Because Pap smear screening was at the time available at different locations within and outside the hospital, albeit on different days, we were not allowed to perform Pap smear screening at the IDC. Furthermore, patients were not willing or able to attend further appointments other than their ART dispensing visits, as they were either too ill, too poor or to overburdened from attending ART and TB appointments.

We therefore initiated a more patient friendly service, largely funded through this research project, but not exclusive to study participants, whereby all women who attended the IDC could undergo Pap smear screening when reporting for cART initiation. This contributed to the improvement of Pap screening coverage to include 96% of our female patients. Since 2012, Pap smear screening has now become part of the standard protocol for all women diagnosed with HIV infection, and most women who present to the IDC now have already had their first Pap smear test done at the referring clinic.

Furthermore, during our initial pilot study, we noticed that many women who had been diagnosed with cervical dysplasia had not met their appointments at Colposcopy Clinic for evaluation and excision treatment as indicated. Some were even unaware of their prior screening result. We therefore decided at the IDC to include cervical dysplasia as an HIV related disease in the Clinic management database data capture sheet (an example is attached as appendix 1) and for this diagnosis also to be automatically included in the electronically

generated patient referral letter (appendix 2). Lastly, but importantly, the funding made it possible to purchase a third and new Colposcope for the Department of Gynaecology which shortened waiting times for appointments.

The improved cooperation and collaboration between the IDC and the CC not only led to a "better deal" for patients, such as fewer visits with combined appointment dates, but enabled us to continue towards finding the answers to the most pertinent question we had regarding HIV infected women: can we expect an increase in cervical cancer cases within the next few years, or will the treatment with antiretroviral agents mitigate or prevent an increase?

In our attempt to answer this question, we rephrased it and refer to the important aspects of cervical dysplasia - the precursor disease of cervical cancer- in women living with HIV disease:

1. *What is the effect of cART on **low-grade cervical dysplasia**?* Can it lower the risk of progression to higher grade (pre-cancer) disease? Chapter 2 describes a retrospective study investigating Progression and Persistence of low-grade disease. It compares these outcomes in HIV diagnosed and undiagnosed women, and investigates the impact of cART on these outcomes in HIV diagnosed women separately.
2. *What the effect of cART on excision treatment of **high-grade cervical dysplasia**?* Can cART reduce the risk of recurrence of cervical dysplasia after excision treatment? Chapter 3 describes a retrospective study of women who underwent excision biopsy treatment of high-grade disease. It compares the excision treatment failure rate and post excision recurrence in HIV infected women with women not diagnosed with HIV. It also describes the impact of cART on these outcomes in HIV infected women separately.
3. *What is the impact of cART on **cervical HPV genotype infection**?* Is this effect, or lack thereof, able to explain the answers to questions 1 and 2? A prospective study which enrolled women initiating cART is contained in Chapter 4.

Chapter 2: Low-Grade Cervical Dysplasia in Women Living with HIV

Introduction

As discussed in the first chapter, cervical cancer is caused by Human Papillomavirus (HPV) infection. Most HPV infections clear within months in women with normal immune status. Progression of Low-grade SIL to pre-cancer (high-grade SIL) or cancer can however occur and is linked to persistent infection with one or more high-risk oncogenic types of Human Papillomavirus (HPV) (26-28).

There is evidence that women who are co-infected with HIV develop cervical cancer at a younger age. Possible reasons are an increased persistence of genital HPV infection due to weakened immune system (29-32) and a resulting higher risk for progression of LSIL to high-grade lesions (33). Prior to 2010, when we initiated our own investigation, there were few reports of an association of cART with a reduction in the burden of HPV and SIL (34-36). These studies had however been done in low HIV prevalence settings.

To investigate the factors that influence the persistence and progression of low-grade cervical intraepithelial lesions, we did a large retrospective cohort study that compared these outcomes in a setting of high HIV prevalence. We needed to confirm in our study population that there is an altered course of LSIL outcomes in HIV infected compared to uninfected women. Specifically, we were also interested to know if there is an association between the behaviour of cervical lesions with the antiretroviral treatment status of women living with HIV infection.

Materials and Methods

Study Design, Population, Setting and Outcomes Definition

This retrospective cohort analysis used electronic records of 3,963 women who attended the Colposcopy Clinic and Infectious Diseases Clinic located in Tygerberg Hospital, Parow, Western Cape during the period July 2004 to July 2009. Explanatory and outcomes variables such as histopathology and cytology data as well as information regarding use of antiretroviral therapy were collected from 1 January 1992 to 31 December 2010, extracted anonymously and linked to a study-specific identifier. Demographic data such as age and race were included.

For the analysis of progression and persistence of intraepithelial lesions we identified 1960 cases that had a low-grade cervical intraepithelial lesion (LSIL) as first abnormal smear. Of these women, 1720 (87.8%) had follow-up available. Women who had a HSIL at first abnormal smear were not included. We defined progression of LSIL as the detection of HSIL. Only women with a follow-up visit >24 weeks after the first LSIL were used for the analysis. Clearance was defined as two serial normal cytology results at least 4 weeks apart.

Women were assigned to one of three groups. A patient was taken to be *HIV positive* if there was evidence that an HIV Enzyme-linked immunosorbent assay (ELISA) test or rapid test was performed, or a HIV viral load determination done that had virus detected. *HIV negative status* was taken as a negative ELISA or rapid test. If there was no evidence of HIV testing, the patient's status was taken as *HIV unknown.* The choice of HIV group was made according to the *status as known at first LSIL,* as this was the most common point at which a patient's status was identified. It was decided, in view of the retrospective nature of the study and lack of repeat HIV tests in the majority of women included, not to disregard the HIV unknown status group in this analysis, as the possibility existed that women not tested could represent women who were considered of very low risk for contracting HIV, whereas women who were tested negative, possibly represented a group considered at risk for HIV infection and were therefore tested but not re-tested during follow-up. *Combination antiretroviral therapy (cART)* was defined as the study subject receiving at least three antiretroviral medications and for the purpose of the study is equivalent to HAART (highly active antiretroviral therapy). *Virological failure* was defined as two consecutive HIV viral load determinations of >1000 copies/ml at least 4 weeks apart.

Squamous abnormalities were classified as *LSIL, HSIL or cancer* according to the Bethesda Classification System. Cytology was performed with routine Papanicolaou (Pap) smear. For the purpose of the analysis, ASC-US and Atypical squamous cells –low-grade (ASC-L) were grouped with *LSIL*, and Atypical squamous cells – cannot exclude HSIL (ASC-H) grouped with *HSIL*. The quality of cytology was assessed by determining the percentage of smears with endocervical cells present. Conventional Pap smear screening was in use for most of the time as the span of this study, but this has been previously found to be just as effective as liquid-based cytology in detecting abnormalities (37). Cytology was read by a cytopathologist and checked by a second cytopathologist, as per usual procedure in our institution.

In our setting, referral of HIV positive women for colposcopy evaluation is encouraged after the first abnormal smear. If a lesion suspicious of a high grade abnormality is visualized during colposcopy evaluation, a diagnostic biopsy is performed. If the biopsy confirms a high grade abnormality- that is CIN II or more on histology - an excision procedure is performed. Excision is by Large Loop Excision of Transformation Zone (LLETZ) under local anaesthesia in an outpatient setting. Cold knife Cone Excision (CKC) is performed in cases where there are either suspected early invasive carcinoma or adenocarcinoma in situ or large lesions or distorted anatomy not suitable for LLETZ. There is a move towards a see-and-treat approach: excision treatment may be performed at the first visit if the colposcopy appearance is suggestive of high grade histology (CIN II or above). In young women, where there is still a wish for future pregnancy, confirmatory biopsies are required before LLETZ or CKC.

Pathology was considered to be more specific than cytology and the histology result was used if a smear was performed on the same day. Excision treatment in this analysis refers to the surgical removal of the cervical lesions with the loop electrosurgical excision (LLETZ or LEEP), cold-knife cone (CKC or conisation), or hysterectomy.

In this study, we compared the survival of LSIL without progression to HSIL here referred to as *progression-free time*, and the survival of LSIL or higher lesion without clearance of the lesion as *persistence of SIL*. Our study was approved by the Stellenbosch University Human Research Ethics Committee (ref nr N11/01/007). Due to the fact that this study was a retrospective cohort analysis from an anonymous database (without patient identifiers), exemption for obtaining informed consent was granted.

Statistical Analysis

STATA Version 11 was used for the statistical analysis. The t- test was used to compare the significance of the difference between the mean ages of women of HIV positive and unknown HIV status with the HIV negative group. Other group characteristics were compared using two-tailed chi square tests. Survival analysis of progression-free time of low-grade cervical lesions, or for persistence of SIL used the Kaplan Meier method and log-rank test to compare the HIV status groups. Multivariable Cox regression was applied to identify independent risk factors for disease progression or persistence. Covariates investigated were age, the type of excision treatment performed and the duration of cART received. The effect

of excision treatment in this study applies only if the treatment was not done at the last visit: if excision was done at last visit, it is considered only as a histological observation and not as a therapeutic procedure.

For both the analysis of progression and the analysis of persistence, the **time 0** was taken as the first date that LSIL was detected. The **end point** for ***progression free- time*** was taken as the first date that the patient had a HSIL or higher lesion detected (event), or the last date that the patient was progression-free (censored if last date of follow-up). Concluding from our earlier definition of *excision treatment effect* on progression, a patient was classified as not having undergone excision treatment if it was performed on or after the day of first diagnosis of progression (HSIL or higher), as this would be on or beyond the end point of the progression end point. This automatically implies that, in our analysis of progression-free time, we adjusted for excision treatment performed for the indication of LSIL and not if done for HSIL. Figure 2.1.a provides an example of a time line of a patient in the study of progression-free time.

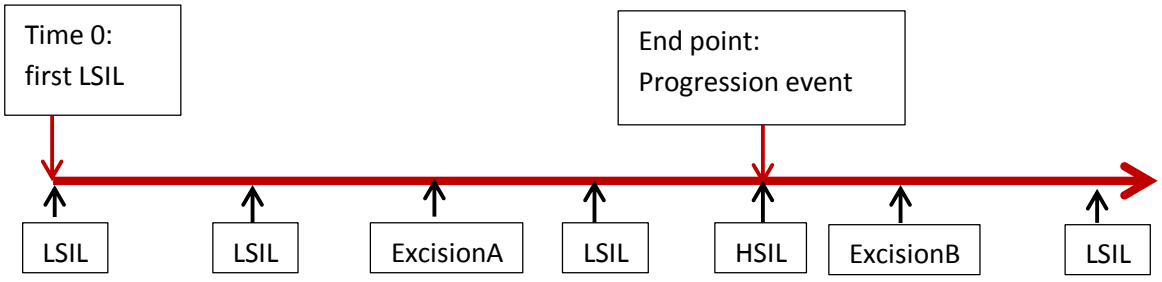


Figure 2.1.a*. Time line of a patient in the study of progression-free time. If an excision was performed at the time point "Excision A", it was considered a therapeutic procedure. If it was performed at time point excision B, it was not.*

For analysis of *persistence of LSIL*, the **end point** was taken as the last date *any* SIL was detected as the event, if followed by a normal smear, or the last date that the patient had SIL detected, and censored if still present at last follow-up. The term *persistence* means the same as a *lack of clearance* for the purpose of this study.

The analysis of progression and the analysis of persistence were conducted separately and a patient who developed HSIL was also considered as experiencing persistence (non-clearance) of SIL. Regarding the effect of excision treatment on persistence of SIL, a patient

was therefore regarded as having undergone the procedure if the date thereof was between time zero and before (but not on) the end point date, regardless of the indication of LSIL or HSIL. Figure 2.1.b provides an example of a time line of a patient in the study of persistence.

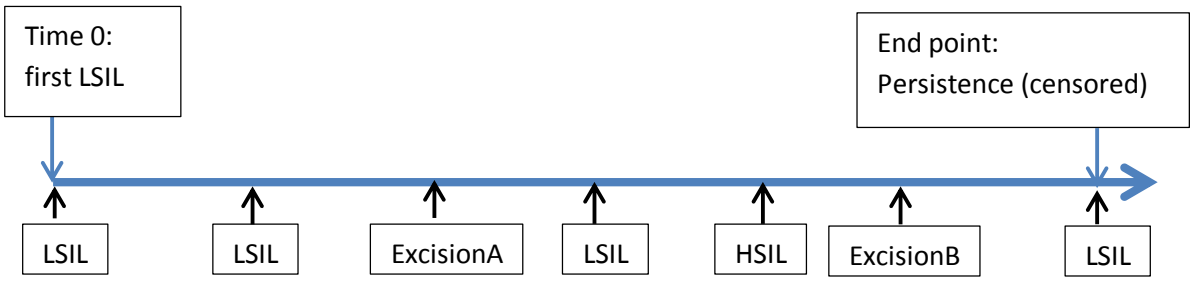


Figure 8 Time line of a patient in the study of persistence.

If an excision was performed at the time point "Excision A" or at time point Excision B", it was considered a therapeutic procedure.

The same methods were followed for a separate analysis of the effects of antiretroviral treatment and CD4 count *within the HIV positive status group*.

Results

I. Baseline Demographics and Clinical Characteristics

Table 2 provides demographics data by HIV status. The mean age of women first diagnosed with LSIL for all three HIV status groups was compared and the HIV unknown status group found to be slightly older. The follow-up time in HIV-infected women compared to HIV-uninfected women was shorter. The quality of Pap smears performed on the HIV positive group was found to be marginally inferior to the other groups as measured by the presence of endocervical cells. Of the total smears that had endocervical cell (EC) presence reported, in the HIV positive group the percentage of smears with EC not present was higher at 19.1%, compared to 15.6% in HIV negative *(p=0.006)* and 18.2% in the HIV unknown status groups *(p=0.05)*. HIV positive women did not undergo more *excision of LSIL* during the follow-up period after first LSIL, and the number that did undergo excision of LSIL was small for all HIV groups.

Table 2 Demographic and Clinical Characteristics

	Total	HIV negative n (%)	HIV unknown n (%)	p-value[1]	HIV positive n (%)	p-value[2]
Age at First LSIL (mean,SD)	**1720**	33.1±10.14	35.6±11.10	0.006	32.5±7.73	0.34
Ethic Group[3]						
Black	**520**	19 (9.5)	31 (6.6)	0.19	470 (44.9)	<0.001
Mixed Race	**1200**	181 (90.5)	441 (93.4)		578 (55.2)	
Total	**1720**	200 (100)	472 (100)		1048 (100)	
Follow-up Time[4] (median,IQR)	**1720**	26.8 (14.9-49.0)	31.4 (13.2-56.4)	0.17	17.5 (5.4-35.6)	<0.001
CD4 count at first LSIL[5]						
0-199					241 (34.8)	
200-349					213 (30.8)	
≥350					239 (34.5)	
Total with CD4 available	**693**	N/A	N/A	N/A	693 (100)	N/A
ART duration						
None					433 (41.3)	
Started Before LSIL					185 (17.7)	
Started After LSIL					430 (41.0)	
Total	**1048**	N/A	N/A	N/A	1048 (100)	N/A
Excision of LSIL[6]						
Performed	**226**	28 (14.0)	88 (18.7)	0.15	110 (10.6)	0.15
Not performed	**1494**	172 (86.0)	384 (81.3)		938 (89.4)	
Total	**1720**	200 (100)	472 (100)		1048 (100)	

1 t-test compares significance between means and Chi-square significance between two proportions (HIV unknown to HIV negative group)
2 t-test compares significance between means and Chi-square significance between two proportions (HIV positive to HIV negative group)
3 Number of patients with LSIL and ethnic group known as Mixed Race or Black (% of Ethnic Group)
4 From First LSIL to date of first progression or last follow-up, in months, for women who had at least 24 week follow-up available
5 Done in year of first LSIL, for HIV infected women who had CD4 count available
6 Includes all method of excision (excision biopsy and hysterectomy) of LSIL

II. Progression of LSIL

Association of Progression of LSIL with HIV status

Overall, there was no difference between HIV groups as to progression of LSIL to HSIL. Progression was observed in 94/200 (47.0%) of HIV negative women compared to 226/472 (47.9%) of women HIV unknown status (*p=0.83*) and 447/1048 (42.7%) of HIV positive women (*p=0.26*)**.** Time to progression from LSIL to HSIL was found to be not different for HIV-infected compared to uninfected women (Figure 9, *Log-rank: p=0.120*). Cox regression showed no increased risk for progression from LSIL to HSIL by HIV status in both univariate and multivariate analysis, after adjusting for the effects of age and whether excision treatment was performed or not (Table 3).

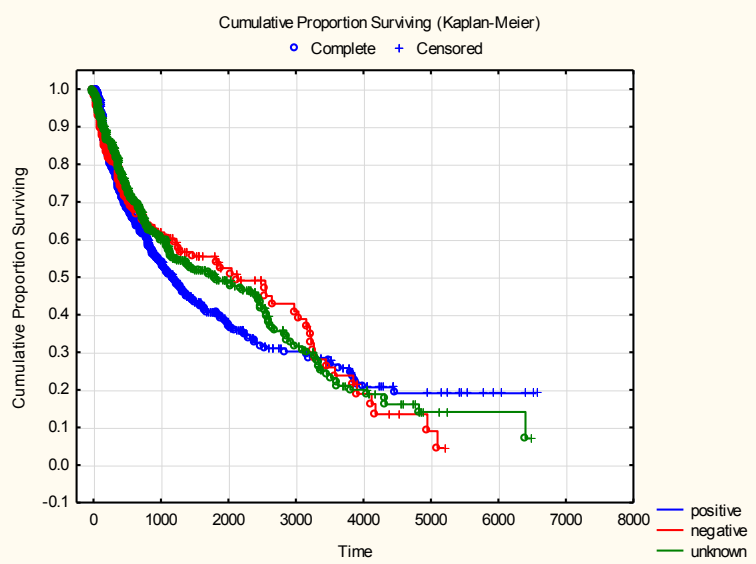
Table 3 Factors associated with Progression of LSIL to HSIL for All Women in Cohort

	Univariate analysis		Multivariate analysis	
Variable	HR (95%CI)	p-value	HR (95%CI)	p-value
HIV Status				
Negative	1.00	-	1.00	-
Positive	1.15 (0.92-1.44)	0.22	1.11 (0.88-1.38)	0.38
Unknown	0.98 (0.77-1.25)	0.89	0.96 (0.75-1.22)	0.75
Age at first LSIL				
15-24	1.00	-	1.00	-
25-29	1.16 (0.91-1.47)	0.23	1.18 (0.93-1.50)	0.18
30-34	1.55 (1.22-1.97)	<0.001	1.55 (1.22-1.96)	<0.0001
35-39	1.67 (1.29-2.14)	<0.001	1.83 (1.42-2.35)	<0.0001
40-44	1.10 (0.81-1.48)	0.54	1.28 (0.95-1.73)	0.10
≥45	1.55 (1.16-2.07)	0.003	2.12 (1.58-2.88)	<0.001
Excision of LSIL‡				
Not performed	1.00	-	1.00	-
Performed	0.10 (0.60-0.16)	<0.001	0.09 (0.06-0.16)	<0.001

‡ Excision includes all method of excision (excision biopsy and hysterectomy) done during follow-up

Figure 9 Kaplan-Meier Plot for Progression-Free Status, According to HIV Groups

Log Rank Test: X^2=4.233, p=0.12



Association of Progression of LSIL with Excision Treatment

Excision of the LSIL during follow-up did however impact on progression of the lesion. In a breakdown of the HIV groups, most women who underwent excision without a history of a prior HSIL lesion did not subsequently progress to HSIL and this was true for all groups by HIV status. For women who did not undergo excision treatment, progression was similar for the HIV groups **(**Table 4). Cox regression analysis confirmed decreased risk for progression if LSIL was excised. Adjusting for HIV status and age, there remains a strong association between the rate of progression and whether excision treatment was performed (Table 3).

Table 4 Impact of Excision of LSIL on Progression, According to HIV Groups

	HIV negative	HIV unknown	p-value*	HIV positive	p-value**
Excision Performed‡					
Progression	0 (0.0)	8 (9.1)	0.20	7 (6.4)	0.34
No Progression	28 (100.0)	80 (90.9)		103 (93.6)	
Total	**28 (100)**	**88 (100)**		**110 (100)**	
No Excision Procedure‡					
Progression	94 (54.7)	218 (56.8)	0.65	440 (46.9)	0.07
No Progression	78 (45.3)	166 (43.2)		498 (53.1)	
Total	**172 (100)**	**384 (100)**		**938 (100)**	
Total	**200**	**472**		**1048**	

‡Excision includes all method of excision- excision biopsy and hysterectomy
** Fisher's exact test to compare significance between two proportions (Comparing HIV unknown to HIV negative group)*
*** Fisher's exact test to compare significance between two proportions (Comparing HIV positive to HIV negative group*

Association of Progression of LSIL with cART

Progression was observed in 62/185 (33.5%) of HIV positive women who had started antiretroviral therapy before the first LSIL on smear, compared to 186/433 (43.0%) of women who received no ART (*p=0.023*) or 192/430 (44.7%) of women who initiated ART after the first LSIL (*p=0.01*). In the multivariate Cox regression model, there was a decreased risk for progression (HR 0.66, 95% CI 0.54-0.81) for the HIV positive patients if ART was started before first LSIL, and this association remained strong when adjusted for age and whether excision treatment was performed (Table 5).

Table 5 Risk Factors for Progression of LSIL for HIV positive Women

	Univariate analysis		Multivariate analysis	
Variable	HR (95%CI)	p-value	HR (95%CI)	p-value
Age at first LSIL				
15-24	1.00	-	1.00	-
25-29	1.19 (0.89-1.62)	0.23	1.30 (1.96-1.76)	0.09
30-34	1.34 (1.00-1.82)	0.06	1.47 (1.08-2.01)	0.01
35-39	1.57 (1.14-2.17)	0.006	1.82 (1.32-2.51)	<0.001
40-44	1.01 (0.67-1.53)	0.96	1.23 (0.81-1.87)	0.34
≥45	1.62 (1.07-2.46)	0.02	1.90 (1.25-2.90)	0.003
Duration of ART				
None	1.00	-	1.00	-
Pre-First LSIL	0.67 (0.55-0.83)	<0.001	0.66 (0.54-0.81)	<0.001
Post-First LSIL	0.90 (0.68-1.20)	0.49	0.90 (0.67-1.20)	0.47
CD4 count at first LSIL				
0-199	1.00	-	-	-
200-349	1.08 (0.81-1.44)	0.60		
≥350	0.94 (0.71-1.24)	0.67		
Virological Failure				
No	1.00	-	-	-
Yes	1.18 (0.84-1.65)	0.35		
Excision of LSIL‡				
Not performed	1.00	-	1.00	-
Performed	0.10 (0.05-0.21)	<0.001	0.09 (0.04-0.19)	<0.001

‡ Excision includes all method of excision (excision biopsy and hysterectomy) done during follow-up

III. Persistence of SIL

Association of Persistence of LSIL with HIV status

Persistence was observed in 669/1048 (63.8%) of HIV positive women (*p<0.001*) and 137/472 (29.4%) of women of unknown HIV status (*P=0.13*) compared to 70/200 (35.0%) of HIV negative women, respectively.

Table 6 Factors that Influence Clearance of LSIL in All Women in Cohort

	Univariate analysis		Multivariate analysis	
Variable	HR (95%CI)	p-value	HR (95%CI)	p-value
HIV Status				
Negative	1.00	-	1.00	-
Positive	0.66 (0.54-0.81)	<0.001	0.69 (0.57-0.85)	<0.001
Unknown	1.14 (0.93-1.40)	0.21	1.09 (0.89-1.34)	0.39
Age at first LSIL				
15-24	1.00	-	1.00	-
25-29	1.01 (0.80-1.27)	0.96	1.01 (0.80-1.28)	0.91
30-34	1.32 (1.05-1.66)	0.02	1.33 (1.06-1.67)	0.01
35-39	1.45 (1.13-1.85)	0.003	1.41 (1.10-1.79)	0.006
40-44	1.60 (1.24-2.08)	<0.001	1.44 (1.11-1.87)	0.006
≥45	2.16 (1.67-2.80)	<0.001	1.81 (1.39-2.35)	<0.001
Excision of LSIL‡				
Not Performed	1.00	-	1.00	-
Performed	1.58 (1.33-1.88)	<0.001	1.39 (1.16-1.65)	<0.001

‡ Excision includes all method of excision (excision biopsy and hysterectomy) done during follow-up

Association of Persistence of LSIL with Excision Treatment

For women of HIV negative or unknown status, persistence of LSIL was strongly influenced by excision treatment, with few experiencing persistence of LSIL after such treatment. For HIV positive women, however, excision had a smaller effect on persistence. With or without excision of LSIL, persistence was much more common amongst HIV positive women than for the other groups (table 7).

Table 7 Impact of Excision of LSIL on Clearance, According to HIV Groups

	HIV negative	HIV unknown	p-value*	HIV positive	p-value**
Excision Performed‡					
Persistence	2 (7.1)	12 (13.6)	0.51	50 (45.5)	<0.001
Clearance	26 (92.9)	76 (92.7)		60 (54.5)	
Total	**28 (100)**	**88 (100)**		**110 (100)**	
No Excision Procedure‡					
Persistence	68 (39.5)	125 (32.5)	0.12	619 (66.0)	<0.001
Clearance	104 (60.5)	259 (67.5)		319 (34.0)	
Total	**172 (100)**	**384 (100)**		**938 (100)**	
Total	**200**	**472**		**1048**	

‡Excision includes all method of excision- excision biopsy and hysterectomy
** Fisher's exact test to compare significance between two proportions (Comparing HIV unknown to HIV negative group)*
*** Fisher's exact test to compare significance between two proportions (Comparing HIV positive to HIV negative group*

Association of Persistence of LSIL with cART

If cART was started before the first diagnosis of LSIL, clearance was improved. Clearance was observed in 141/433 (32.6%) of HIV positive women who did not receive ART, compared to 164/430 (38.1%) of women who started ART after the first LSIL (*p=0.09*) and 76/185 (41.1%) of women who had started ART before the first LSIL (*p=0.04*). This was confirmed with survival analysis (figure 10). In the multivariate Cox regression model, HIV-infected women who started ART before first LSIL had an improved clearance (HR 1.71, 95% CI 1.29-2.27) compared to women not on ART and if excision treatment was performed (HR 1.39, 95% CI 1.05-1.83) (table 8).

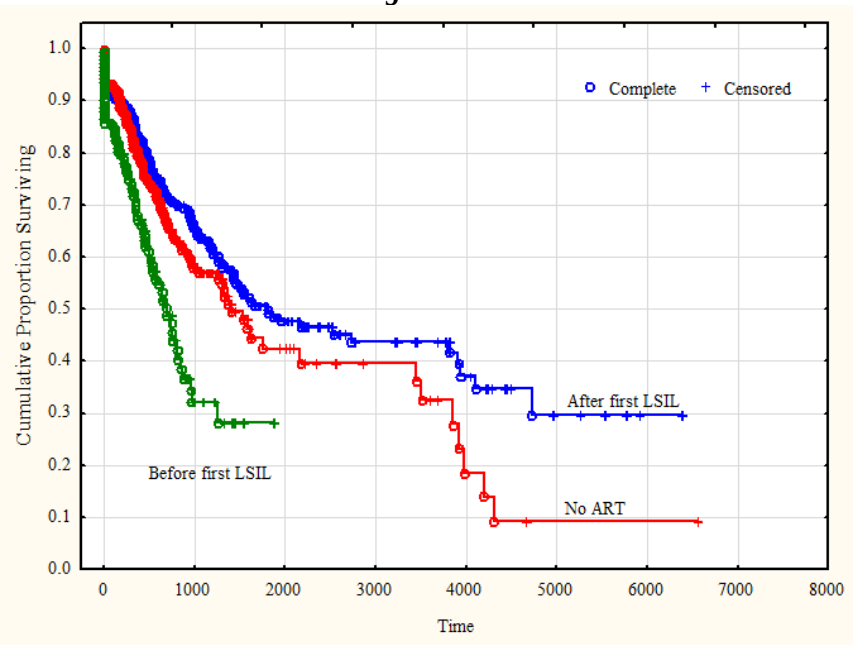
Table 8 Factors that Influence Clearance of LSIL in HIV Infected Women

	Univariate analysis		Multivariate analysis	
Variable	HR (95%CI)	p-value	HR95%CI	p-value
Age at first LSIL				
15-24	1.00	-	1.00	-
25-29	1.14 (0.82-1.59)	0.44	1.10 (0.79-1.54)	0.56
30-34	1.44 (1.03-2.00)	0.03	1.37 (0.98-1.92)	0.07
35-39	1.51 (1.06-2.16)	0.02	1.46 (1.02-2.08)	0.04
40-44	1.58 (1.04-2.39)	0.03	1.48 (0.97-2.25)	0.07
≥45	1.76 (1.12-2.76)	0.02	1.60 (1.02-2.53)	0.04
Duration of ART				
None	1.00	-	1.00	-
Pre-First LSIL	1.80 (1.36-2.38)	<0.001	1.71 (1.29-2.27)	<0.001
Post-First LSIL	0.79 (0.63-0.99)	0.04	0.77 (0.61-0.97)	0.03
CD4 count at first LSIL				
0-199	1.00	-	-	-
200-349	0.77 (0.56-1.05)	0.10		
≥350	1.00 (0.75-1.33)	0.99		
Virological Failure				
No	1.00	-	-	-
Yes	0.58 (0.39-0.87)	0.008		
Excision of LSIL‡				
Not Performed	1.00	-	1.00	-
Performed	1.49 (1.13-1.96)	0.005	1.39 (1.05-1.83)	0.02

‡ Excision includes all method of excision (excision biopsy and hysterectomy)done during follow-up

Figure 10 Survival of Persistence of SIL, for HIV positive group according to when ART started in relation to first LSIL diagnosis

Figure 2.2.



Discussion

Most previous studies have investigated the effect of HIV infection on incidence of SIL and HPV infection, and have found higher occurrence of both in HIV infected women. In these studies, the proportion of women with LSIL at baseline was small, or included only women with normal cytology (38-40) (41, 42). We were however interested in the behaviour of LSIL in HIV infected women compared to HIV uninfected women, and specifically the risk of progression of LSIL. Such an analysis by Nappi in a cohort of 150 women found the risk for progression 4-5 times higher than for HIV uninfected women (33). In contrast, Denny, from Cape Town, examined the risk for progression in a study of 400 HIV infected women (of whom 137 had LSIL at baseline) and found percentage of women with progression very low (4% over 3years) and similar to that for HIV uninfected women; over 30% of women started ART within 2 years of participation in the study. Adjustments for ART initiation were not made due to delayed availability of ART in state-sponsored programmes and the small numbers involved(43). In our study, we also found no overall difference in progression of LSIL to HSIL between HIV infected and uninfected groups before adjusting for other covariates and therefore decided to further investigate the effect of CD4, ART and excision treatment on progression and persistence in our HIV infected women separately.

As for the effect of CD4 count, most previous reports have concluded that a decrease in CD4 count could be linked to higher incidence of HPV infection and/or SIL (41-45). With regards to progression of LSIL, Nappi and Schuman found higher occurrence of progression with lower CD4 counts(33, 38), and Omar, who enrolled 225 HIV infected women with LSIL, reported decreased risk for progression if the CD4 count was over 500 cells/ql(46). Our study, as a retrospective cohort, investigated the real-life scenario of availability of CD4 counts in HIV infected women - these are seldom repeated if already done in the preceding 6 months. Since fewer than 20% of women in our cohort had CD4 count above 500 cells/ql, we decided to group CD4 as is currently under negotiation for initiation of ART per national treatment protocol from 200 to 350 cells/ql, and found it had no predictive value as to progression or persistence of SIL.

We were therefore still intrigued as to the apparent lack of difference of progression of LSIL between our HIV status groups, and if cART could have contributed in reducing risk for the HIV infected women. The results of previous studies have been contradictory. Numerous reports showed no effect of cART on the HPV infection or SIL pattern (33, 39, 45), but others indicated reduced SIL occurrence or progression and persistence of LSIL when receiving cART. Of note, a more recent report by Omar of over 1000 HIV infected women in Soweto, South Africa (of whom 225 had LSIL at baseline) found that ART protected against LSIL progression. We found a similar ART-reduced hazard for LSIL progression compared to their study in our more than 1000 LSIL-diagnosed HIV infected women (0.66 versus their 0.72) (46). This finding concurs also with the Minkoff findings already in 2001, and again in 2010, as well as the small cohort of Heard in 1998 (34, 35, 47).

The question remained whether excision treatment in women with LSIL played a role in preventing progression or persistence of LSIL. Although the number of women who underwent excision of LSIL was small but similar for all groups, the decision to do excision was based on colposcopy appearance and therefore could have contributed to the prevention of progression and/or persistence. This was indeed the case- none of the HIV uninfected women who underwent excision treatment experienced progression, and the effect was similar for HIV infected women. The effect of excision was however smaller on persistence in HIV infected women than HIV uninfected women. We therefore included the effect of excision of LSIL in our multivariate analysis of progression and persistence, and still found a decrease in the risk for both progression and persistence with ART use; the influence of CD4 remained not significant.

To interpret the results of our findings, the conditions under which women entered study have to be understood. The current local management protocol preselected women who have had LSIL for at least one year, as they would have only been referred for colposcopy after a repeat LSIL. This may explain the high progression of LSIL in the HIV uninfected group- previous prospective studies which included HIV uninfected women have shown a much lower risk for progression of CIN I lesions (48).

We expected women of unknown HIV status to be of low risk for HIV infection and therefore not tested, or of normal to high risk but to have declined HIV testing, and that their risk for persistence to be somewhat between the HIV infected and uninfected groups. Unexpectedly,

the persistence risk for this group was similar to the HIV negative group, implicating similar risk for HIV acquisition than the uninfected group. Also, the risk for progression in HIV uninfected women was higher than for HIV infected women who had started ART before the first LSIL, and we relate it to the high prevalence of HIV infection in our communities. Women who were HIV negative at first LSIL, possibly did not remain so during follow-up time. Unfortunately, due to the retrospective nature of the study, we do not know to what extent subsequent acquisition of HIV infection could have contributed to the progression of LSIL in the HIV negative group, and it is something we would only be able to adequately answer in a prospective cohort study. We therefore advise at regular intervals re-testing of HIV negative women who present with cytological abnormalities. In our institution, facilities for HIV screening have dramatically improved and we expect an increased screening frequency in HIV negative women and women of HIV unknown status than was experienced during the study period.

Overall, we found that there was no difference between the progression of LSIL to higher grade cervical dysplasia between the HIV groups. Importantly, patients who started ART before first LSIL were associated with a significant decreased risk for progression. Of note, CD4 count did not have an influence on the risk for progression.

We conclude that ART protects against progression of LSIL to HSIL and that this effect was independent of the CD4 count at which the LSIL was first diagnosed. From this finding, we postulate that cART exposure –by the protection it offers- may partly be responsible for the overall perception that HIV infected women are not at higher risk for progression of LSIL to HSIL than HIV uninfected women. However, one has to bear in mind that women who underwent regression of LSIL would not have been referred for colposcopy evaluation, and this selection bias may further explain the high rate of progression in the HIV uninfected women in this cohort.

We also found persistence (absence of clearance of SIL) to be markedly increased in the HIV positive group. ART was independently associated with improved clearance of LSIL. On the other hand, a higher CD4 count at the time of first LSIL was not associated with improved clearance of the lesion, but virological failure was associated with increased persistence.

Finally, it can be concluded that since the effect of cART is to reduce progression and persistence of LSIL, and because LSIL is caused by HPV of the cervical epithelium, the association of cART with the detection of HPV also warrants further investigation.

Chapter 3: High-Grade Cervical Dysplasia in Women Living with HIV

Introduction

As described in Chapter 1, the development of cervical cancer depends on the persistence of oncogenic HPV and the progression of the associated low-grade lesions to more severe lesions. In our investigation of the progression and persistence of LSIL, we found that cART reduces the risk of both in HIV infected women, and that this is independent of the CD4 count.

Once progression of LSIL to HSIL has occurred, the lesion very seldom regresses, and the management of confirmed HSIL is always that of excision, regardless of the patient's HIV status. As such, the "see and treat" approach, which refers to excision treatment without histological confirmation in women who have completed their families, seems to rarely lead to overtreatment (49).

In the background of increased persistence of LSIL in women living with HIV infection, which we have postulated is caused by more persistent HPV infection, it may be assumed that the success of the treatment of HSIL – excision treatment – will be also affected by HIV status and possibly even cART.

Previous studies of excision treatment outcome of HSIL as described by Tate (50), Reimers (51), Massad (52) and Wright (53), have involved small cohorts of HIV positive women in low HIV prevalence settings. They found treatment failure very common at 36·2 to 73% in women affected by HIV. Treatment success was associated with higher CD4 count (50, 51, 53), but not with antiretroviral treatment (51). Reimers also found an improved outcome with conisation (CKC) over loop excision (LEEP or LLETZ) for HIV infected women (51).

Furthermore, as to the recurrence of lesions post excision, reports were also conflicting. Lesions observed after excision treatment were either mostly low-grade (52) or high-grade (51).

We therefore proceeded to investigate the factors that influence outcome of excision treatment and the recurrence of cervical intraepithelial lesions in a large cohort in our setting of high HIV/ HPV prevalence. Specifically, we were interested to find out if there exists again an association of HIV status with excision treatment failure, and if cART in HIV infected women could reduce recurrence of lesions after excision treatment.

Materials and Methods

Study population

For this retrospective cohort analysis we extracted electronic records from our database of women who attended the Colposcopy Clinic at Tygerberg Hospital, Parow, Western Cape region of South Africa during the period July 2004 to July 2009. We identified 1848 women for this analysis that underwent excision treatment of squamous intraepithelial lesions of the cervix and that had >24 weeks follow-up available. Women were assigned to one of three groups: HIV- infected, HIV- uninfected, or HIV status unknown. The choice of HIV group was made according to the status as known at the time of excision biopsy (EB).

Regulatory Approvals

The study was approved by the Stellenbosch University Human Research Ethics Committee (ref nr N11/01/007).

Operational Definitions, Explanatory and Outcome Variables

Histopathology and cytology data as well as information regarding use of antiretroviral therapy (ART) were extracted and anonymously linked to a study-specific identifier. Antiretroviral therapy exposure was defined as follows: "*ART started before* EB" and "*no ART"* indicate the start time or absence of ART respectively. For the analysis of excision treatment failure, "*after EB"* means that ART was started during the first 365 days after EB; for the analysis of recurrence, it indicates that ART was started after EB but before end point of survival analysis. Baseline CD4 count was stratified in three groups according to the current South African guidelines for initiation of antiretroviral therapy: <200, 200-350 or >350 cells/ql.

Squamous abnormalities were classified as LSIL, HSIL or cancer according to the 2001 Bethesda Classification System(54). We again for the purpose of this analysis included

ASCUS in the LSIL category, and ASC-H as HSIL. Cytology evaluation was performed with routine Papanicolaou (Pap) smear. The quality of cytology was assessed by determining the percentage of smears with endocervical cells (EC) present. Pathology was considered to be more specific than cytology and the result of histology was used if a smear, performed on the same day, was of lesser severity.

Excision biopsy refers to the surgical removal with the loop electrosurgical excision (LLETZ or LEEP) or the cold-knife cone (CKC or conisation) method. For completeness of excision, we set strict criteria at *LSIL present at any margin* to agree with previous reports (51). *Excision treatment failure* is the presence of at least CIN I (LSIL) at first follow-up visit. *Post excision recurrence (at one year or later)* refers to the proportion of the population with disease after EB at a given time.

Statistical Analysis

Mean or median and the interquartile range were calculated as a measure of spread of the baseline characteristics. The t- test was used to compare statistical significance of baseline continuous variables such mean ages of women by HIV status. Dichotomous baseline variables were evaluated using proportions and compared by performing two-tailed chi-squared test.

For the analysis of excision biopsy treatment failure, 1620 women out of the total 1846 in the cohort were selected as women who had the first post-excision follow-up evaluation within 365 days. Logistic regression was used to evaluate the association of factors such as HIV status with excision treatment outcome. Of the 1620 women analysed, the 653 HIV infected women were analysed separately as well, using univariate and multivariate logistic regression to evaluate the association of each risk factor separately with treatment failure. In the multivariate analysis the selected covariates were included because of clinical relevance. Subsequent excision and subsequent hysterectomy were not included in multivariate analyses of excision treatment failure because, although there was a strong association, the subsequent procedure was performed as a result of excision treatment failure and could not be considered a cause thereof.

For survival analysis of recurrence-free time in the post-excision period we used the Kaplan-Meier method and log-rank test to compare the HIV status groups. All 1846 women in the

cohort were included in the recurrence analysis. For the analysis the time 0 was taken as the date that the first excision biopsy treatment was performed. The end point for recurrence-free survival was taken as the first date that the patient had LSIL or higher (event). If there was no lesion after excision, the date of last follow-up evaluation (Pap or histopathology) during the post excision period was used (censored). Cox regression analysis was applied to investigate the influence of covariate factors such as excision type and completeness of excision. The analysis was also done separately for the 778 HIV infected women to further include the covariates CD4 count and ART.

Results

I. Baseline Demographics

Table 9 provides demographics data by HIV Status. Of note, the quality of Pap smears performed on the HIV infected group was found to be marginally inferior (with the percentage of smears with no EC present higher at 19·1%), compared to 15·6% in HIV uninfected *(p=0·006)* and 18·2% in the HIV unknown status groups *(p=0·05)*.

Table 9 Demographic and Clinical Characteristics of Women who underwent Excision Biopsy treatment

	Total	HIV neg n (%)	HIV unk n(%)	p-value[1]	HIV pos n(%)	p-value[2]
Age at Excision Biopsy (mean,SD)	**1848**	37·3±10·19	38·2±9·99	0·14	34·1±7·39	<0·001
Race[3]	**1848**	**335 (100)**	**735 (100)**		**778 (100)**	
Black	662	59 (17·6)	98 (13·3)	0·03	505 (64·9)	<0·001
Mixed Race	1122	248 (74.0)	606 (82·5)		268 (34·5)	
Other	64	28 (8.4)	31 (4.2)		5 (0.6)	
Follow-up Time[4] (median, IQR)	**1848**	1·24 (0·54-2·35)	1·52 (0·56-2·81)	0·008	1·31 (0·59-2·24)	0·22
Excision treatment reported[5]	**1848**	**335 (100)**	**735 (100)**		**778 (100)**	
Complete	682	129 (38·5)	306 (41·6)	0·57	247 (31·8)	0·003
Incomplete	790	128 (38·2)	279 (38·0)		383 (49·2)	
Not Applicable[6]	186	40 (11·9)	95 (12·9)		51 (6·6)	
Not Stated	77	13 (3·9)	21 (2·9)		43 (5·5)	
Cannot be determined	113	25 (7·5)	34 (4·6)		54 (6·9)	
Cytology: LSIL	**331**	**45 (100)**	**97 (100)**		**189 (100)**	
Diagn Biopsy Result LSIL	102	15 (33·3)	35 (36·1)	0·75	52 (27·5)	0·44
Diagn Biopsy Result HSIL	229	30 (66·7)	62 (63·9)		137 (72·5)	
Cytology: HSIL	**310**	**52 (100)**	**151 (100)**		**117 (100)**	
Diagn Biopsy Result LSIL	47	5 (9·6)	25 (16·6)	0·26[#]	17 (15·9)	0·34[#]
Diagn Biopsy Result HSIL	263	47 (90·4)	126 (83·4)		90 (84·1)	
Pre-excision diagnosis	**1848**	**335 (100)**	**735 (100)**		**778 (100)**	
LSIL or less	615	104(31·0)	226(30·8)	0·92	285 (36·6)	0·07
HSIL or higher	1233	231 (69·0)	509 (69·2)		493 (63·4)	
Type of Excision	**1848**	**335(100)**	**735 (100)**		**778 (100)**	
CKC	146	41 (12·2)	71 (9·7)	0·20	34 (4·4)	<0·001
LLETZ	1702	294 (87·8)	664 (90·3)		744 (95·6)	
Pre-excision diagnosis for CKC	**146**	**41 (100)**	**71 (100)**		**34 (100)**	
HSIL or higher	116	36 (87·2)	51 (72·5)	0·06[#]	29 (84·8)	1·000[#]
LSIL or less	30	5 (12·8)	20 (27·5)		5 (15·2)	
Subsequent Excision[7]	**1848**	**335 (100)**	**735 (100)**		**778 (100)**	
Performed	147	28 (8·4)	48 (6·5)	0·28	71 (9·1)	0·68
Not Performed	1701	307 (91·6)	687 (93·5)		707 (90·9)	
Subsequent Hysterectomy[8]	**1848**	**335 (100)**	**735 (100)**		**778 (100)**	
Performed	249	67 (20·0)	115 (15·6)	0·08	67 (8·6)	<0·001
Not Performed	1599	268 (80·0)	620 (84·4)		711 (91·4)	
CD4 count at Excision Biopsy[9]	**778**				**778 (100)**	
<200	131				131(16.8)	
200-350	218	N/A	N/A	N/A	218 (28.0)	N/A
≥350	230				230 (29.6)	
Not available	199				199(25.6)	
ART	**778**				**778 (100)**	
Started Before EB	244	N/A	N/A	N/A	244 (31·4)	N/A
Started After EB	189				189(24·6)	
No ART	345				345 (44·3)	
Log Pre-ART viral load (mean, SD)	**130**	N/A	N/A	N/A	4.95±0.99	N/A

1 t-test used to compare significance between means and Chi-square test used to compare significance between two proportions (Comparing HIV unknown to HIV-negative group)
2 t-test used to compare significance between means and Chi-square test used to compare significance between two proportions (Comparing HIV+ to HIV-negative group)
3 significance of difference between Black and Mixed Race
4 from EB to last FU date
5 significance of difference between Complete and Incomplete
6 there was no lesion seen on specimen
7 Number of all Patients that underwent excision biopsy (Percentage of HIV status group)
8 Number of all Patients that underwent subsequent EB hysterectomy (Percentage of HIV status group)
 # value calculated with the Fisher's exact test
9 CD4 done in year of excision

II. Excision Treatment Failure

Association of Excision Treatment Failure with HIV Status

HIV infected women experienced markedly higher excision treatment failure than HIV uninfected women at first follow-up after excision biopsy treatment. Overall, treatment excision failed in 53·8% of HIV infected women *(p<0·001)* and 23·8% of women of unknown HIV status *(p=0·31)* compared to 26·9% of HIV uninfected women. In cases where excision was reported as *complete*, treatment failure occurred in 41·1% of HIV infected women *(p<0·001)* and 11·7 % of women of unknown HIV status *(p=0·38)* respectively, compared to 14·9% of HIV uninfected women. Treatment failure for incomplete excisions was 65·9% for HIV infected women *(p<0·001)* and 34·5% for women who were of unknown HIV status *(p=0·68)* compared to 36·7% in HIV uninfected women.

The results of univariate and multivariate logistic regression of excision treatment outcome in all women are listed in table 10. When adjusted for age, completeness of excision, excision type and excision result (a reflection of indication for the excision), HIV infection was associated with an increased risk for excision treatment failure (RR=3.66, 95% CI 2.67-5.01, *p<0.001*).

Table 10 Factors Influencing Excision Treatment Failure for All HIV groups

	Total	Univariate analysis		Multivariate analysis	
Variable		HR (95%CI)	p-value	RR95%CI	p-value
Age at EB	**1620**	1·02 (1·00-1·03)	0·008	1·03 (1·02-1·04)	<0·001
Age at EB	**1620**				
15-24	108	0·67 (0·47-0·95)	0·03		
25-29	253	0·87 (0·63-1·20)	0·39		
30-34	333	0·83 (0·61-1·13)	0·25	-	-
35-39	391	0·86 (0·61-1·22)	0·40		
40-44	244	0·64 (0·40-1·02)	0·06		
≥45	291	1·00	-		
HIV Status	**1620**				
Neg	309	1·00	-	1·00	-
Pos	652	3·18 (2·36-4·26)	<0·001	3·66 (2·67-5·01)	<0·001
Unk	659	0·85 (0·63-1·16)	0·31	0·87 (0·63-1·20)	0·39
Excision Complete	**1620**				
Yes	597	1·00	-	1·00	-
No	703	3·34 (2·62-4·25)	<0·001	3·07 (2·37-3·97)	<0·001
Not Stated	62	1·88 (1·08-3·28)	0·03	1·60 (0·89-2·88)	0·12
Cannot Say	99	2·22 (1·42-3·48)	<0·001	1·89 (1·18-3·03)	0·01
NA‡	159	1·48 (1·00-2·18)	0·05	1·57 (0·99-2·50)	0·06
Pre-Excision Diagnosis	**1620**				
LSIL	561	1·00	-		
HSIL	858	1·16 (0·92-1·44)	0·21	-	-
Not Done	186	1·13 (0·80-1·60)	0·48		
CA	15	2·20 (0·76-6·15)	0·13		
Diagnostic Biopsy Result	**1620**				
LSIL	114	1·00	-		
ND	1093	1·12 (0·75-1·69)	0·59	-	-
HSIL	411	1·26 (0·81-1·94)	0·31		
CA	2	0·40 (0·01-16·7)	0·63		
Excision Type	**1620**				
LLETZ	1495	1·00	-	1·00	-
CKC	125	1·09 (0·75-1·59)	0·64	1·16 (0·76-1·78)	0·49
Excision Result	**1620**				
LSIL	264	1·00	-	1·00	-
HSIL	1211	1·22 (0·92-1·62)	0·16	1·11 (0·81-1·52)	0·53
Normal	121	0·90 (0·56-1·43)	0·64	1·01 (0·59-1·73)	0·98
CA	24	2·03 (0·88-4·71)	0·10	1·90 (0·77-4·64)	0·16
Subsequent EB*	**1620**				
Not Performed	1457	1·00	-	-	-
Performed	163	11·64 (7·55-18·0)	<0·001		
Subsequent Hysterectomy*	**1620**				
Not Performed	1331	1·00	-	-	-
Performed	289	3·98 (3·05-5·19)	<0·001		

‡*Not applicable: no lesion*

**after 1 year*

Association of Excision Treatment Failure with cART

Looking at HIV infected women alone, the effect of already receiving cART before excision treatment was not significant, although a lower CD4 count was associated with increased risk (table 11).

Table 11 Factors Influencing Treatment Failure for HIV positive Women

		Univariate analysis		Multivariate analysis	
Variable	Total	RR (95%CI)	p-value	HR (95%CI)	p-value
Age at EB	**653**	1·01 (0·99-1·03)	0·52	1·01 (0·99-1·03)	0·35
Age at EB	**653**				
15-24	140	1·00	-		
25-29	138	0·56 (0·29-1·08)	0·08		
30-34	196	0·61 (0·32-1·16)	0·13	-	-
35-39	78	0·61 (0·31-1·19)	0·15		
40-44	36	0·77 (0·38-1·59)	0·48		
≥45	65	0·60 (0·28-1·27)	0·18		
Excision Complete	**653**				
Yes	210	1·00	-	1·00	-
No	321	2·73 (1·96-3·79)	<0·001	2·63 (1·87-3·69)	<0·001
Not Stated	28	0·87 (0·42-1·81)	0·71	0·80 (0·38-1·69)	0·56
Cannot Say	44	1·36 (0·75-2·48)	0·32	1·30 (0·71-2·40)	0·39
NA‡	48	1·18 (0·65-2·13)	0·59	1·13 (0·59-2·15)	0·71
Pre-Excision Diagnosis	**653**				
LSIL	296	1·00	-		
HSIL	305	1·62 (1·18-2·24)	0·003	-	-
Not available	50	2·01 (1·08-3·74)	0·03		
CA	2	5·72 (0·14-237)	0·36		
Excision Type	**653**				
LLETZ	625	1·00	-	1·00	-
CKC	28	0·81 (0·40-1·63)	0·55	0·64 (0·31-1·33)	0·23
Excision Result	**653**				
LSIL	124	0·73 (0·51-1·04)	1·00	0·92 (0·63-1·35)	0·66
HSIL	491	1·00	-	1·00	-
Normal	33	0·77 (0·41-1·45)	0·69	0·99 (0·49-2·01)	0·98
CA	5	2·02 (0·39-10·51)	0·40	2·05 (0·37-11·26)	0·41
Subsequent EB*	**653**				
Not Performed	570	1·00	-	-	-
Performed	83	9·99 (4·73-21·08)	<0·001		
Subsequent Hysterectomy*	**653**				
Not Performed	570	1·00	-	-	-
Performed	83	4·60 (2·57-8·23)	<0·001		
CD4	**653**				
<200	105	2·50 (1·60-3·92)	<0·001		
200-350	184	1·50 (1·03-2·18)	0·03	-	-
≥350	200	1·00	-		
Not Done	164	1·19 (0·82-1·75)	0·36		
ART	**653**				
Started Before EB	199	1·42 (1·02-2·00)	0·04	1·37 (0·96-1·94)	0·08
Started After EB	154	1·62 (1·14-2·29)	0·007	1·58 (1·10-2·27)	0·01
No ART	300	1·00	-	1·00	-

‡ *Not applicable: no lesion*
**after 1 year follow-up*

Lesion at Excision Treatment Failure

At treatment failure the lesion detected was most likely to be LSIL in HIV infected compared to HIV-uninfected women. Of HIV infected women, 62·7% had LSIL, compared to 33·0% of HIV uninfected women *(p<0·001)*.

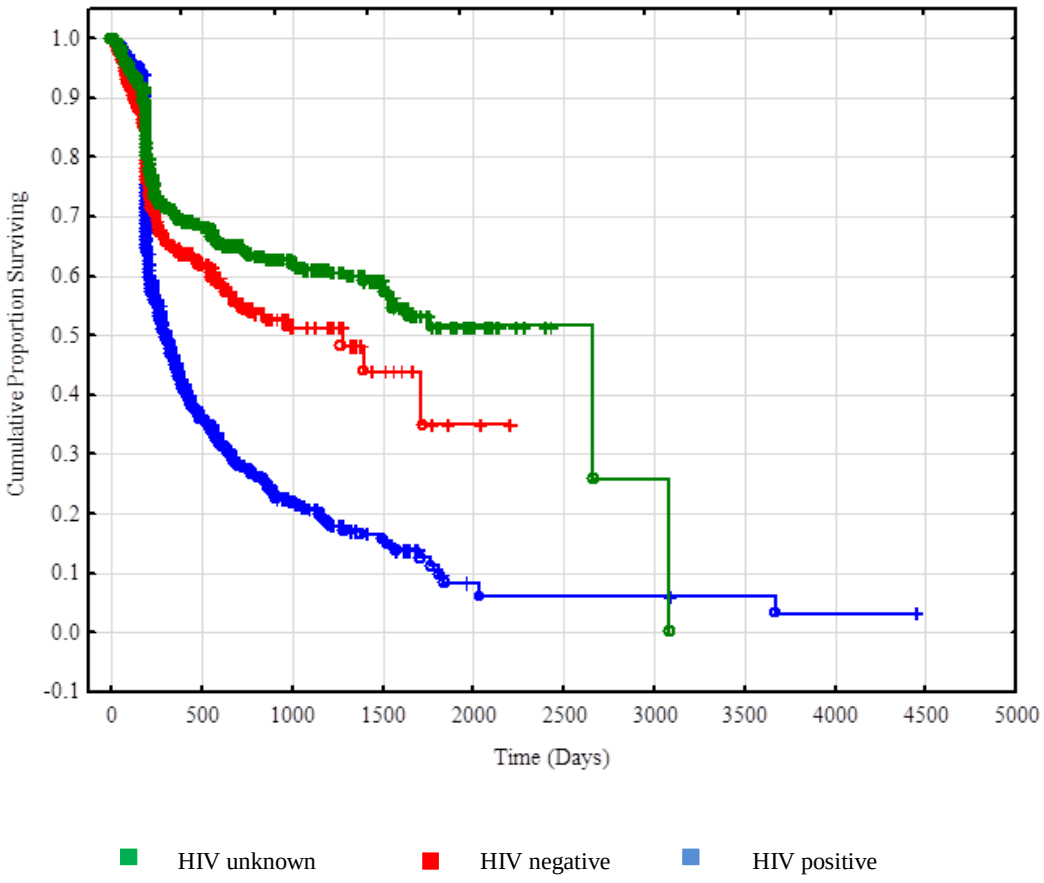
III. Post excision Recurrence

Association of Recurrence with HIV Status

More HIV infected women experienced recurrence of lesions after excision treatment than HIV uninfected women or women of unknown HIV status (figure 11).

Figure 11 SIL Recurrence-free survival After Excision Biopsy

(log rank p<0.001)



Association of Recurrence with HIV Status

The results of univariate and multivariate survival analysis of post excision recurrence of lesions are listed in table 12. As can be seen, HIV uninfected women had a lower risk for recurrence, and this association remained after adjusting for age, excision type and subsequent excision.

Table 12 Factors Associated With Post Excision Recurrence of SIL in All Women

Variable	Total	Univariate analysis HR (95%CI)	p-value	Multivariate analysis HR (95%CI)	p-value
Age at EB	**1848**	1·01 (1·00-1·01)	0·05	1·01 (1·00-1·01)	0·19
Age at EB	**1848**				
15-24	128	1·00	-		
25-29	294	0·83 (0·60-1·15)	0·22		
30-34	447	0·90 (0·66-1·23)	0·90	-	-
35-39	386	0·93 (0·69-1·26)	0·70		
40-44	275	1·14 (0·84-1·56)	0·38		
≥45	318	1·19 (0·88-1·62)	0·15		
HIV Status	**1848**				
Neg	335	1·00	-	1·00	-
Pos	778	1·86 (1·52-2·26)	<0·001	1·95 (1·59-2·39)	<0·001
Unk	735	0·75 (0·60-0·93)	<0·001	0·81 (0·65-0·99)	<0·001
Excision Complete	**1848**				
Yes	682	1·00	-		
No	790	1·90 (1·42-2·54)	<0·001	-	-
Not Stated	77	1·37 (0·86-2·20)	0·51		
Cannot Say	113	1·68 (1·25-2·25)	0·22		
NA‡	186	1·39 (1·07-1·81)	0·69		
Excision Type	**1848**				
CKC	146	1·00	-	1·00	-
LLETZ	1702	0·99 (0·78-1·28)	0·96	1·08 (0·83-1·41)	0·54
Excision Result	**1848**				
LSIL	197	1·00	-		
HSIL	1605	0·79 (0·51-1·23)	0·57	-	-
Normal	9	1·02 (0·41-2·50)	0·80		
CA	37	1·45 (0·88-2·38)	0·20		
Subsequent EB*	**1848**				
Not Performed	1701	1·00	-	1·00	-
Performed	147	3·19 (2·70-3·78)	<0·001	2·25 (1·89-2·67)	<0·001
Subsequent Hysterectomy*	**1848**				
Not Performed	1599	1·00	-	1·00	-
Performed	249	2·79 (2·40-3·24)	<0·001	2·50 (2·12-2·95)	<0·001

‡ *Not applicable: no lesion*
**after 1 year follow-up*

Association of Recurrence with cART

When looking at HIV infected women alone, cART that was started before excision treatment reduced risk for recurrence of lesions, even after adjusting for completeness of excision, excision type, and subsequent excision (HR 0.70, 95%CI 0.55-0.89, 95%, p=0.006) (table

13). The impact of cART that was started after excision treatment was not significantly different from the effect it had in women who never started cART.

Table 13 Factors Associated With Recurrence of SIL in HIV Positive Women

		Univariate analysis		Multivariate analysis	
Variable	Total	HR (95%CI)	p-value	HR (95%CI)	p-value
Age	**778**	1·00 (0·99-1·01)	0·93	1·00 (0·98-1·01)	0·47
Age at EB	**778**				
15-24	48	1·00	-		
25-29	163	0·87 (0·58-1·30)	0·79		
30-34	232	0·75 (0·49-1·15)	0·32	-	-
35-39	170	0·79 (0·52-1·21)	0·36		
40-44	94	0·89 (0·59-1·34)	0·69		
≥45	71	0·84 (0·54-1·31)	0·94		
Excision Complete	**778**				
Yes	243	1·00	-	1·00	-
No	394	1·57 (1·05-2·32)	<0·001	1·41 (0·95-2·11)	0·004
Not Stated	35	0·76 (0·40-1·42)	0·21	0·80 (0·42-1·49)	0·41
Cannot Say	52	0·96 (0·65-1·44)	0·39	0·90 (0·60-1·35)	0·31
NA‡	54	1·27 (0·87-1·85)	0·37	1·20 (0·82-1·75)	0·41
Excision Type	**778**				
CKC	33	1·00	-	1·00	-
LLETZ	745	1·17 (0·76-1·80)	0·47	1·24 (0·80-1·92)	0·35
Excision Result	**778**				
LSIL	100	1·00	-		
HSIL	668	0·98 (0·45-2·13)	0·56	-	-
Normal	2	0·82 (0·20-3·34)	0·62		
CA	8	1·32 (0·53-3·29)	0·56		
Subsequent EB*	**778**				
Not Performed	680	1·00	-	1·00	-
Performed	98	2·17 (1·73-2·71)	<0·001	1·89 (1·49-2·38)	<0·001
Subsequent Hysterectomy*	**778**				
Not Performed	685	1·00	-	1·00	-
Performed	93	2·30 (1·82-2·91)	<0·001	2·01 (1·55-2·60)	<0·001
CD4 at EB	**778**				
<200	131	1·00	-		
200-350	218	0·94 (0·73-1·22)	0·68	-	-
≥350	230	0·81 (0·62-1·05)	0·09		
Not Done	199	-	-		
ART	**778**				
Started After EB	189	1·00	-	1·00	-
Started Before EB	244	0·80 (0·64-1·01)	0·11	0·70 (0·55-0·89)	0·006
No ART	345	0·89 (0·72-1·10)	0·92	0·86 (0·69-1·06)	0·78
Virological failure	**778**				
Observed	89	1.00		-	-
Not Observed	344	0.96 (0.72-1.28)	0.79		
No ART	345	-	-		
Pre-ART HIV-RNA	**130**	1.22 (0.97-1.53)	0.09	-	-

‡ Not applicable: no lesion
**after 1 year follow-up*

Discussion

In this retrospective study we wanted to know if women living with HIV had worse outcome after undergoing excision biopsy treatment of SIL, and if cART protected against disease

recurring. We found that HIV infected women experienced much higher excision treatment failure than HIV uninfected women, but that failure occurred mostly as low-grade lesions. Factors that improved outcome were higher CD4 count and complete excision. Recurrence was much more common in HIV infected women than uninfected women. Importantly, initiating antiretroviral therapy before excision treatment reduced risk for recurrence.

Treatment failure in HIV infected women was previously reported to be more LSIL (52), but Reimers found most (>70%) treatment failure lesions to be HSIL, regardless of the grade of lesions at the time of treatment (51). Like Reimers, we set the minimum lesion for treatment failure conservatively at CIN1, but found 62·7 % of lesions at treatment failure to be LSIL in the HIV infected women. We sought possible explanations for this difference in outcome, especially the timing of first follow-up visit and the influence of possible progression of LSIL to HSIL in the post-excision period. Reimers included *all* women who had an evaluation after excision treatment in her analysis, even if it occurred > 12 months. She did note, however, that "in most cases, the time from initial follow-up to failure occurred within the first year after the procedure", and found higher risk for excision treatment failure for women with first follow-up *after* 6 months than if it had occurred *before* 6 months after excision procedure. In contrast, we only included women who had follow-up within 6-12 months post excision treatment. Early follow-up (<6 months) therefore would not have contributed to over diagnosing LSIL at treatment failure in our cohort. One would, however, consider it possible that the few women who were only evaluated *after* 6 months by Reimers contributed to their HSIL proportion of excision treatment failure, especially in their comparatively small cohort of 136 women.

Others have previously already reported better excision treatment outcome with higher CD4 count, (50, 51, 53) which we could confirm. We found no association of CD4 count with recurrence. The CD4 count is not only influenced by disease HIV disease progression; many conditions, amongst other concomitant diseases (e.g. the HPV infection) and medications may play a role. The CD4 count also may not accurately reflect the presence of CD4 cells at mucosal level. Further investigative studies that focus on cervical immunological factors, such as dentritic cell density, could be of more value.

ART did not significantly reduce risk for excision treatment failure, which was not surprising for women who initiated cART only after the excision treatment procedure. The follow-up

time would have been too short. More unexpected was the lack of protection it provided to women who had started cART before the excision treatment. A possible explanation could be found in the small number of women who were already on cART prior to the LLETZ/CKC. We had few women who had been treated with ART for longer periods and presented with HSIL as the indication for excision treatment. Indeed, as we (55) and others (46) have shown, the risk of progression of LSIL to HSIL is reduced by cART. It is possible that cART had protected women from progression of low-grade disease (46), thereby reducing the number of study subjects who had been treated with cART available for our analysis.

ART was associated with a reduced risk for recurrence of lesions after excision treatment. Because our definition of recurrence refers to any lesion after one year, it again reflects the importance of the timing of the initiation of cART with the protection it may offer.

The strength of this study lies in large sample size and the use of a combination of cytology and histopathology results in determining and comparing outcome of excision treatment of cervical intraepithelial lesions. Set in high HPV/HIV prevalence area, it describes the clinical scenario physicians and their patients are faced with in limited-resource countries.

This investigation had some limitations. First, as for all observational data, our study may be subject to unknown confounding factors that we are not aware of and therefore were not controlled for in our multivariate models. Furthermore, the quality of cytology was inferior for the HIV group as measured by the percentage smears without EC present. Judging the completeness of a Pap smear, the presence of endocervical cells on cytological reporting is considered important, as cervical abnormalities usually occur in the region of the endo-ectocervical junction. The presence of these cells is routinely used to compare quality of smears between groups of patients. If more patients in one group have incomplete smears (endocervical cells not seen), such as with the HIV infected group in this study, it means that cervical lesions could have been missed more often in the HIV infected group. Without the possible attenuation of effect, the results could have been more pronounced.

For completeness of excision, we set strict criteria at *LSIL present at any margin* to agree with previous reports (51). Completeness overall was low, and also significantly less in HIV infected women. However, comparing only the excisions reported as either complete or incomplete, treatment failure still occurred more in HIV infected women. The higher

occurrence of incomplete excision in HIV infected women could have been due to extensive HPV infection in the immune deficient cervical milieu (56).

Also, there was a selection bias toward HIV infected women entering care earlier. For HIV infected women, local guidelines request referral for colposcopy evaluation after their first LSIL. In addition, Pap screening is available free to all women over the age of 30 years, but HIV infected women qualify for this benefit regardless of age. The younger age at which HIV infected women underwent first excision treatment reflects referral policy but does not necessarily implicate faster progression of low-grade lesions.

We included women of unknown HIV status in our analysis. The clinical significance of including the HIV unknown group is high. As can be seen in the total patient numbers, this group is larger than the HIV negative group. The reason is that HIV testing for the largest part of the study period was not routinely done for women with cervical dysplasia. Importantly, the study did not find increased risk for recurrence of SIL post excision in the HIV unknown status group compared to the HIV uninfected group. We think that women regarded by physicians as at risk for HIV infection were more likely to be tested, and that women not tested, were considered low- risk for acquiring HIV infection. Testing for HIV infection has become standard practice since 2009 for all women who attend the Colposcopy Clinic.

Our data suggest that careful follow-up should be maintained after cervical excision treatment, especially in countries with high HIV/HPV prevalence. Women on ART may be expected to have overall better excision treatment outcome and this supports earlier initiation of ART than current guidelines stipulate in vulnerable communities. It also underlines the importance of HIV screening in all women with cervical dysplasia.

In conclusion, we found that post excision disease occurs more as LSIL than as HSIL in HIV infected women, but that cART protects against this recurrence. In chapter 2, we already described the impact of cART in reducing risk of progression and persistence of LSIL. As our next step we needed to proceed to examine the effect of cART on the ***cause*** of low-grade cervical dysplasia, that being cervical HPV infection.

Chapter 4: Cervical HPV Infection in Women Living with HIV

Background

Introduction

We have described in the preceding chapters that cART reduces the risk for progression and persistence of LSIL. These findings have been confirmed by others (46, 57, 58). After excision of existing lesions, it also reduces risk of recurrence of SIL. We therefore wanted to proceed by doing a study which would investigate directly the effect of cART on cervical HPV infection.

In order to plan a robust study with the best chance of an undisputable verdict as to the effect of cART, an understanding of HPV epidemiology was first required. This would enable us to choose the most appropriate detection method for the presence of cervical HPV infection. Also, we looked at previous studies done, specifically not only the outcomes achieved, but the success with which their outcomes have been duplicated by others. If not, we would try to identify possible reasons, and how these could be improved upon in our methodology.

Human Papilloma Virus Infection in HIV Uninfected Women

The progression from HPV infection to moderate and severe dysplasia and eventually invasive cancer is a slow process. In the Human Immunodeficiency Virus (HIV) uninfected patient, mild dysplasia as well as moderate dysplasia almost always regresses spontaneously, with 1% annual progression of mild cervical dysplasia to moderate or worse dysplasia. (59). Ninety per cent of women may clear HPV infection of the cervix within 60 months (60). Even infection with High-Risk (HR)-HPV clears naturally in most HIV uninfected women, as was recently again described by Kim et al in Korea (61) HR-HPV types tend to persist longer, particularly HPV16, which can cause larger lesions (62), although some authors recently reported better HPV16 clearance rate than for HR- HPV types such as 31, 39 and 73 (63).

Before technological advances enabled identification of the viral aetiology of cervical cancer, the focus of research was on environmental and socioeconomic factors as causative agents. Although HPV infections resolve mostly spontaneously without causing symptoms, some

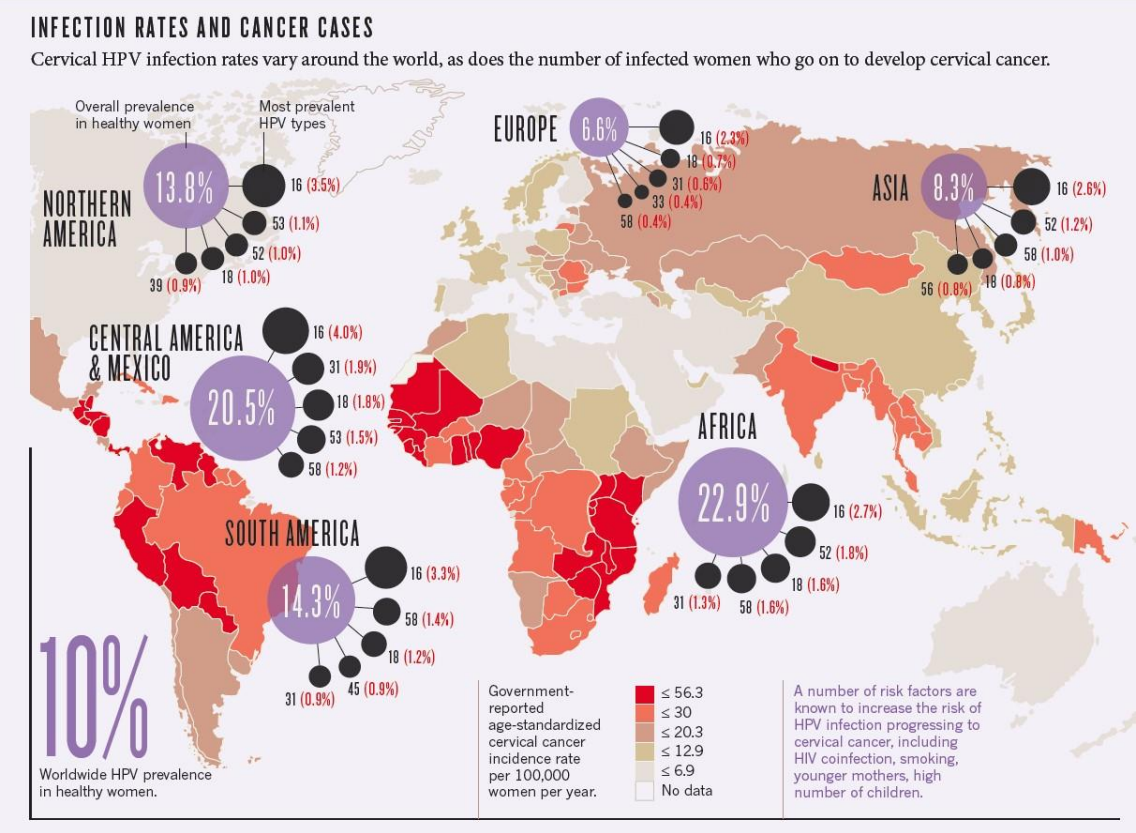
women develop cancer. In the past decade, an important aspect of cervical HPV infection in the pathogenesis of cervical cancer emerged: the *persistence* of HPV infection, especially *type specific persistence* (60, 64-67).

More than 120 human strains of HPV have been identified. Fifteen are classified as oncogenic types (68, 69). Literally hundreds of epidemiological studies of cervical HPV DNA genotype distribution have been done worldwide. It is therefore best to focus on meta-analyses that summarize these findings.

The first meta-analysis from 2005 which included only published studies of women with normal cervical cytology, found the overall HPV prevalence at 10.4%, with Africa highest at over 22%. HPV16 and -18 were the commonest subtypes observed, but their relative contribution was smaller for women with normal Pap smears than in women with CIN (70).

Figure 12 HPV prevalence world-wide

(Reproduced with permission from (4))



On the opposite side of the spectrum of HPV related disease, in a meta-analysis of HPV genotypes associated with invasive cervical cancer (ICC) published in 2011, 79% of cancer was associated with only one genotype HPV, and in 56.6% of these cases it was HPV-16. Second most common was HPV-18 at 13.2%, followed by HPV-58 , -33, -45, -31, -52, -35, -59, -39 and -56, each contributing 1% or more. Other subtypes represented less than one per cent of detections. This pattern was also found in African studies (71).

A meta-analysis published in 2012 that combines the full spectrum of cervical disease, summarized the association of the different genotypes with the severity of disease. They found the relative contribution of HPV 16/18/45 to the combined lower spectrum of cervical disease to remain stable, but that HPV16 was detected significantly more in higher grades to cancer (table 14) (72). A large study combining the results of 8050 HIV uninfected women from Khayelitsha, Cape Town, also found HPV16 most common, with the other main contributors to CIN 2/3 to be HPV35 and HPV45 (73).

	HPV16	**HPV18**	**HPV45**
Normal/ASCUS	20.4%	8.4%	4.8%
CIN1	27.1%	9.0%,	4.2%
CIN2	39.8%	10.0%	5.0%
CIN3	58.2%	7.4%	3.6%
ICC	62.8%	15.7%	5.3%

Table 14 Relative contribution of HPV16 in increasing severity of cervical dysplasia and cancer

To summarize, of the 70 strains, HPV 16 and 18 are strongly linked with the development of invasive cervical cancer. Together they account for approximately 70% of invasive cancer (69). Specifically, HPV-16 causes larger and more persistent lesions (62), is less likely to regress (74) and more likely to cause squamous cell carcinoma of the cervix (75). What needs to be remembered, that although HPV16 contributes most to cases of cervical cancer, the contribution of other genotypes to *cervical dysplasia* is substantial (76), and the behaviour of each genotype potentially varied. Added to this, prevention of HPV16 infection only would decrease risk of cervical cancer, but the impact on cervical dysplasia could be expected to be substantially smaller.

HPV Infection in HIV infected women

In studies done internationally and in South Africa, HIV positive women have been found to have higher incidence of Human Papilloma Virus infection (31, 77-84) and of recurrence thereof after treatment (85) than HIV negative women. The HPV prevalence in HIV infected South African women is among the highest ever reported at 95%, (82), with the risk for HR-HPV infection estimated at 5 times that of HIV uninfected women (86). HIV positive women also have a higher risk for the development of invasive cancer and the cancer occurred at a younger age. It is thought to be due to persistence of HPV infection in HIV positive women, reactivation of latent HPV infection (77, 85) or reinfection from their male partners. HIV positive women may be more likely to be infected with HPV from their male partners than HIV negative women (87) .

As to the HPV genotypes that most contribute to cervical cancer in HIV infected women, there seems to be geographic variation in reports. As in HIV-negative women, subtypes 16, 18, 31, 13 and 45 were found to most likely to lead to cancer by certain investigative groups (81, 88), or just HPV-16 and -18 in an Italian study (89), or HPV-16 and HPV33 in Norway (90) . In Australia, HPV-16 and -52 and -58 were most common (91). A Brazilian study found HPV 16, -18-, 61 and -53 to be most commonly detected in HIV positive women (92), and in Honduras HPV 16, 51, 84, 66, 39 were most common (93) .Clifford in the US found HIV positive women are less likely to harbour HPV 16 in HSIL (81), but more commonly have multiple HPV types, especially HPV 18, 33, 51, 52, 58, 11, 53, and 61 (88, 94).

There seems to be variation of HPV genotype distribution and relative contribution to invasive cervical cancer also within the African continent. A meta-analysis of Medline listed studies showed HPV-16 to be the commonest subtype in Africa, although no Sub-Saharan country study was included in this review (95). Numerous regional studies in Sub-Saharan Africa have however since been done. In Uganda HIV positive women were 4 times more likely to have cervical HPV infection (96), in Rwanda HPV-16 was most prevalent and persistent (97), and in Botswana, HPV-58 was more common than HPV-16, followed by HPV-39 in third place (98). In another study done in Mozambique, no difference was found between HPV type detected in the cervical cancer between HIV negative and positive women (99).

Even within South Africa, there has been variation of reports of HPV genotype distribution amongst HIV infected women. The commonest types detected in HIV positive women in Johannesburg were HPV 16, 35, 53 and 18 (82). In Cape Town, HPV-16 was also still found to be the most common type, with HPV-18 only the fifth commonest (43). Nevertheless, no matter what company it seems to keep, HPV-16 features predominantly in all reports as having the strongest prevalence in those cases of cancer (100). The subtype distribution of HPV and the role of specifically HPV-16 in women living with HIV infection have not been investigated in the Tygerberg Hospital referral population. Also, what has to be kept in mind that over the years, HPV testing has become more expansive in their ability to detect the different genotypes, which contributed to the variation in and increasing number of genotypes reported to be associated with cervical disease.

Apart from the role of specific HPV genotype infection in progression of cervical lesions to pre-cancer and cancer, the association of HPV infection with increasing immunosuppression caused by HIV infection has been reported in numerous studies. In a Johannesburg study of 148 HIV infected women, 54% had abnormal smears (of which 66% LSIL and 33% HSIL), 95% of the 148 women had HPV detected and 83% more than one subtype, with increased risk with CD4<200 (82). This was also seen in a Cape Town study, where abnormal cytology and high-risk HPV positivity were strongly correlated with low CD4 counts and high HIV viral loads (43). The association of HPV infection with immunosuppression may be explained by the commitment of local immune mediators towards clearance of incident HPV infection, and that non-transient HPV infection was most associated with elevated levels of these immunological markers (101). In contrast to all these reports, Mbulawa in Cape Town grouped all HPV-HR types together, analysed outcomes in a Poisson regression model and found that no variables (such as CD4 count) except abnormal cytology had an impact on HPV clearance (84).

Latency and Reactivation of HPV

Just what happens when HPV is no longer detectable - or as authors mostly describe the phenomenon - the infection is “cleared”, is not certain. As with most DNA tests based on PCR technique, the level of detection is extremely sensitive. The question remains that if HPV-DNA is not detected, is it just because the number of copies of the virus has dropped to below the level of detection, or has the viral infection been completely cleared? A few studies have tried to explain other coincidental findings on the basis of clearance not always being

absolute, but with regression of HPV viral shedding to include presence of the virus in a latent, asymptomatic and undetectable state in the basal cells (102).

In a study examining the association of incident HPV detection with immunological markers (CD4 count) and HIV-viral load, there was a peak hazard ratio for HPV incident detection with the CD4 count <200 and HIV Viral Load over 100,000 copies/ml. Because this was also observed in women who were sexually *inactive,* the author postulated that increased detection of HPV in such instances could be due to reactivation from a latent state (103).

A rapid rise in HPV detection within the first years of HIV infection, a period of intense immune modification, especially shortly after incident HIV infection, has also been described. The authors thought that local immune dysfunction after HIV acquisition may play a role in this increased detection of HPV. (104).

Theiler et al went further to investigate reactivation events for five HPV genotypes. Prior exposure was determined by serologic testing and HPV shedding observed with PCR methods at 6 monthly intervals. The author found reactivation of latent HPV infection higher in HIV infected when compared with uninfected women, who had few reactivation events. The risk factor associated with reactivation was the CD4count <200. Total HPV shedding (reactivation plus recurrence) was 1.8 to 8.2 times higher in HIV infected than uninfected women (105). This finding has to be seen in the light that re-appearance of type-specific HPV is extremely rare in HIV uninfected women (106).

A study published very recently was done in women in California, and here the authors do not report HIV status in the followed subjects. They found reappearance of HPV DNA in 18% of women, that it was linked to sexual exposure, even in women without serological evidence of prior HPV exposure. Their explanation was that not all women who acquire HPV16 infection seroconvert, or that tests serological testing is still inadequate (107). This study is interesting, especially when read in together with the findings of Theiler, and we need to keep in mind that it is additionally possible that in immunocompromised women, there exists a possibly greater chance of insufficient development of memory immune responses to HPV16, leaving HIV infected women at greater risk for reinfection or redetection of HPV.

Although there is still paucity of proof as to whether HPV may persist in a latent state in the basal layer of the cervix, the possibility thereof may explain at least in some part the difficulty researchers are having in adopting definitions of persistence, clearance, and prevalence - especially in the immunocompromised population- from research done in the field of cervical dysplasia and cancer, in spite of expansion of the repertoire of definitions to include *incident detection of HPV*.

Monitoring HPV infection

There are a number of commercially available tests for detecting the presence of HPV, of which the Roche Linear Array is able to detect the larger number of genotypes:

HPV DNA detection test

1. **Roche Linear Array HPV Genotyping Test**
   This qualitative in vitro test uses amplification of target DNA by polymerase chain reaction (PCR) and nucleic acid hybridization and detects **37** anogenital HPV DNA types in cervical cells collected in PreservCyt® Solution.
2. The **Cobas4800®** (Roche Molecular Systems, Pleasanton, CA) detects **12** HR-HPV types also with individual HPV-16 and HPV-18 detection.
3. **Hybrid Capture 2 (HC2) test** (Qiagen, Gaithersburg, MD) is an amplification assay which uses a combination of antibody capture and chemiluminescent signal detection. It can detect **13** HR- and **5** LR-HPV types.

HPV mRNA

Apart from identifying persistent HPV infection with HPV-DNA screening tests and/or the specific genotype present on cervical smear, a more specific test has been developed which may predict the development of high grade lesions (108-110). These tests are flow cytometry-based in-situ tests that identify the presence of HPV E6/E7 mRNA in intact ectocervical cells. Oncogenic genotypes of HPV over express E6 and E7 mRNA after integration of HPV into genomic DNA (111).The E6/E7 proteins inactivate two tumour suppressor proteins, p53 and pRb. Increasing levels of E7 mRNA appear to be associated with an increased risk of developing cervical cancer (112).

The mRNA tests may be especially helpful in patients under the age of 30 years, when the prevalence of HPV infection is the highest, in identifying oncogenic activity in the HPV

subtypes most linked with dysplasia (113). Further application may be to triage women with normal cytology but positive HR-HPV to Colposcopy evaluation: a positive mRNA test relating to increased CIN2 risk. Also clinically validated for ASC-US triage, the negative predictive value of an mRNA test result for CIN2 or higher is 99% after ASC-US cytology, and the positive predictive value for CIN2 is between 8-14% (114). The mRNA detection tests do not however contribute diagnostically in cases of mild dysplasia or higher cytological abnormality (115).

Commercially available tests for detecting mRNA include:

1. **PreTect Proofer Assay (NorChip AS, Klkkarstua, Norway)** is a multiplex nucleic acid sequence based amplification (NASBA) can detect mRNA of five (5) HR subtypes.
2. **NucliSENS EasyQ™ HPV (bioMérieux Clinical Diagnostics, France)** can detect mRNA of five (5) HR subtypes.
3. **APTIMA HPV Assay (Hologic Gen-Probe Incorporated, San Diego, CA)** can detect mRNA of fourteen (14) HR subtypes.

Although the potential value of mRNA for the *detection of oncogenic activity* exists, two factors excluded its use for monitoring cervical HPV infection in this study: the limited number of genotypes that may be detected, and that the focus of this study is the detection of *specific HPV genotype infection* and not that of the study of *oncogenic activity*. To put it more plainly: we were not just interested in the association of cART with certain oncogenic types, but with all types that could cause an abnormal Pap smear.

HPV Viral Load

Advances in molecular techniques have enabled scientists to not only detect the presence of HPV in cervical tissue, but also to quantify the amount of HPV shedding. There is an association of the level of cervical HPV shedding with patient age and the progression of cervical dysplasia. Cervical HPV viral loads are consistently higher in women under 25 years of age (116), with HPV viral load declining with increasing age. HPV viral load has in some reports been found to be significantly associated with degree of cervical lesions (83, 117).

Specifically, HPV Type 16 and 18 viral load levels in cervical smears are higher in women with HSIL than with LSIL, even after excision treatment, in women aged 30-40 years. In younger women, a high HPV viral load can also be detected in some women with LSIL or

even normal cervix (118). Severely immunocompromised patients (CD4<100cells/ql) are at highest risk for high HR-HPV viral load and cytological abnormality (119). HR-HPV viral load may also be influenced by transmission from the sexual partner. A study previously showed that circumcised males have a lower prevalence of HPV-associated penile lesions (120). Supporting this, the risk for higher HPV viral load in the female partner has been found to be reduced by circumcision intervention in the male partner, primarily for incident HPV infections, but not persistent HPV (121).
The association of cervical HPV viral load with severity of cervical disease is still under investigation, however. A recent study saw no association of HPV viral load at baseline with progression to CIN3+, and the slight increase in those that did progress was not a significant change. What was interesting, though, was that the women who developed persistence of HPV infection- which is considered the most important factor contributing to risk for developing cervical cancer- had lower HPV viral loads, while clearance of the infection was associated with high cervical HPV viral load levels at baseline (122) .

When the disease has progressed to invasive cancer, no correlation of pre-treatment HPV viral load with prognosis of early stage cervical carcinoma was found (123). HPV-16 could however be detected in *plasma* of women with CIN III (16.7%), Cancer stage I (25%), Stage II (61.3%), stage III (74.3%), stage IV (100%). In this publication, the Clinical Stage was correlated with HPV 16 viremia. No association could be found for HPV 18 viremia and advancing stage of cancer (124).

As with the detection of HPV mRNA in cervical tissue, monitoring the amount of HPV shedding from the cervix seems to have highest value in the study of progression of cervical dysplasia to cancer. Unfortunately, it would only be possible to monitor HPV viral load levels for one or two genotypes. The purpose of the study is to investigate the efficacy of cART to eliminate, or at least drive to below the level of detection, the HPV genotype population. In addition the aim is to compare the relative effect of cART on those subtypes, but not to focus on the amount of shedding of just one or two types.

We have therefore chosen as most suitable for the purpose of this investigation, with proven high sensitivity for detection of the most HPV genotypes, the **Roche Linear Array HPV Genotyping Test.**

Antiretroviral Therapy and HPV infection

Several studies that examined the effect of cART on cervical HPV infection - from as early as 1998 - have been executed, and they reported some conflicting findings. Firstly, earlier studies used less sensitive HPV tests- based on all or none detection- that possibly could not detect the variety of genotypes that can be detected with tests more recently. Furthermore, HPV genotypes were often grouped together as HR-HPV. Most studies were based on confusing study end points that had been adopted from cervical dysplasia research. These endpoints were possibly also used in order to apply statistical methods available at the time (Xhi-square tests, logistic regression, and cox regression and survival analysis). These tests ignore the repeated measures effect, resulting in loss of statistical power by ignoring a large proportion of observations. Finally, some were done on very small cohorts; some had very few patients initiated on cART, or very few patients not exposed to cART. Most studies, but not all, failed to show an effect of cART on HPV infection, even if some could demonstrate an effect on SIL, or vice versa.

One of the first studies was done in France. Early regression of cervical lesions was seen in 49 women initiating ARV therapy, but clearance of the HPV infection was not always observed in those cases (47). In another small study that enrolled 45 MSM, a high prevalence of anal HPV and high-grade SIL remained despite immune restoration under cART (125). When looking more specifically at incident HPV detection, two more recent study reports also failed to show a protective influence of cART (39, 126). One was focused on women with genital warts, the other included only adolescents (39).

In contrast, in an observational study by the Minkoff group of women initiating cART, these women were 40% more likely to experience regression of their cervical lesions after adjustment for CD4 counts (34). ARV therapy was found to alter the course of HPV-related disease, reducing progression and increasing regression of lesions (34). Paramsothy et al included 537 women in their study and found that HAART was associated with improved HPV clearance in women with pre-existing cervical squamous intraepithelial lesions (Hazard ratio was 4.5, 95% CI 1.2-16.3), but not with abnormal Pap test regression. Also, it did not lead to improved clearance of HPV infection in women with normal Pap smears. (36).

Similarly, another larger study was done by Konopnicki et al. Sustained suppression of HIV RNA plasma load for >40 months and CD4count >500 cells/µl for >18 months reduced risk

of HR-HPV persistence. The group did not distinguish between the different the HR-HPV genotypes in their analysis (127).

Minkoff did another prospective study of 286 women which was published in 2010. His team again found that HAART use in women was significantly associated with a reduced burden of HPV infection and SIL. Specifically, there was a reduced prevalence (OR= 0.60 (0.44-0.81), incident detection (HR=0.49 (0.30-0.82) and more rapid clearance of HPV positive SIL (HR 2.35, 1.07-5.18). The effect was bigger if the women were adherent to the medication. He did not find a difference if a stricter definition of adherence, that of undetectable viral loads, was used for adherence. Minkoff did however apply frailty models instead of standard Cox proportional hazards models, but still used traditional definitions of prevalence, persistence and incident detection as outcomes. In both the 2001 and 2010 studies, a PCR method was used. In the 2001 study, no distinction was made between the different oncogenic HPV subtypes as outcomes. Rather, the presence of any HR-HPV was seen as a positive outcome. In the 2010 study, oncogenic and non-oncogenic groups were evaluated separately as two outcomes (35). Table 4.2 summarizes the methods applied in these reports.

These conflicting findings, leading to a lack of consensus on the effect of cART on cervical HPV infection, therefore provided us with the impetus for this research question to be revisited. We decided to design a more robust study which enrolled women with a wide range of immunological as well as cervical cytological status, and to apply the most appropriate analytical method that utilizes maximum statistical power suited to the design of the study. We also chose to also evaluate the effect of cART on each individual HV genotype and not restrict ourselves to the overall effect on all HPV types together.

Author	Year	Study Population	Number HIV+ Subjects (Number cART)	HPV Method	Number Genotypes tested	Outcome HPV	Statistical Method	Outcomes tested	cART effect tested	Significant effect
Heard	1998	Adult women	49 (49)	PCR SBH	9	HPV types together	Proportions	Persistence, regression	Before and after 5 months cART	SIL: Yes HPV: No
Minkoff	2001	Adult women	741 (741)	PCR	14	HR-HPV types together	Proportion of *paired* samples	Progression, Persistence, of SIL/HPV	Time On ART (1yr) vs not on ART	SIL: Yes
Pikkety	2004	MSM	45 (45)	PCR	>26	Any HPV HR-HPV types together	Proportions and MU test	Prevalence	Before and after 6 months cART	No
Paramsothy	2009	Adult Women	537(252)	PCR	26	HR-HPV types together	Cox proportional hazards	Progression, Clearance	On ART vs not on ART	SIL Clearance: No HPV: Yes
Shreshta	2010	Adolescents	227 (100)	PCR	30	Some individual types	Proportions, Time to end point	Prevalence, clearance, incidence	Before/ after cART	No
Minkoff	2010	Adult Women	286 (286)	PCR	>40	HR-HPV and non-HR-HPV	Mixed model random effect	Prevalence, persistence, incident detection	Adherent/non-adherent cART vs. pre-ART	Yes
Low	2011	High risk Adult Women	273 (26)	INNO-LiPA	28	Incident detection and warts	Multivariate logistic regression	Prevalence, incidence	On ART vs not on ART	No
Konopnicki	2013	Adult Women	652 (158)	Hc2 HR	13	HR-HPV types together	Multivariate Logistic regression.	Prevalence, persistence, incident detection	On ART >40mths vs not on ART	Yes

Hc2 = Hybrid Capture HR HPV DNA Test; SBH = Southern blot hybridization; MSM = Men who have sex with men; MU test = Mann Whitney U test

Table 15 Summary of Studies investigating the effect of cART on cervical or anal HPV Infection

Methods

Study Design, Population, Setting and Outcomes Definition

In this prospective study women known with HIV infection were approached for enrolment to the study if they were cART naïve or had recently (<1 month) initiated cART. Two study sites - both situated in Tygerberg Hospital, Parow, South Africa- were used. The first site was the Infectious Diseases Clinic (IDC), a treatment centre for the management of HV infection, which includes provision of antiretroviral therapy. The second site was the Colposcopy Clinic (CC) of the Department of Obstetrics and Gynaecology. The twin enrolment approach was needed to ensure inclusion of study subjects already known with cervical dysplastic lesions, whilst also including women with normal cytology from the Infectious Diseases Clinic. Patients known with cervical or other cancers, however, were excluded, as were pregnant women. Each study site contributed approximately one half of study participants. Enrolment commenced November 2009 and ended October 2011. The study was approved by the Stellenbosch University Human Research Ethics Committee (ref nr N09/04/106).

Follow-up visits were conducted twice yearly, either at the IDC, or both the IDC and the Colposcopy Clinic, depending on whether colposcopy evaluation and a procedure such as biopsy or excision treatment was indicated. At each visit, the medical history, including the obstetric history, sexual behaviour and substance (alcohol or drugs) use were recorded.

A cervical Pap test was performed for each study subject at every visit, except on the days that a cervical biopsy was taken or an excision biopsy was done. Cytology and pathology were read by Pathologists according to standard protocol at the routine laboratory service located in Tygerberg Hospital. Classification of results was based on the 1988 Bethesda scoring system for cervical pathology: normal, LSIL and HSIL. For simplification for statistical analyses, ASC-US and ASC-L were grouped with LSIL, and ASC-H was grouped with HSIL. For the analyses, a combination of Cytology and Pathology results were used, but Pathology that was performed on the same date was used in the case of conflicting results.

Management of HSIL cervical lesions was not altered for the purpose of this protocol, but continued unchanged regardless of HIV status of the patient. We did, however, have a lower threshold for referral of LSIL for women known with HIV infection. Referral for colposcopy

evaluation occurred already after a single LSIL Pap smear result, whereas HIV uninfected women were only referred after the second abnormal smear showed LSIL or a higher lesion. If a suspicious lesion was seen on aceto white stain, a biopsy was taken to confirm or exclude HSIL. If confirmed, an excision biopsy treatment was scheduled at the next available appointment at Colposcopy Clinic, usually a week later. If the diagnostic biopsy excluded HSIL but showed LSIL, the Pap test would then be repeated 6 months later for the purpose of this protocol, and not two years later as would normally be standard for non-study participants.

The decision to initiate antiretroviral therapy was not influenced by the study, but was based on the Government Protocol for the Initiation of Antiretroviral Therapy. In 2009, the protocol prescribed commencement of cART when the CD4 count dropped under 200 cells/ql. This was revised in 2010 to include initiation of cART when the CD4 decreased to under 350 cells/ql in pregnant women and persons with Tuberculosis, and finally in 2012 again revised to recommend initiation in all persons with a CD4 count less than 350 cells/ql. The standard initiating regimen of antiretroviral therapy was stavudine, lamivudine and efvavirenz or nevirapine in 2009, but was changed to replace stavudine with tenofovir in April 2010. The Protease inhibitor, ritonavir boosted lopinavir, was and still is used only in the second-line regimen in almost all patients treated on this programme.

Antiretroviral therapy use was recorded at each visit. Interruption of the prescribed regimen was ascertained by a combination of the pharmacy pickup and patient self-report methods. No distinction was made whether interruptions were physician or patient-initiated.

Response to antiretroviral therapy was measured by testing the CD4 count at study enrolment and at each six monthly follow-up visit. A viral load determination was also done at baseline and is referred to as the viral set point. The Abbott m2000 RealTime HIV-1 Assay was used throughout the study and performed at the Department of Virology, NHLS, Tygerberg Hospital. For the patients that have commenced cART, the viral load test was repeated at six monthly intervals. If an unscheduled viral load test done between study visits was at a detectable level and higher than at the next study visit (for example in the case of suspected poor adherence not reported by the patient or revealed by missed pharmacy appointments), the higher value was used to reflect that viral replication was not suppressed for the time between study visits.

Cervical HPV samples were collected with a Cervexbrush® at baseline and 6 monthly intervals, placed in PreservCyt® (Hologic, Bedford, MA) solution and stored at -80deg Celsius for later analysis by Roche linear array, according to the manufacturer's instructions. Specimen preparation was done using the AmpiLute Medica Extraction Kit which yields HPV target DNA and human HPV suitable for PCR amplification. The Master Mix reagent contains primers for the amplification of DNA from 37 HPV genotypes and of the ß-globulin gene. The test procedure performs target DNA amplification with AmpliTaq Gold DNA Polymerase, and AmpErase for further selective amplification. Genotyping was then performed by hybridization technique on the extracted DNA. Test strip results were read independently by the two molecular scientists. Both were blinded as to the ART and cytological status detected in any of the study subjects. Samples that were ß-globulin negative were excluded from the analysis. HR-HPV subtypes are HPV-16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. The rest of the HPV subtypes detected by the Roche array are considered LR-HPV and include HPV 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, CP6108 and IS39. For simplification, we did not consider the probable HR types 67, 69 and IS39 in the HR group (68). A study subject was considered HPV positive if the specific HPV genotype was detected on a cervical sample. The test results were reported keeping in mind the cross-reactions that occur: a positive test result for subtype 33* means that HPV 33 is positive, but the presence of HPV 52 cannot be excluded; the same for HPV 35* and HPV 58*.

Statistical analysis

A SQL database (Microsoft SQL Server 2008) was created to capture, store and retrieve study data (Appendix 1-5). The database was securely stored on the University of Stellenbosch server with a double layered per-user defined password access system. Data extraction was done from an Access front end (Microsoft Access 2010). Once extracted, the data were analysed using Microsoft Excel 2010, XLSTAT (version 2013.3.01) and Stata (version 11). Baseline demographic variables were assessed using Pearson's $\chi 2$ or Fisher exact tests to compare proportions, and Student's paired-t tests to compare means between groups.

For the purpose of this analysis, we defined antiretroviral treatment in two ways. For the first variable, ***time on cART***, the cART start date was recorded and the time since first date of

administration calculated at each visit. This variable would not be influenced by adherence to the cART regimen. The ***cART treatment status*** variable was used as a binary variable to indicate if the patient was still using cART at a visit; never started or an interruption of more than one month were labelled "not on treatment" at that visit.

Historically, analysis of the factors that influence detection of HPV subtypes, and this includes the influence of cART, were done using logistic regression or survival analyses. Unfortunately, artificial simplification of data always had to be done in order to accommodate these methods. Firstly, HPV infection outcomes had to be defined, mostly as HPV *prevalence*, HPV *incident detection* and HPV *persistence* (the time to clearance of an HPV test result following its initial detection, with clearance defined as the first or the first *subsequent negative* result). This simplification presents opportunity for bias and human error: for example how would the detection of a subtype, previously detected at baseline, not detected at two subsequent visits and then detected at the next, be defined? Minkoff (35) used frailty models to evaluate incident detection and time to clearance, but the artificial diagnosis of both incident detection and clearance would duplicate the within-participant correlation already provided by the statistical method of analysis, namely that of different snapshots in time of the same study subjects. Frailty analysis already takes into account the presence or absence of the subtype at another time point and it is therefore not necessary to create these additional classifications of HPV detection outcomes.

A second simplification was made in almost all of these analyses. Because of the multiple subtypes that are tested for, the subtypes were roughly grouped as any high-risk HPV (HR-HPV). This implied that the factors that influence HPV infection are equal for all HR-subtypes, ignoring possible differences between the subtypes and the effect of the presence of any HR-or LR-HPV subtype might have. LR-HPV types were mostly ignored. A third and important simplification was that time-dependent factors were simplified or classified in order to include them as covariates in the analyses, thereby ignoring the statistical effect of repeated measurements on study subjects. For example, the time treated with cART was traditionally taken as <1yr, 2 yrs etc; sexual activity was stated as at baseline without taking into account that it might change during follow-up period; CD4 counts were taken as either baseline or pre-cART and ignored the effect of changes during follow-up.

Independent predictors of HPV detection before or after cART can be assessed by performing random effect modelling which controls for the fact that data collected include repeated observations measures of the same women over time and possibly multiple different HPV types. These multiple HPV subtypes presented separate outcomes which are possibly not equal: it is known that HPV16 causes most cases of cancer and larger and more persistent CIN lesions. Immunity conferred during infection with one subtype also may not confer complete immunity to another (128). The various other factors that influence outcomes may also not be independent from each other; cART status can be expected to have a strong association with HIV-RNA level.

Time-to-event analyses were therefore performed using detection of HPV outcomes by performing frailty models which incorporate a random effect to adjust for within-participant correlation. Indeed, they are the most commonly used time-to-event method that permits the comparison of women with themselves (i.e. before and after cART initiation) and are therefore more appropriate for our study (129). All models were adjusted for excision treatment of cervical neoplasia and the CD4+ cell count, using time-dependent variables. Other time-dependent factors adjusted for in our models include: time on cART, cART treatment status and sexual activity. Age was included as a non-time dependent variable due to the limited follow-up time. P-Values reported were two-tailed and alpha level 5%.

Approach to missing data

Missing data are unavoidable under even the most strictly controlled studies. There are several methods to deal with missing data. A common approach is to exclude all observation sets with missing data points. We followed this approach as regards to the **outcome variable** (successful DNA sample) and included only observation sets from those visits that had complete HPV detection results available; four (4) of 1115 cervical specimens on which no DNA could be extracted were excluded from the analysis, even though it also led to the complete exclusion of one study subject (see *Results*).

To deal with missing data from **factors influencing** the outcome variable, the data set was examined as to determine the type of missing points. All of the remaining 1111 data set variables were found to be complete, except for the HIV-RNA and CD4 count determinations. The missing data points were not missing at random, but represented tests not done for very specific clinical reasons. In the case of the CD4 count test, this was not

done at time points between six monthly visits, as this value can be expected to vary very little in between visits. A commonly used approach was applied here by single imputation with the average of the preceding and following value. There were 27 such single imputations done of the total of 1111 for this variable, which is further discussed in the *Results* section.

HIV-RNA plasma level determination presents a unique type of variable. In the time before cART is started, it behaves as a non-time dependent variable, with little log-variation. In clinical medicine this is called the viral set point. A HIV-RNA determination was therefore in our study done only once, prior to initiation of cART, and an assumption could be made that it would remain at viral set point value until the cART initiation date. Furthermore, this variable starts behaving like a time-dependent variable after cART is initiated, with a dramatic decrease in plasma HIV-RNA level which is only sustained for as long as the subject remains treatment adherent and the virus susceptible to the regimen. An HIV-RNA level was thus not done on days of supplemental cervical HPV sampling if the study subject remained on cART and remained adherent to the regimen. If the subject remained adherent between scheduled visits and the HIV-RNA level at the previous as well as the following visit was below the level of detection, a very strong assumption could be made that at the time point between the visits, the HIV-RNA level would also be undetectably low. Lastly, if the cART was interrupted for more than 30 days, another strong assumption could be made that the HIV-RNA level would have returned to the viral set point.

The approach of blindly excluding observation sets with missing HIV-RNA levels would be highly inappropriate in this setting, as it would omit most observation sets of study subjects who have not started cART, leading to serious bias and loss of statistical power. We therefore performed single imputations for missing HIV-RNA levels in the following manner:

1. If the study subject had not started on cART, an assumption was made that the HIV-level remained at viral set point.
2. If the study subject had an undetectable HIV-RNA level at the visit before and after the visit with the missing HIV-RNA level, and remained adherent to the regimen throughout the time period in between, it was assumed that that HIV-RNA level was also undetectable.

3. If a study subject interrupted cART for more than 30 days prior to the missing data point, it was assumed that the HIV-RNA level had returned to viral set point.
4. If none of the above could be applied, the observation data set was excluded from the analysis.

There are other options for dealing with missing data points available in statistical software. Most have to be used with caution, especially in the case of data ***not*** missing at random, as in this study. The most commonly used is that of multiple imputation. This method (in Stata the **mi** command) calculates and imputes values for the missing observations – the process is usually repeated 3-5 times, resulting in as many data sets. The desired analysis is then performed on each set, and the average of the parameter estimates with standard errors calculated. Of note, it is important to also include in the MI calculation all the variables that influence the imputed values (130). In the background of the systematic and intentional pattern of missing values in only two variables, namely that of CD4 count and HIV-RNA levels for HPV samples that were taken between scheduled dispensing visits, it was decided that substituted calculated values based on average predictors would be inferior to the expected value at that point- and concluded that single imputations that fit the rules described a nearest to accurate reflection of the maximum likelihood of those values.

Results

From November 2009 to October 2011, a total of 304 participants were enrolled to the study, of whom two were excluded from the analysis as having exceeded 30 days of cART exposure prior to enrolment (figure 13). One patient was consented but withdrew consent immediately after colposcopy evaluation. In total, 1115 HPV DNA specimens were received from 301 patients of which four (4) no DNA could be extracted, including one patient who, having presented only the one time for evaluation, was then subsequently not included in the analysis. We therefore had 1111 specimens from 300 women which could be tested for HPV DNA.

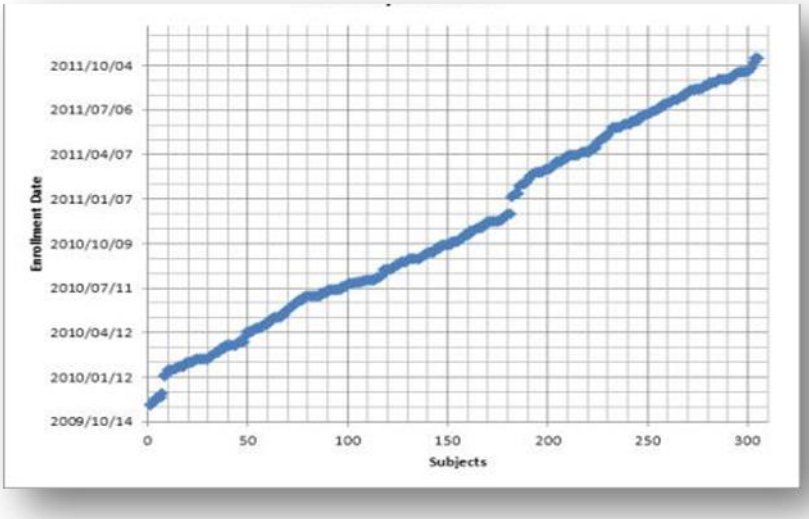


Figure 13 Prospective Study enrolment

Single imputation of missing values for CD4 counts and HIV-RNA levels were then performed, according to the rules stipulated in "Methods-Statistical Analysis-Missing Data". For missing CD4 count values, 27 values were imputed, which represents 2.4% of the 1111 data sets. The mean±SD of the imputed values were 360±147 (*n=27*) compared to the 371±199 (*n=1084*) for non-imputed data.

For missing HIV-RNA levels, applying the rules stipulated, 261 single imputations for subjects not on cART, 22 for subjects that had interrupted cART longer than 30 days, and 27 imputations for subjects that were adherent to cART together with undetectable HIV-RNA levels at the preceding as well as at the following visit, were done. Ten (10) data sets were excluded because no assumption of value could be made, leaving 1101 data sets included in the analysis. The mean±SD of the imputed log values were 3.77±1.28 (*n=310*) compared to the 2.61±1.83 (*n=791*) for non-imputed data. The higher mean of the imputed viral loads reflects the fact that most imputations were done for patients that had not yet initiated cART, and that imputing population-based cohort averages would have been inappropriate. Due to the clinical nature of HIV infection, the expected viral set point variation in each study subject is small, whereas large variations exist between subjects. Indeed, in our cohort, the viral set point varied from 29 copies/ml to 7.2 million copies/ml for the 290 out of the 300 women who had a viral load test done before initiating cART. Furthermore, conditions that could have caused a small variation in the viral set point, such as an AIDS-related illness, would

have been unlikely in the women who did not need to initiate cART. We were therefore confident that our method for patient-based imputation was superior to averaged inputs, and we avoided serious bias based on exclusion of pre-cART- initiated visits.

We analysed the quality of Pap smears throughout the study period and found that overall, the percentage of smears that did not contain endocervical cells, were 11% of the total of 1458 cytology results. The quality of smears at the start of the study was similar to that seen with our previous analysis of LSIL and HSIL (Chapters 2 and 3) for HIV infected women, at 17%, and as study site personnel performed the follow-up Pap smears, the incomplete smears dropped to below 10% (figure 14).

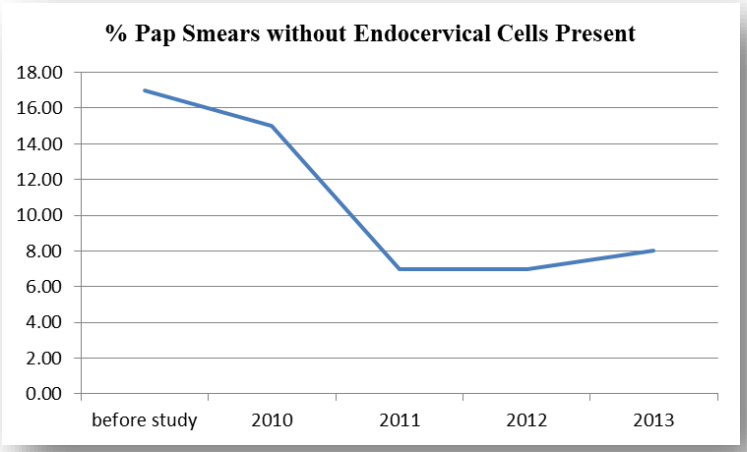


Figure 14 Percentage of Pap Smears reported as no endocervical cells present

A biopsy from a single site can underreport the severity of a lesion (131, 132). Standard procedure was however used at Colposcopy clinic which was to take diagnostic specimens from more than one area on the cervix to improve sensitivity of the test. We were therefore confident that the sensitivity of diagnostic biopsy was high enough to consider it of overriding diagnosis in determining the cytopathology status of a patient. For this analysis, a combination of Cytology and Pathology results were used, but Pathology that was performed on the same date, was used in the case of differing results.

Patient Demographics

We compared certain characteristics of women who were initiated on cART with those who were not initiated on cART, as to describe the differences in risk factors in our study cohort (Table 16). The differences that were found reflect the design of the study and the participants included. These include the reasons why cART was *not initiated in the untreated group*- a higher CD4 count at study enrolment and the lower viral set point. Besides being younger, the time from coitarche to study enrolment was also significantly shorter. The shorter follow-up time in the non-cART treated women may be another reason why cART was not started as these women possibly moved to another cART site to start their treatment.

Other factors were more equally represented. The women not initiated on cART had a slightly lower history of smoking and alcohol use, and fewer reported abstinence from sexual intercourse at study baseline. Excision treatment during study follow-up was equal both in proportion, completeness and indication. Only one patient underwent cold-knife cone (CKC) excision treatment; the distinction as to the type of excision treatment was thus not included in the analyses of factors influencing cervical HPV infection.

Exposure to cART was a sum of 98442 days for the 204 study subjects that were initiated on cART. All patients treated were initiated on standard regimen of non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) -containing regimen. Only seven patients were exposed to a Lopinavir-containing regimen for a total of 2435 days during the study, which represents 2.48% of the total time of regimen exposure. The type of cART regimen was therefore not included in analysis.

Table 16 Demographic and Clinical Characteristics: Prospective Study

		Total	ART n (%)	No ART n(%)	p-value[1]
Age	(mean,SD)	**300**	35·9±9·26	31·4±7·75	<0·001
Age study entry		**300**	**204 (100)**	**96 (100)**	<0·001
(years)	15-24	27	11 (5·4)	16 (16·7)	
	25-29	79	47 (23·0)	32 (33·3)	
	30-34	68	45 (22·1)	23 (24·0)	
	35-39	46	34 (16·7)	12 (12·5)	
	40-44	37	30 (14·7)	7 (7·3)	
	≥45	43	37 (18·1)	6 (6·3)	
Ethnicity		**300**	**204 (100)**	**96 (100)**	
	Black	156	94 (46·1)	62 (64·6)	0·01
	Mixed Race	143	109 (53·4)	34 (35·4)	
	Indian	1	1 (0·5)	0 (0·0)	
Time Coitarche to Enrolment (Sexual Age)	(mean,SD)	**286**	18·0±8·99	14·5±7·06	<0·001
Follow-up Time	(mean,SD)	**300**	578±154.9	367 ±314.1	<0·001
Life Time Sexual Partners		**300**	**204 (100)**	**96 (100)**	0·92
	1-3	195	132 (64·7)	63 (65·6)	
	4-6	85	59 (28·9)	26 (27·1)	
	>6	20	13 (6·4)	7 (7·3)	
Partner Status		**300**	**204 (100)**	**96 (100)**	0·04
	Married/partner	217	140 (68·6)	77 (80·2)	
	Abstinence>1 year	83	64 (32·4)	19 (19·8)	
Ever Smoker		**300**	**204 (100)**	**96 (100)**	0·005
	Ongoing	79	59 (28·9)	20 (20·8)	
	Previous	32	28 (13·7)	4 (4·2)	
	Never	189	117 (57·4)	72 (75·0)	
Ever Alcohol Use		**300**	**204 (100)**	**96 (100)**	0·04
	Ongoing	36	21 (10·3)	15 (15·6)	
	Previous	105	81 (39·7)	24 (25·0)	
	Never	159	102 (50·0)	57 (59·4)	
Ever Substance Abuse		**300**	**204 (100)**	**96 (100)**	0·24
	Ongoing	2	2 (1·0)	0 (0·0)	
	Previous	25	20 (9·8)	5 (5·2)	
	Never	273	182 (89·2)	91 (94·8)	
Excision treatment prior to study enrolment		**300**	**204 (100)**	**96 (100)**	0·14
	Yes	26	21 (10·3)	5 (5·2)	
	No	274	183 (89·7)	91 (94·8)	
Excision treatment prior to enrolment Result		**26**	**21 (100)**	**5 (100)**	0·74
	Normal	3	2 (9·5)	1 (20·0)	
	LSIL	4	3 (14·3)	1 (20·0)	
	HSIL	19	16 (76·2)	3 (60·0)	
Cytopathological diagnosis study enrolment		**300**	**204 (100)**	**96 (100)**	
	Normal	98	84 (41·2)	14 (14·6)	<0·001
	LSIL	143	87 (42·7)	56 (58·3)	
	HSIL	59	33 (16·2)	26 (27·1)	
Cytopathological diagnosis ART initiation		**204**	**204 (100)**		
	Normal	96	96 (47·1)	**NA**	**NA**
	LSIL	75	75 (36·8)		
	HSIL	33	33 (16·2)		
Excision Treatment on Study		**300**	**204 (100)**	**96 (100)**	
	Performed	91	64 (31·4)	27 (28·1)	0·57
	Not Performed	209	140 (68·6)	69 (71·9)	
Excision Treatment on Study Result		**91**	**64 (100)**	**27 (100)**	
	Normal	3	3 (4·7)	0 (0·0)	0·25
	LSIL	28	17 (26·6)	11 (40·7)	
	HSIL	60	44 (68·8)	16 (59·3)	
Excision treatment on Study[5]		**91**	**64 (100)**	**27 (100)**	
Completeness	Complete	35	21 (32·8)	14 (51·9)	0·37
	Incomplete	33	24 (37·5)	8 (29·6)	
	Not Applicable[6]	4	4 (6·3)	0 (0·0)	

	Not Stated	6	5 (7·8)	1 (3·7)	
	Cannot be determined	15	10 (15·6)	4 (14·8)	
CD4 count		**300**	**204**	**96**	
	at study enrolment[9]	**300**	209±128·0	398±224·3	<0·001
	at ART initiation	**204**	194±117·0	NA	NA
CD4 at study enrolment		**300**	**204 (100)**	**96 (100)**	
	<200	140	117 (57·4)	23 (24·0)	<0·001
	200-350	80	61 (29·9)	19 (19·8)	
	≥350	80	26 (12·8)	54 (56·3)	
CD4 at cART initiation		**204**	**204 (100)**		
	<200	125	125 (61·3)	NA	
	200-350	61	61 (29·9)		
	≥350	18	18 (8·8)		
Study Enrolment HIV-RNA		**300**	**204 (100)**	**96 (100)**	
	<5,000	43	19 (9·3)	24 (25·0)	
	5,000-50,000	97	62 (30·4)	35 (36·5)	
	50,000-100,000	32	23 (11·3)	9 (9·4)	<0·001
	100,000-500,000	79	65 (31·9)	14 (14·6)	
	≥500,000	39	30 (14·7)	9 (9·4)	
	ND	10	5 (2·5)	5 (5·2)	
Study Enrolment Log HIV-RNA	(mean,SD)	**290**	4·8±0·95	4·3±1·08	<0·001
Days cART	(mean,SD)	**300**	482 ±199.5	N/A	

Frequency of Detection of HPV genotypes

Of a total of 40737 HPV genotype observations, 1101 for each genotype, there were 4455 HPV positive detections. The most frequently detected HPV genotype was HPV84, followed by HPV62 and HPV16. There were fewer than twenty detections of HPV40 and HPV64 (Figure 15).

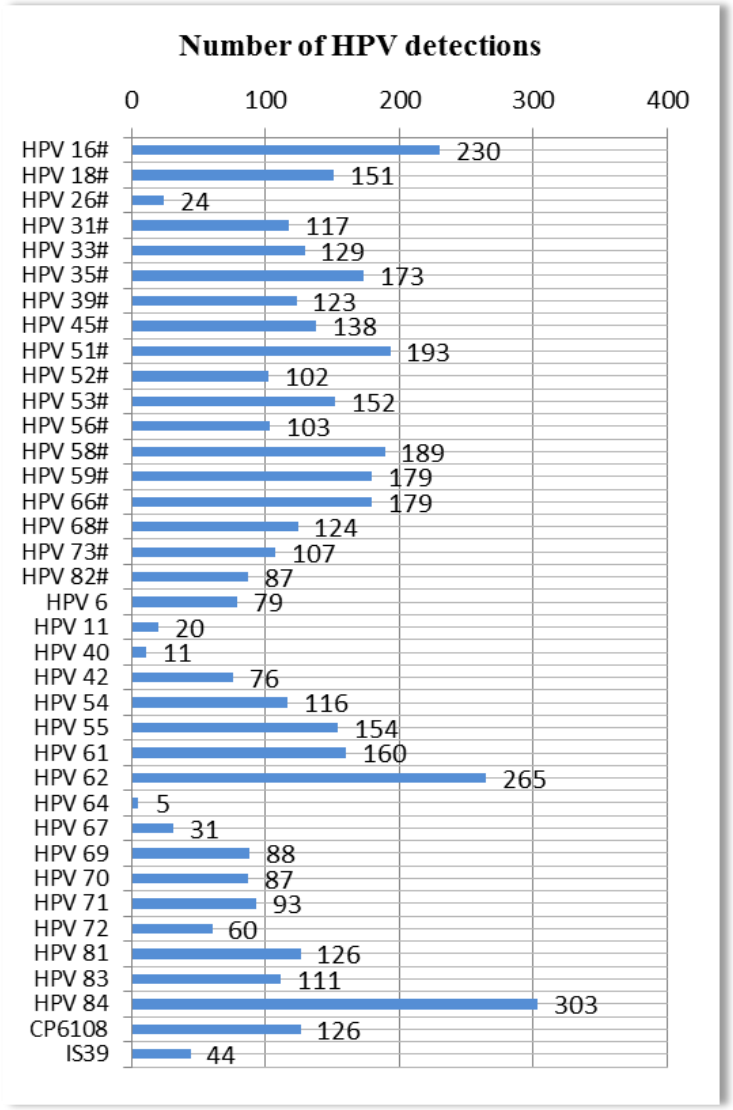


Figure 15 Total Number of HPV Genotype Detections During Follow-up Time

HPV Prevalence in Study Subjects

The prevalence of HPV types was calculated as the number of study subjects that *ever* had a positive detection of the subtype during the time of follow-up, as depicted in table 17.

We tested 1101 samples for each of the 37 HPV genotypes. The most frequent oncogenic type was HPV16. Any High-Risk Type was detected at least once in 94.3% of patients during follow-up time. Only 18 (6.0%) of study participants had only one HR type, 115 (38.3%) had between two to four HR types, 25 (8,3%) women had ten or more HR types. Of the LR-types, the most common was HPV84, -62 and -66 (Table 17).

Table 17 HPV types Most Detected Per Patient during follow-up time

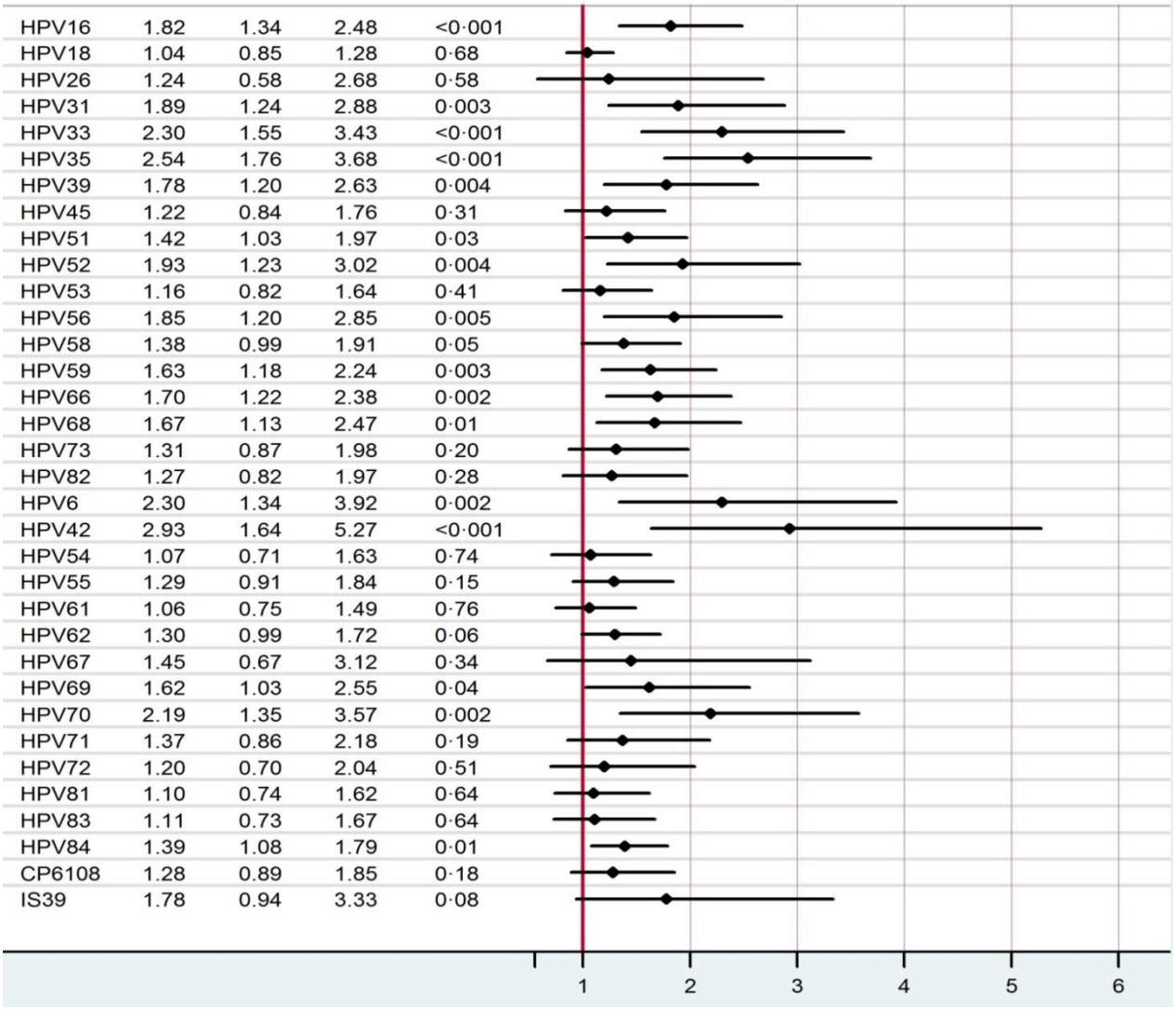
HR-HPV type	Number	Percentage of Patients	LR-HPV type	Number	Percentage of Patients
HPV 16	127	42.3%	HPV 84	183	61.0%
HPV 51	112	37.3%	HPV 62	145	48.3%
HPV 59	111	37.0%	HPV 66	106	35.3%
HPV 58	108	36.0%	HPV 53	92	30.7%
HPV 35	97	32.3%	HPV 61	90	30.0%
HPV 39	83	27.7%	CP6108	85	28.3%
HPV 33	81	27.0%	HPV 55	84	28.0%
HPV 68	81	27.0%	HPV 69	69	23.0%
HPV 18	79	26.3%	HPV 81	67	22.3%
HPV 45	76	25.3%	HPV 73	66	22.0%
HPV 31	69	23.0%	HPV 82	65	21.7%
HPV 56	65	21.7%	HPV 54	59	19.7%
HPV 52	57	19.0%	HPV 83	58	19.3%
HPV 82	65	21.7%	HPV 70	52	17.3%
HPV26	21	7.0%	HPV 71	50	16.7%
			HPV 6	43	14.3%
			HPV 42	40	13.3%
			HPV 72	39	13.0%
		Any HR risk HPV Type Detected		**287**	**94.3%**
		Any Low Risk HPV Type Detected		**284**	**94.7%**
		No HPV Detected		**2**	**0.7%**
		HPV 16 and/or HPV 18		**160**	**53.3%**
		HPV 16 and HPV 18		**46**	**15.3%**
		Only one High-Risk type detected		**18**	**6.0%**
		High-Risk types- two to four		**115**	**38.3%**
		High-Risk Types- five to nine		**129**	**43.0%**
		High-Risk Types -ten or more		**25**	**8.3%**

Association of HPV subtype detection with Cytopathology Results

Using a GEE population averaged model to test the association of any abnormal cytopathology result (compared to normal) separately for each HPV genotype, we found that HPV 16, -31, 33, -35 and -39 were significantly linked to abnormalities. Of the non-oncogenic types, HPV 6 *(OR 2.30, 95% CI 1.34-3.92, p=0.002)*, HPV42 *(OR 2.93, 95% CI 1.64-5.27, p<0.001)*, HPV70 *(OR 2.19, 95% CI 1.35-3.57, p=0.002)* and HPV84 (*OR 1.39, 95% CI 1.08-1.79, p=0.01)* were significantly associated with abnormal cytopathology (Figure 16).

Figure 16 Independent Association of all HPV types with Abnormal Cytopathology (Unadjusted)

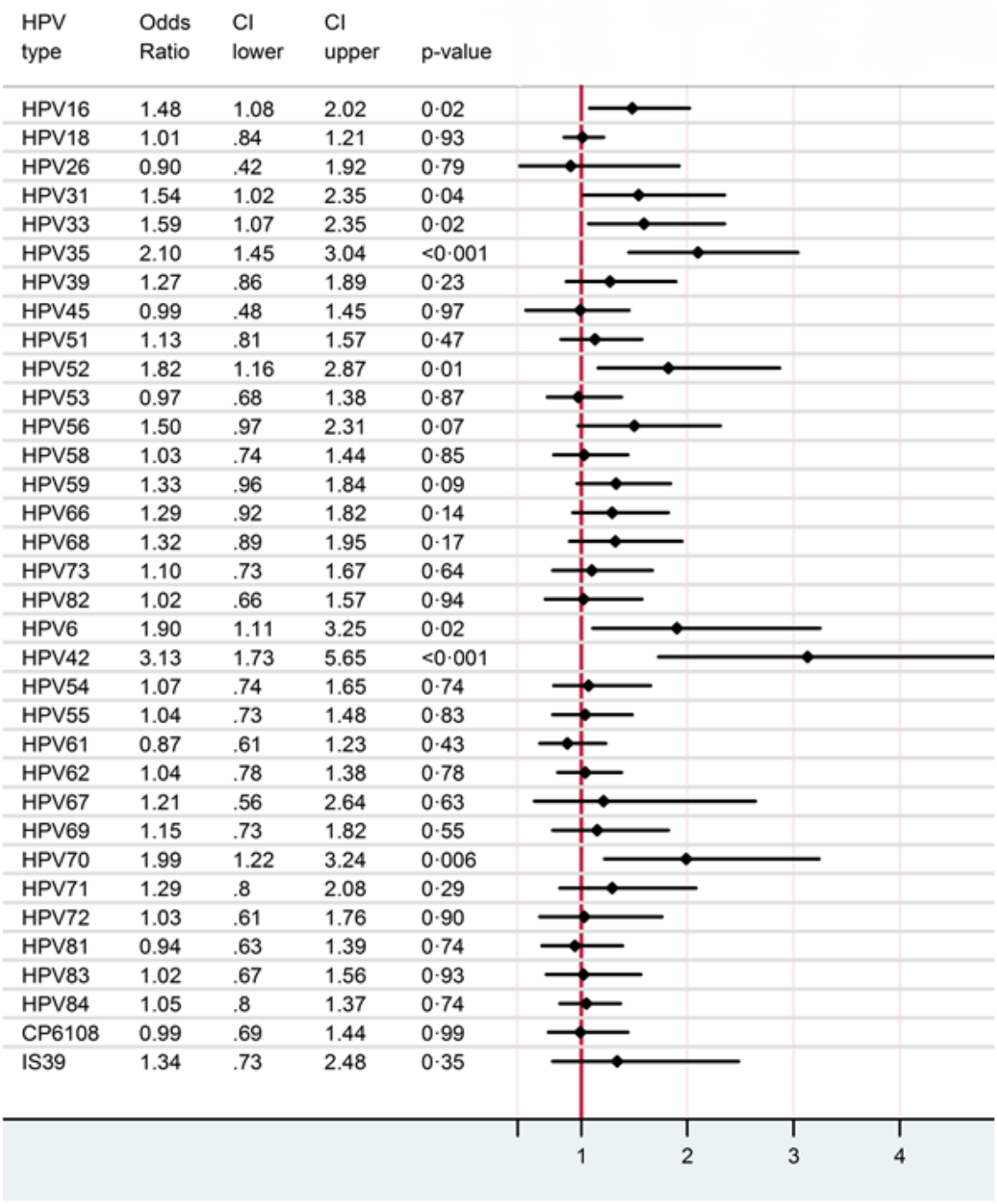
HPV type	Odds Ratio	CI lower	CI upper	p-value
HPV16	1.82	1.34	2.48	<0·001
HPV18	1.04	0.85	1.28	0·68
HPV26	1.24	0.58	2.68	0·58
HPV31	1.89	1.24	2.88	0·003
HPV33	2.30	1.55	3.43	<0·001
HPV35	2.54	1.76	3.68	<0·001
HPV39	1.78	1.20	2.63	0·004
HPV45	1.22	0.84	1.76	0·31
HPV51	1.42	1.03	1.97	0·03
HPV52	1.93	1.23	3.02	0·004
HPV53	1.16	0.82	1.64	0·41
HPV56	1.85	1.20	2.85	0·005
HPV58	1.38	0.99	1.91	0·05
HPV59	1.63	1.18	2.24	0·003
HPV66	1.70	1.22	2.38	0·002
HPV68	1.67	1.13	2.47	0·01
HPV73	1.31	0.87	1.98	0·20
HPV82	1.27	0.82	1.97	0·28
HPV6	2.30	1.34	3.92	0·002
HPV42	2.93	1.64	5.27	<0·001
HPV54	1.07	0.71	1.63	0·74
HPV55	1.29	0.91	1.84	0·15
HPV61	1.06	0.75	1.49	0·76
HPV62	1.30	0.99	1.72	0·06
HPV67	1.45	0.67	3.12	0·34
HPV69	1.62	1.03	2.55	0·04
HPV70	2.19	1.35	3.57	0·002
HPV71	1.37	0.86	2.18	0·19
HPV72	1.20	0.70	2.04	0·51
HPV81	1.10	0.74	1.62	0·64
HPV83	1.11	0.73	1.67	0·64
HPV84	1.39	1.08	1.79	0·01
CP6108	1.28	0.89	1.85	0·18
IS39	1.78	0.94	3.33	0·08



Result for HPV11 not shown, 95% CI too wide

When adjusting for age, CD4 count, days on cART and days since excision treatment, the association remained, but the time since excision was performed also had a strongly significant contribution. The time on cART and CD4 count did not (Figure 17).

Figure 17 Independent Association of all HPV types with Abnormal Cytopathology (Adjusted)

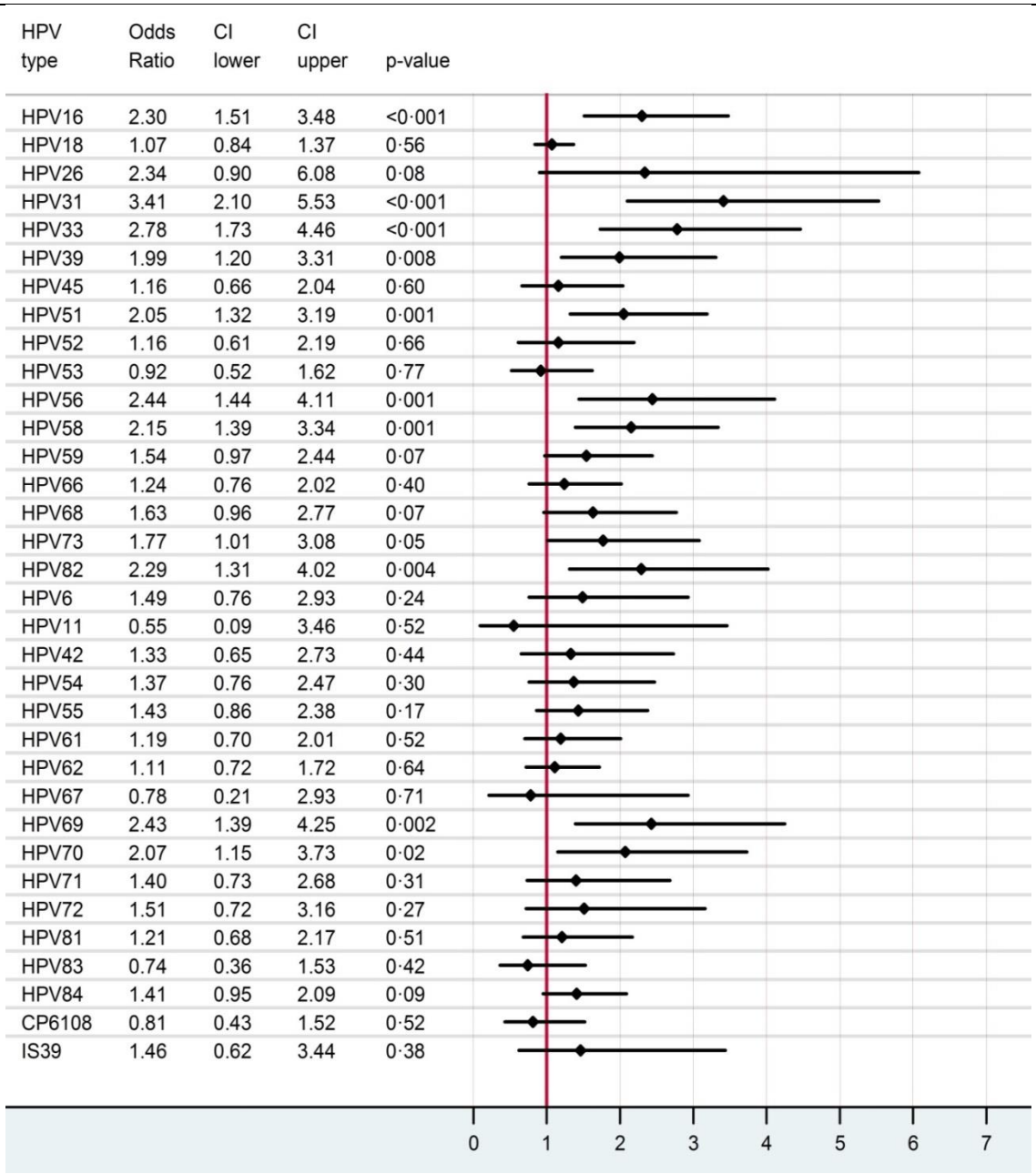


Result for HPV11 not shown, 95% CI too wide

Association of HPV subtype detection with HSIL

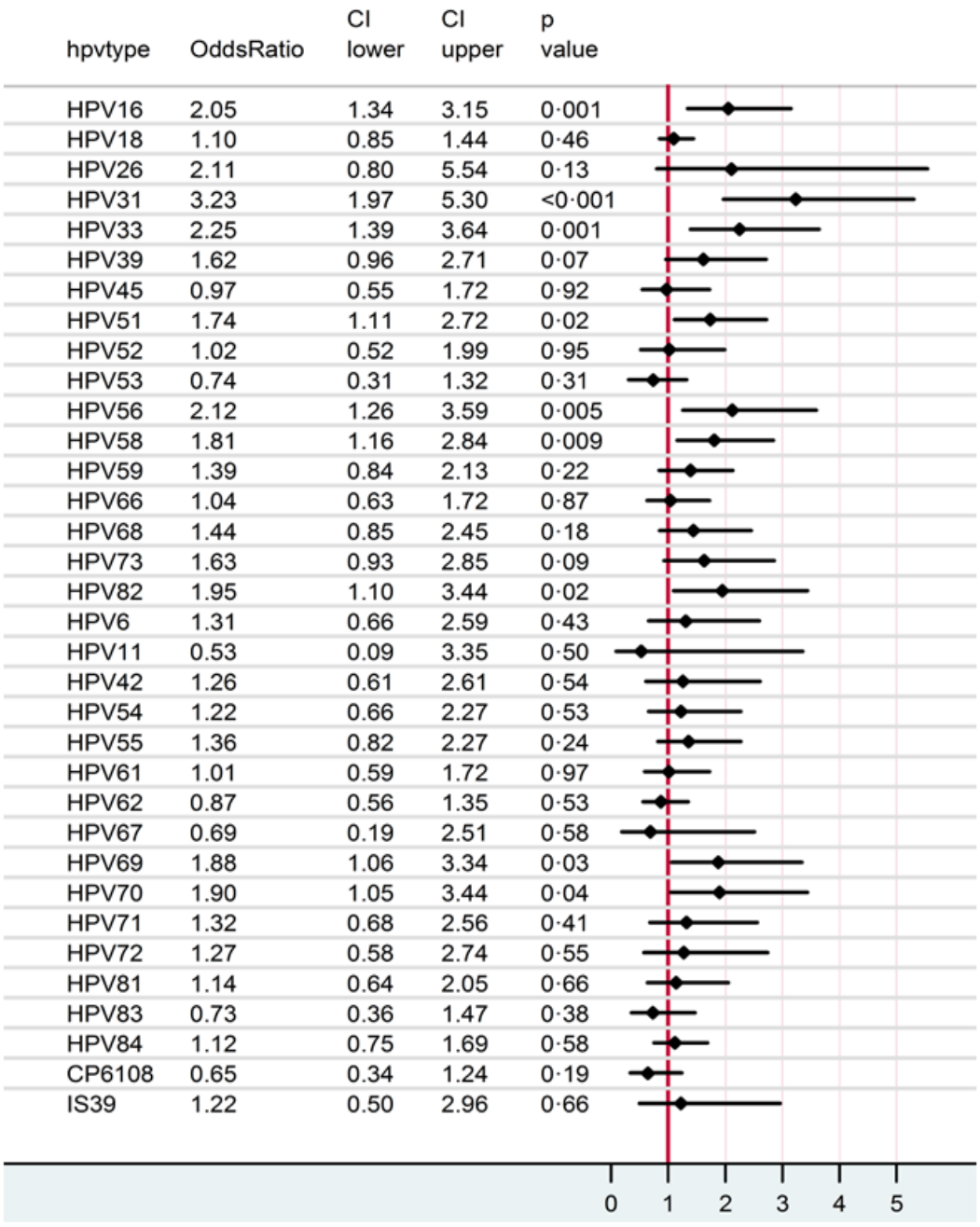
Again using a GEE population averaged model to test the association of a HSIL cytopathology result (compared to LSIL or normal combined) separately for each HPV genotype, we found that HPV 16, -31, 33, -35, -39, -56, -58, -59 and -82 were significantly linked to HSIL. Of the non-oncogenic types, HPV 69, and -70 were significantly associated with HSIL (Figure 18).

Figure 18 Independent Association each HPV type with HSIL, unadjusted



When adjusting for age, CD4 count, and days on cART, the association remained. The days since excision was not included, as this did not reach convergence: too few patients developed HSIL after having had excision treatment. The time on cART was significantly associated for each genotype except HPV16. The association with CD4 count was not significant (Figure 19).

Figure 19 Independent Association each HPV type with HSIL, Adjusted



Influence of cART status on HPV infection

Association of cART treatment status with detection of all HPV subtypes

During the unadjusted GEE population-averaged model analysis of the detection of any HPV genotype, and cART status (receiving cART or not) as only predictor, the risk for detection was reduced by 47% (table 18).

Table 18 Association of cART treatment status with detection of all HPV subtypes

Variable	OR (95%CI)	p-value
Receiving ART	0·53 (0·49-0·58)	<0·001

After adjusting for the other covariates, the time since cART was first started and the CD4 count had a stronger effect. Other factors that had a significant influence were age, excision treatment and whether the patient was sexually active (table 19).

Table 19 Association of cART treatment status with detection of All HPV Infection

Receiving ART	0·95 (0·80-1·13)	0·59
Age	0·99 (0·98-0·99)	0·003
Months since cART first started	0·99 (0·98-0·99)	0·003
Days Since Excision Treatment	0·99 (0·99-0·99)	<0·001
Sexually active	1·13 (1·01-1·26)	0·03
CD4 count at visit	0·99 (0·99-0·99)	0·02
Log HIV-RNA level	0·97 (0·99-0·99)	0.12

Association of cART treatment status with detection of HPV-16 genotype

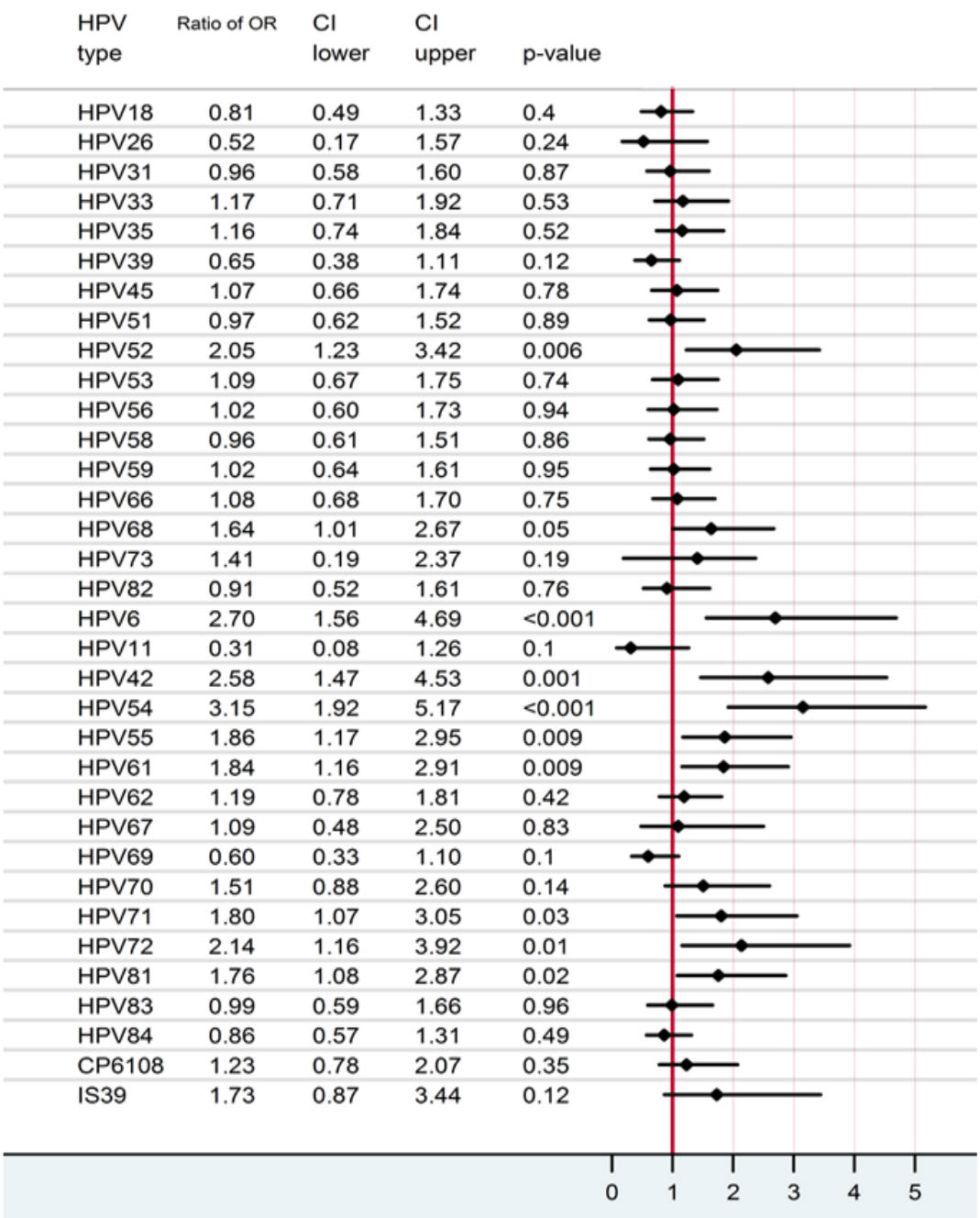
For the analysis of the association of cART with the detection of HPV16, again using cART treatment status as primary predictor, the time since cART was first started and CD4 count were stronger predictors of risk of detection. Other covariates that influenced the outcome were age, excision treatment and whether the patient was sexually active (table 20).

Table 20 Association of cART Treatment status with detection of HPV16

Receiving ART	0·99 (0·84-1·17)	0·94
Age	0·98 (0·97-0·99)	<0·001
Months since cART first started	0·93 (0·90-0·95	<0·001
Days Since Excision Treatment	0·99 (0·99-0·99)	<0·001
Sexually active	1·12 (1·01-1·24)	0·04
CD4 count at visit	0·99 (0·99-0·99)	0·001
Log HIV-RNA level	0·97 (0·94-1·01)	0.12

The influence of cART on all HPV types was expressed as the ratio of the OR of HPV16. Of the oncogenic types, only HPV52 was associated more strongly with cART treatment status than HPV16. Several non-oncogenic types were influenced more strongly by cART treatment status. No HPV genotype was influenced less than HPV16. The ratio of the OR for HPV40 and HPV 64 could not be interpreted as the number of observations was very low (Figure 20).

Figure 20 Association of cART Treatment status with detection of HPV16 compared to other HPV types



Influence of Time Since cART first started on HPV infection

Association of Time Since cART first started with detection of all HPV subtypes

During the unadjusted GEE population-averaged model analysis of the detection of any HPV genotype, the risk for detection was reduced by 6.4% for each month since cART was started for the first time (table 21).

Table 21 Association of months since cART first started with detection of all HPV subtypes

Variable	OR (95%CI)	p-value
Months since cART first started	0·94 (0·93-0·94)	<0·001

After adjusting for the other covariates, the time since cART was first started and the CD4 count still remained significant. Other factors that had a significant influence were age, excision treatment and whether the patient was sexually active (table 22).

Table 22 Association of months since cART first started with detection of all HPV subtypes

Months since cART first started	0·94 (0·94-0·95)	<0·001
Receiving ART	0·95 (0·80-1·13)	0·59
Age	0·98 (0·98-0·99)	0·003
Days Since Excision Treatment	0·99 (0·99-0·99)	<0·001
Sexually active	1·13 (1·01-1·26)	0·03
CD4 count at visit	0·99 (0·99-0·99)	0·02
Log HIV-RNA level	0·97 (0·94-1·01)	0.12

Association of Time since cART first started with detection of HPV-16 genotype

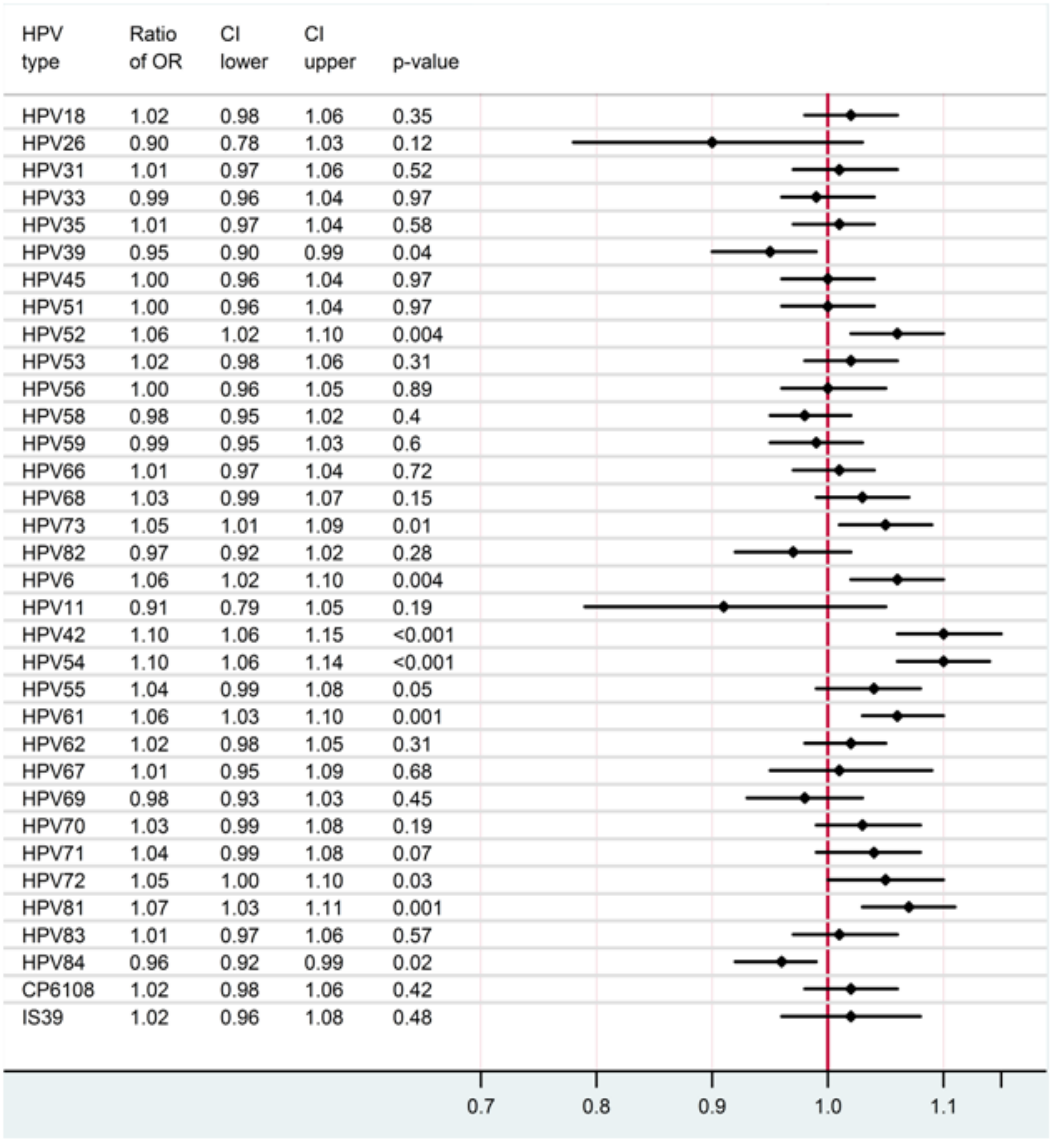
For the analysis of the association of *time since cART first started* with the detection of HPV16, again using months since the cART start date as primary predictor, the association with cART time and CD4 count was also significant. Other covariates that influenced the outcome were age, excision treatment and whether the patient was sexually active (table 23).

Table 23 Association of months since cART first started with detection of HPV16 infection

Months since cART first started	0·93 (0·90-0·95	<0·001
Receiving ART	0·99 (0·84-1·17)	0·94
Age	0·98 (0·97-0·99)	<0·001
Days Since Excision Treatment	0·99 (0·99-0·99)	<0·001
Sexually active	1·12 (1·01-1·24)	0·04
CD4 count at visit	0·99 (0·99-0·99)	0·001
Log HIV-RNA level	0·97 (0·94-1·01)	0.12

The influence of time since cART was first started (cART Time) on all HPV types was expressed as the ***ratio of the OR*** of HPV16. Of the oncogenic types, the detection of HPV52 and HPV73 was associated more strongly with cART Time than HPV16 was. Several non-oncogenic types were associated more strongly with cART time, of which HPV6. Only HPV39 and HPV84 genotypes were influenced less than HPV16. The ratio of the OR for HPV40 and HPV64 could not be interpreted, as the number of observations was very low (Figure 21).

Figure 21 Association of months since cART was first started with detection of HPV16 compared to other HPV types



The Influence of CD4 count on HPV infection

Association of CD4 count<200 with detection of all HPV subtypes

During the unadjusted GEE population-averaged model analysis of the detection of any HPV genotype, and with CD4 count less than 200 as only predictor, the risk for detection was increased by 63% (table 24).

Table 24 Association of CD4<200 with detection of all HPV subtypes

Variable	OR (95%CI)	p-value
CD4<200 at visit	1·63 (1·50-1·77)	<0·001

After adjusting for the other covariates, the time since cART was first started was stronger. Other factors that had a significant influence were age, excision treatment and whether the patient was sexually active (table 25).

Table 25 Association of CD4<200 with detection of All HPV Infection

CD4<200 at visit	1·12 (1·00-1·23)	0·06
Receiving cART	0·97 (0·82-1·15)	0·72
Age	0·99 (0·98-0·99)	0·005
Months since cART first started	0·94 (0·93-0·95)	<0·001
Days Since Excision Treatment	0·99 (0·99-0·99)	<0·001
Sexually active	1·13 (1·01-1·26)	0·03
Log HIV-RNA level	0·98 (0·94-1·01)	0.17

Association of CD4 count<200 with detection of HPV-16 genotype

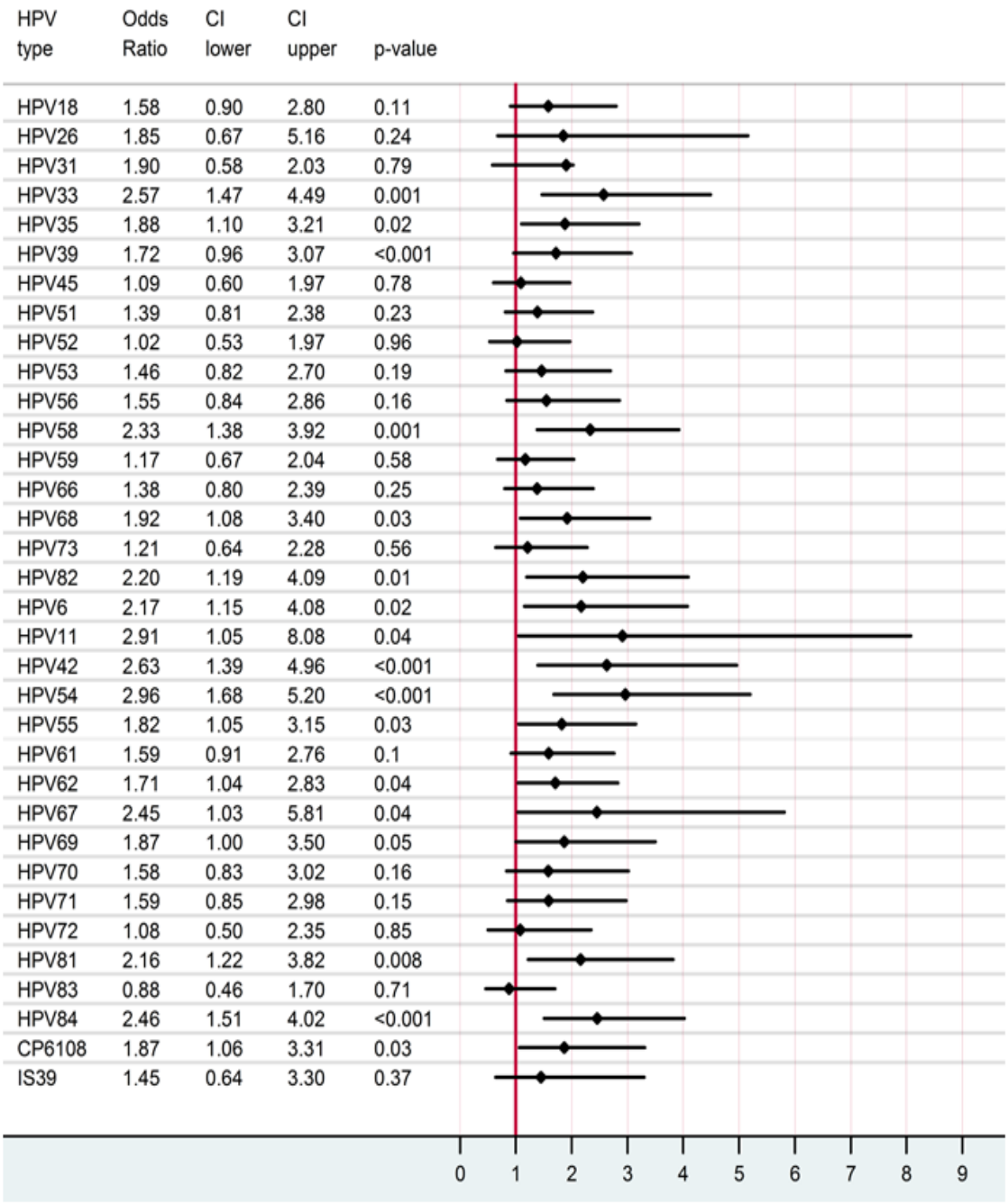
For the analysis of the association of CD4 count<200 with the detection of HPV-16, again using months since the cART start date as primary predictor, the association with the time since cART was first started was stronger. Other covariates that influenced the outcome were age, and whether the patient was sexually active (table 26).

Table 26 Association of CD4<200 with detection of HPV16 infection

CD4<200 at visit	0·69 (0·47-1·02)	0·94
Age	0·98 (0·98-0·99)	<0·001
Receiving cART	0·98 (0·83-1·16)	0·82
Months since cART first started	0·94 (0·93-0·94	<0·001
Sexually active	1·15 (1·03-1·28)	0·01
Log HIV-RNA level	0·98 (0·95-1·02)	0.32

The influence of CD4<200 on all HPV types was expressed as the ratio of the OR of HPV-16. Of the oncogenic types, HPV33,-35,-39,-58 -68 and -82, were associated more strongly with CD4<200. Several non-oncogenic types were influenced more strongly than HPV16, of which HPV6. Only HPV39 and HPV84 genotypes were influenced less than HPV16. The ratio of the OR for HPV40 and HPV64 could not be interpreted as the number of observations was very low (Figure 22).

Figure 22 Association of CD4<200 with detection of HPV16 infection, compared to other HPV types



Discussion

We know that HIV disease increases the prevalence and persistence of cervical HPV infection and resulting cervical dysplasia. Conversely, HPV infection of the cervix may increase the risk of HIV acquisition (133-138). The respective interactions are thought to be due to not only shared risk factors, but due to alteration of the systemic as well as cervical immunological milieu. In spite of registered effective vaccines for the prevention of infection with oncogenic subtypes HPV16 and -18, the effect of improved availability of HPV vaccination programmes will, in countries with highest burden of cervical cancer, not be felt for the next decade at least, as there will be no catch-up efforts attempted for adolescent girls. This is in spite of an assessment that including 12-15 year olds in a once-off effort may reduce the cumulative probability of HPV 16/18 prevalence at the age by age 35years by about 30% in medium to low-income countries (139). HIV infection, on the other hand, is a largely manageable disease, and the question remains as to why a dramatic increase or re-emergence of cervical cancer due to increasing longevity in HIV infected women has not become evident in countries with effective HIV treatment programs.

To study the early effect of initiating antiretroviral therapy on cervical HPV subtype infection, we enrolled 304 women known with HIV infection in a prospective observational study at Tygerberg hospital. To ensure that women with varying levels of immune suppression and cervical dysplasia lesions were included, women were recruited from both the Infectious Diseases Clinic (IDC) – expected to contribute women with higher chance of more severe immune suppression but possibly also women with normal Pap smears- and the Colposcopy Clinic, where women with cervical dysplasia would be seen but not necessarily with a degree of immunosuppression that would necessitate initiation of antiretroviral therapy within the following 18 months.

Decision to start cART was purely per standard protocol and not influenced by cervical pathology. The difference in ethnicity found between women who initiated and those who did not, could be explained by the referral areas for the two clinics: the IDC serves mostly areas surrounding the hospital whereas the Colposcopy referral area reaches wider to include larger neighbourhoods with more Black residents. This also may tie in with the slightly lower active smoking, alcohol and other substance use in the women who did not need to initiate cART, as

these habits tend to be less prevalent in Black women in the respective referring areas, and should not necessarily be seen as a reason for better immune status.

The results of previous investigations indicated increased prevalence and persistence of cervical dysplasia in women living with HIV infection, but that the risk for progression and persistence of cervical dysplasia may be reduced by improving immunity during treatment with HIV-suppressing antiretroviral therapy regimens.

Previous studies on the effect of antiretroviral therapy on HPV infection have, however, lacked both power and detail, and many failed to show any effect of such treatment. A possible reason for the lack in statistical power experienced could be the simplification of chosen outcomes of HPV infection, as was probably required for the statistical methods available at that time. Also, authors could have chosen to adopt the familiar terminology from cervical dysplasia research. Clinical endpoints, although easy to understand in such a setting, do not adequately entertain the statistical effect of repeated measures in a group of women. The simplification also ignores the time-dependency of factors that influence HPV detection, such as the change in CD4 count and sexual activity during follow-up. We therefore chose to apply multivariate GEE analysis using the logistic regression model with detection of HPV infection as the primary outcome. The primary predictors were chosen as cART status, time on cART and CD4 count<200 in separate analyses. Sexual activity was included as a time-dependent variable and age as a non-time-dependent variable.

We were also curious as to the possible statistical weakness created by grouping subtypes as low-risk (LR) and high risk (HR), or only HR types analysed, thereby ignoring possible differences between genotypes in the studies previously done. A concern was that the subtypes could be influenced differently by exposure to cART and immune reconstitution. We have therefore also applied multivariate GEE analysis using HPV-16 subtype detection as our main outcome, and cART as the main predictor, with other covariates the time-dependent variables CD4 count, HIV-RNA level, and days since excision treatment. Here again, age was included as a non-time-dependent variable, since the time of follow-up was relatively short. Multivariate GEE analysis incorporates a random effect to adjust not only for within-participant correlation, but can compare the HPV genotypes as a ratio of the odds ratio of as base HPV type, in our case we chose HPV16.

Due to this study design- not that of a randomized study, but of a prospective design where every woman serves as her own control, women who did not start on cART were different from those that did. Mostly, the differences reflect the more advanced HIV disease in women who needed to initiate treatment. They had lower CD4 counts and higher viral set points. This is also reflected in the significant older age and sexual age for the cART treated group, as they had possibly lived with HIV infection a longer time. Poorer health might also have reduced sexual activity at the time of entering the study for women who did need cART. It is possible that the shorter follow-up time for women who did not receive cART reflects at least some of the women who died due to complications of immunosuppression, or moved elsewhere for medical care. A further consequence of the separate Colposcopy clinic enrolment stream was that the cytopathology status at study entry was more LSIL and HSIL in the women who did not need to initiate cART, as many women who present at Colposcopy do so for evaluation of LSIL and diagnostic biopsy for exclusion of HSIL. This was not a concern, as women served as their own controls in follow-up.

There were important similarities between the women regardless of whether cART was initiated. Excision treatment, which is usually done for high-grade dysplasia, was done at the same prevalence, for the same indication and with similar completeness for the women who did initiate cART than those who did not. Although excision treatment may clear HPV infection, it does not always eradicate HPV infection and subtypes may persist afterwards, especially in immune compromised women. The women who underwent excision treatment were thus not excluded from the analysis, but we chose to retain the effect of excision treatment as a time-dependent covariate influencing the detection of HPV subtypes.

Having concluded that the decision-making towards initiation of cART was based on immune status, and that the basic protocol for excision treatment of dysplastic lesions was applied equally regardless of ART or immune status, we proceeded to examine the effect of ART on the detection of HPV types. We included in the analysis two time-dependent variables as indicators of ART administration. The first, a binary variable, was indicative of receiving cART, which was determined by self-reporting (adherence) of ART use, the other that of the viral load at the visit. The latter was important, as the HIV-RNA level, even though mostly strongly correlated to cART use, may under certain exceptional conditions not be an honest reflection of drug use, such as in resistance to cART regimen dispensed.

Because the effect of cART may be longer-lived than the actual administration status of the medication, we chose to include another time-dependent variable, time-since-cART-first started, which depicts the months from the cART initiation date. This variable would therefore be independent of cART adherence or interruptions (and the HIV-RNA level). This variable harbours a *memory effect of immune reconstitution*, maybe even on local cervical level of indicators not measured, or which may not be reflected by the CD4 count in the blood.

As described in some other centres (140), in spite of telephonic outpatient visit reminders and intensive tracing, we had some difficulty in maintaining contact with some study subjects. Besides reasons of poverty and poor health, a number of non-SA residents without the required permits were apprehensive of deportation. Failure to complete study follow-up is however not a concern in mixed model effect analyses, as all visits may be included, with no resulting bias due to lost to follow-up status. For the same reason, observations for patients who died could still be included for the visits completed before such event.

Most prospective observational studies, no matter how rigorous the data collection is, will have to deal with missing data points. In this study, all *outcome* variables (HPV results) were complete. Only two variables, namely that of CD4 count and plasma HIV-RNA level, had missing values. Because of the complete un-randomness of missing values, that is, they represent tests that were intentionally omitted at visits where there could be a very accurate prediction of what their value would be, we decided not to implement statistical methods such as multiple imputations to calculate values based on population averages. Instead, for CD4 counts, we calculated the average of the preceding and following values, a common method to deal with missing such values. For the missing viral load values, single imputations according to maximum likelihood of value were used. The viral load imputations represent fewer than 5% of the total data sets.

We tested 1101 samples for each of the 37 HPV genotypes. The most frequent oncogenic type, as found in other Southern African countries, was HPV16. High-Risk Types were found in the majority of women – 94.3% in our cohort had a HR type at some point. The frequency of HR is types is higher than reported by Firnhaber (82) in Soweto in 2010, but this may be ascribed to the fact that our study purposefully targeted HIV infected women known with cervical dysplasia from the Colposcopy clinic as part of the enrolment stream. Also, our

figures represent a period of follow-up over a year and are therefore not comparable as reflection of prevalence. Nevertheless, the 83% HR prevalence found by Firnhaber, also having used Roche Linear Array HPV genotyping, in their once-off screening at their cART treatment clinic, corresponds with our high number of HR positive results.

We found that the detection of most HPV genotypes was not specifically associated with abnormal cytopathology, except oncogenic types HPV16, -31, 33, -35 and -39 and non-oncogenic types, HPV6, -42, -70 and – 84. From this, we conclude that detection of many HPV subtypes tend to be equally likely in women with normal cytopathology results than abnormal results. This remained so when adjusted for the covariates, but excision treatment had a significant contribution in reducing this association. Similarly, the association with HSIL was not significant for the presence of most HPV subtypes, except for oncogenic types HPV16, -31, 33, -35, -39, -56, -58, -59 and -82 and non-oncogenic types, HPV69, and -70. This means that detection of most HPV types is just as likely in the presence of HSIL as in the absence thereof.

The lack of exclusive association of subtypes with cervical dysplasia, together with the presence of multiple subtypes in most women, moved us to the decision that we needed to examine the effect of cART treatment on each subtype, and not just those that can cause cancer. Most genotypes can contribute to cervical dysplasia. We did not, therefore, analyse the proportional distribution of HPV subtypes associated with lesions. Indeed, the oncogenicity of HPV genotypes been described extensively in literature. Also, the purpose of this study was not to identify which one of the multiple types detected in a patient was causing the lesion that might progress to HSIL. We were seeking to investigate the effect of antiretroviral therapy on the presence of HPV on the cervix, because it is this presence that may cause the abnormal Pap smear. This is also important on a larger scale of cervical disease burden in our HIV population in rural South Africa, where many women have limited access to colposcopy clinics and certainly no availability of HPV genotyping tests.

From our analysis we learned that cART exposure reduced the risk of detection of any HPV type by 47%. When adjusted for covariates, *time* of exposure to cART and CD4 count had a stronger effect. Every month since the treatment was first started, reduced the risk detection of any HPV type with 7%. The effect was also significant on HPV16 alone. All non-oncogenic subtypes were influenced similarly or more strongly than HPV16, as well as the

oncogenic type HPV52. Only one oncogenic subtype, HPV39, was influenced marginally less (ratio of OR 0.95, CI 0.90-0.99, *p=0.04*).

We included *age*, whether the patient was *sexually active* and *time since excision treatment* as covariates in all analyses, and as expected, the effect of all was significant. Increasing age was associated with higher risk of HPV detection, and excision treatment reduced the risk. There was a weak association with sexual status, with a marginally increased risk of HPV detection.

The effect of cART seemed to be immunology-driven. There was an increased risk for any HPV detection at CD4 count<200 (OR 1.63, 1.50-1.77), but when adjusted, the time of cART exposure again remained the strongest predictor of risk. There was a weak association of HPV-16 with very low CD4 count, with no other HPV genotype significantly less influenced and several types more strongly associated with CD4 count<200.

Interestingly, there was a lack of association of HPV detection with HIV-RNA level in our adjusted models. This also supports the stronger effect of *time of cART exposure* than *cART status* at a particular observation point, with the *cART exposure time* as a mirror of immune reconstitution and *cART status* reflected by the *HIV-RNA level*.

As described earlier, we were curious to know why previous reports on the effect of cART on cervical HPV infection have been so conflicting, with several studies failing to show any effect. Firstly, we postulated that HPV subtype differences could have contributed to the confusion. It has become possible to test for an increasing number of subtypes over the past 15 years. Because we were not just interested in the prevention of cervical cancer alone, but the impact on *cervical dysplasia*, we applied the Roche Linear Array Method which can test for the largest number of HPV genotypes. We also included all subtypes separately in our analyses, and did not just analyse the HR types together as a group. From the similar impact of cART on the detection of all subtypes, we could however *exclude subtype differences of the effect of cART on HPV* as a major factor contributing to the authors' different published results.

Our ability to show the impact of cART on HPV infection, where some studies have failed, could however be ascribed to several other factors. We used robust statistical methods that

included all study visit observations, avoiding loss of valuable data and statistical power. Also, we rejected artificial clinical end points such as prevalence, incident detection and persistence in our analyses. These, although applicable and efficient enough in large cohort studies of cervical dysplasia, can be fraught with simplifications, and are not suitable for interpreting HPV results. Deciding when detection of a HPV subtype is part of prevalence, persistence or an incident detection can be subjective, and in any case ignores pre-study infection; if we do not really know whether the presence of the virus is due to reinfection or reactivation at time points during follow-up, more value would not derived from including those definitions in the analysis, however accustomed we have become to them in our research of cervical dysplasia. Using them would have led to omission of a large quantity of data points. Instead, snapshot detections of HPV presence led to full utilization of observations, which again contributed to statistical power. We therefore conclude that the combination of using repeated measurement analysis and of using the most applicable binary outcome of HPV detection as the reason for our success.

Finally, it can be said that cART reduces cervical HPV infection in a time-dependent and immunology-driven manner. In contrast, severe immunosuppression as measured by the CD4 count, is not independently associated with increased HPV detection. Because ARVs as antiviral medication have no direct anti-HPV effect, one question still remains: could residual cervical HIV replication, in spite of undetectable HIV-RNA levels in the blood, play a role? If not, we may have to assume that there are *immune deficiencies or immune reconstitution effects* occurring more on a *local cervical level*, which may not be reflected in the CD4 count, or at least not before the CD4 count dips to below 200.

Chapter 5: Cervical HIV infection and HPV detection

Background

We have previously concluded that cART reduces cervical HPV infection in a time-dependent and immunology-driven manner. Time since cART first started was found to be a stronger predictor of risk for HPV detection than the CD4 count, but even more so than the HIV RNA plasma level. We therefore hypothesized that the mechanism by which the reduced cervical HPV infection occurs is an early immune reconstitution effect in the cervical mucosa.

The life cycle of HPV is entirely intra-epithelial, and the virus can infect no other cells. Cervical infection first occurs in the basal keratinocytes via micro-abrasions. From here, the life cycles follow closely the path of the keratinocytes as they migrate to the surface epithelium, from where viral assembly occurs and mature virions are released. The HPV, however, for most of its replicative life, does not cause cell death; there is no viremia and little exposure of its antigens to start the cascade of cytokine release and adaptive immune response. This cunning ability to remain invisible to the innate immune defences is witnessed as the early persistence of the infection even in non-immunocompromised women (141). Eventually, after some months, there is evidence of an immune response against HPV. Spontaneous regression of HPV-associated lesions is seen in almost all cases. Clearance of the infection is associated with large infiltrates of T cells (both CD4+ and CD8+) and macrophages. This cell-mediated Th1 biased response is characterized by the release of pro-inflammatory cytokines IL-12, IFN-γ and TNF-α (101). In response to HPV infection, there is an *associated concentration of T-lymphocytes subsets, NK cells and macrophages in the transformation zone of the cervix*, in contrast to the normal vaginal mucosa, where there are few T cells and antigen-presenting cells (APCs) (142).

In contrast to HPV, HIV is capable of infecting more than one cell type. Physical abrasions during sexual intercourse puts the virus into close contact with susceptible cells. Virions interact and travel through the epithelium along several cellular pathways, which are not all associated with productive infection. Cervical cell types that are known to be infected by HIV are epithelial cells, CD4 T+ cells, Langerhans Cells (LCs), Dendritic Cells (DCs), macrophages and Natural Killer Cells (143-148) *(figure 23)*.

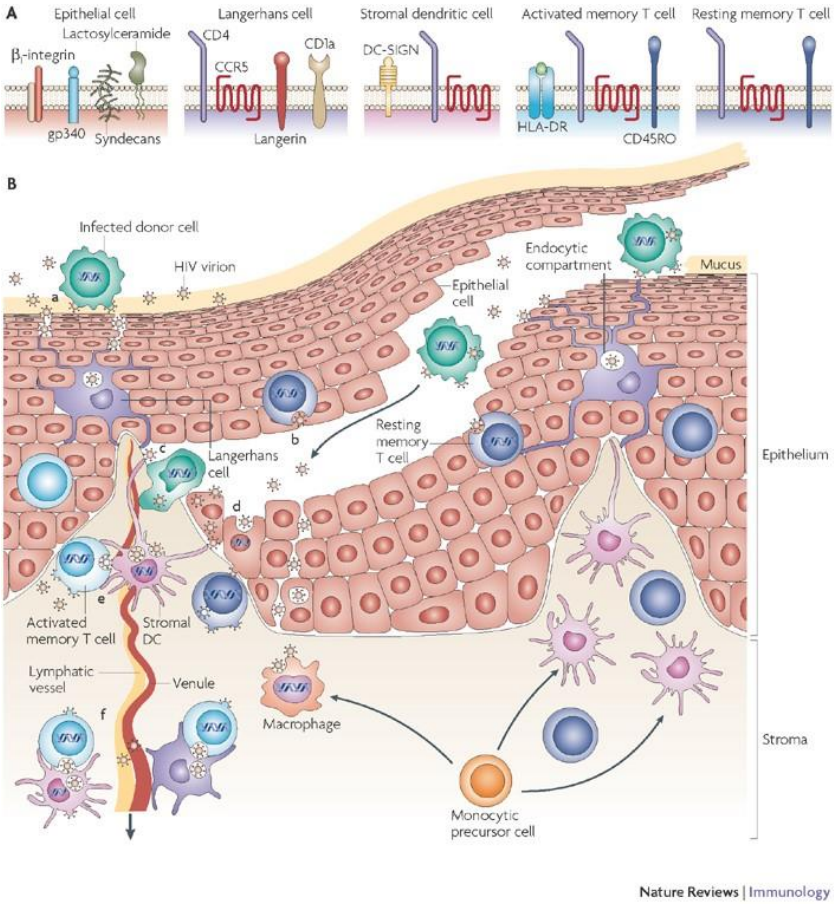


Figure 23 Early host invasion by HIV

(Reprinted with permission from (145))

In women with chronic HIV infection, measured *cervical* immune markers (CD4 cells, macrophages, Langerhans cells, neutrophils, NK cells, and expression of interferon-γ and regulatory cytokines) are lower (149-152). The decreased local cervical immunity does not appear to be influenced by the CD4 count or plasma HIV RNA load (152). There is reduced maturation of HIV-infected monocytes, which may cause decreased antigen presentation capacity and generalized lowered T Cell responses (153) at mucosal level. Even worse, it has been shown that a *subset of activated cervical CD4+ T cells*, responsible for production of IFN-γ and IL-17 normally associated with the clearance of HPV infection, is almost entirely depleted in vivo during HIV infection (154). This could explain, even though not all mechanisms have been understood, the increased persistence and multiple HPV subtype infection in the same anatomical milieu in women living with HIV/HPV co-infection. There is already evidence that cervical HPV infection may increase the risk for contracting HIV infection (155-157) due to the high density of HIV-susceptible cells in the cervix.

There seems to be a good correlation between plasma and genital HIV viral loads. An observational study that followed women after acute infection, saw both measurements decrease in parallel after the early period to a viral set point (158). Similarly, initiation of cART was found to not only cause a rapid decrease of plasma HIV-RNA levels, but also to a dramatic drop, within days (159), in HIV-RNA levels in cervicovaginal samples (160, 161), with good correlation between plasma HIV-RNA and cervical HIV-DNA (162).

Despite this, it has been shown that in as many as half of women who have reached undetectable levels of plasma HIV-RNA, detection of HIV-RNA in cervicovaginal lavage specimens was still possible (163, 164). Even more so, mutant virus that was different from the virus in the blood has been detected in the genital tract of women who were taking non-suppressive therapy (165-167), indicating ongoing viral production in a separate anatomical (i.e. cervical) compartment. Importantly, antiretroviral drug concentrations in the female genital tract have been shown to vary (168-170). Most worrying, however, are the extremely low concentrations of efavirenz reported in all studies. Almost all women enrolled to our prospective study received an efavirenz-containing regimen when initiating cART.

Few studies have been done that have examined the association between *HIV infection* and *HPV detection* in cervical samples, and these have had conflicting results (162, 171-175). It is obvious that evidence for an association between the cervical HIV-Viral Load and the presence HPV infection is lacking. From the current research related to eradication efforts of the HIV, we think that observations taken from the *blood* compartment possibly do not accurately represent what is happening in the *cervical* compartment. We therefore proceeded to perform an **ad hoc sub-analysis**, using ultrasensitive HIV DNA qPCR method, to investigate if an association between the *cervical* ***HIV proviral DNA load*** and ***HPV DNA detection*** can be identified.

Methods

Study Design, Population, Setting and Outcomes Definition

This ad hoc sub-analysis was done on the same cohort as described in Chapter 4. The prospective study had enrolled women known with HIV infection and were cART naïve or had recently (<1 month) initiated cART. Antiretroviral therapy use, CD4 count, HIV-RNA plasma level and HPV Genotyping had been recorded as described in the previous chapter.

HIV DNA Analysis

HIV DNA copy numbers were measured by the real-time quantitative PCR method (qPCR) on the DNA extracted from the cervical specimens for the HPV analysis, as previously described (176). Primers and probes were obtained from Integrated DNA Technologies (South Africa) and included parts of the HIV-1 LTR-*gag* fragment (figure 24). Briefly, The iTaq^{TM} universal probe supermix were used (Bio-Rad Laboratories, Inc.) for the PCRs. Primers were used in a final concentration of 300nM, while probes were used in a final concentration of 150nM (*CCR5: Forward ATGATTCCTGGGAGAGACGC, Probe VIC-AACACAGCCACCACCCAAGTGATCA, Reverse AGCCAGGACGGTCACCTT; HIV-1: Forward TACTGACGCTCTCGCACC, Probe FAM-CTCTCTCCTTCTAGCCTC, Reverse TCTCGACGCAGGACTCG*). The HIV-1 copy number was determined by using the pMJ4 plasmid copy number as reference (177). The cell numbers were calculated by using pc-CCR5 (obtained from the NIH AIDS reference reagent laboratory) as a reference for cellular copy number. Negative- and positive-control DNA were included in all reactions. Samples were run in duplicate on a CFX96 Touch^{TM} Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.), at 95^0C for 15 min, 40 cycles of 95^0C for 1 minute and 60^0C for 1 minute. Analyses were completed using CFX Manager^{TM} software. Data graphs and tables were exported to Microsoft Excel for further analysis. The copy numbers of each sample gene were analysed against the standard curves and the HIV DNA per 10^6 cells extrapolated as described (174). The threshold of detection of real-time PCR for HIV-1 cellular provirus DNA was 10 copy/10^6 cells, and therefore the lower limit of detection was 1.0 log10/10^6 cells.

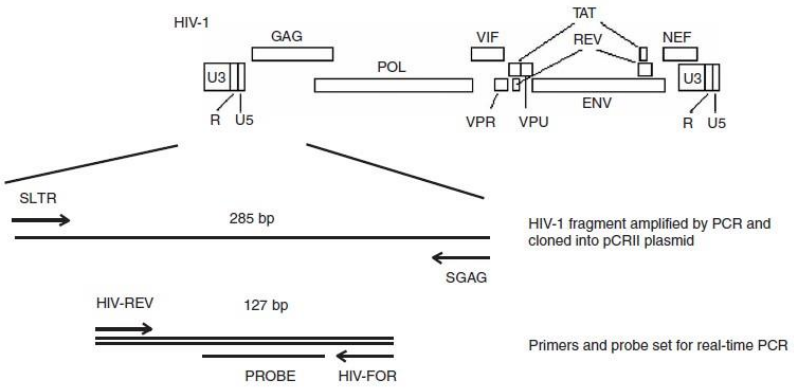


Figure 24 HIV-1 Region detected by the proviral DNA qPCR assay

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Statistical analysis

We used GEE regression analysis to determine the association between the ***cervical HIV-DNA level*** with the ***plasma HIV-RNA level*** and in a separate analysis with the ***detection of cervical infection of each HPV subtype*** respectively. All models were adjusted, using *time-dependent* variables, for the CD4+ cell count, plasma HIV-RNA level, the time since cART was first started and cART treatment status. P-Values reported were two-tailed and alpha level 5%. For the purpose of the analysis, plasma HIV-RNA levels <50 copies/ml were reported as 49 copies/ml. Only women whose cervical smears had successful qPCR HIV-DNA determination performed, were included, leaving on **290** study subjects and the final study observations at **1036**.

Results

Exploratory descriptive analysis: HIV-RNA and -DNA results

There were more than 100 observations at quarter 0, 2, and 4 after cART was first started, indicating adequate representation of ART- exposed subjects in the analysis. The large sample size taken during quarter zero represents women at baseline as well as those samples taken during the time initiators were still naïve to cART. The small sample sizes at quarter 1, 3, and 5 represent subjects that had to initiate cART between the scheduled 6 monthly study visits. Since follow-up for this study was 18 months, there are very few observations taken after quarter 6 of ART (figure 25).

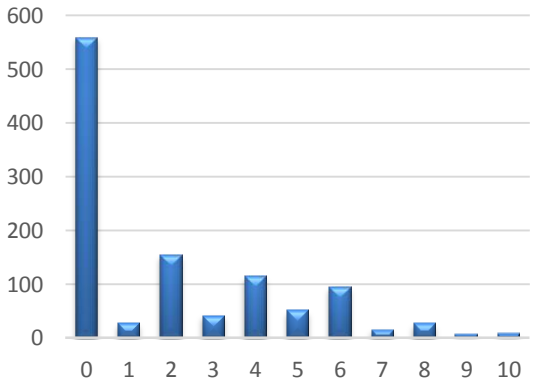


Figure 25 Number of Observations at observation points for quarters 1-10 after cART was first started.

Results plasma HIV-RNA levels

There was a rapid decrease in the log HIV-RNA level for observations for each quarter after cART was initiated (figure 26).

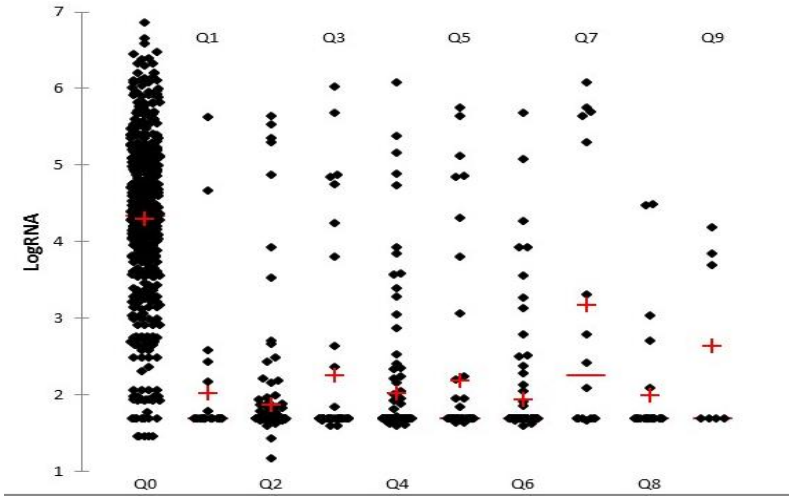


Figure 26 Log plasma HIV-RNA level at each quarter after cART first started.

Observations taken at quarter 0 had wide range of log plasma HIV-RNA determinations, with the largest share falling in the range of 4-5 log RNA copies/ml. At each quarter 1-6 after ART was first started, the % observations that obtained a log value <2.0 exceeded or was close to 80%. This indicates a maintained response for most study subjects during the period after initiation of cART. At quarters 7-10, the number of observations was smaller and this could have contributed to the aberrant distribution. Also, some of the observations quarter 7-10 were repeat samples in women who had interrupted cART or developed virological failure (figure 27).

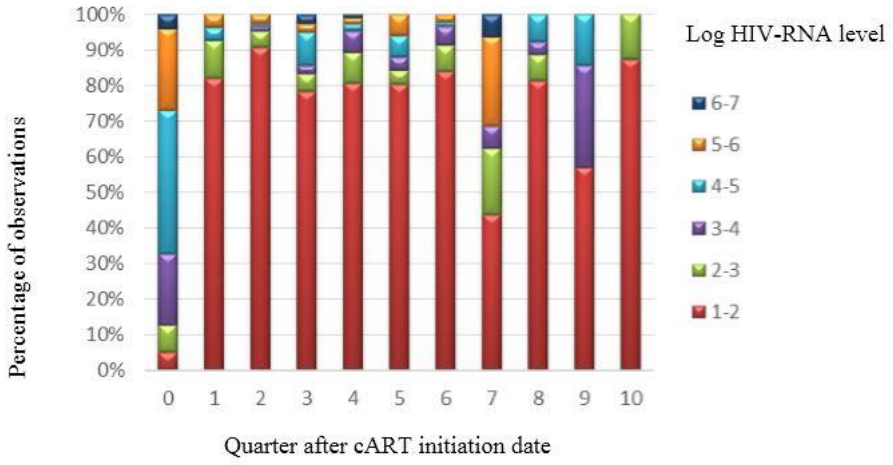


Figure 27 Distribution of Log Plasma HIV-RNA Level Results at observation points for quarters 1-10 since cART was first started.

Results: Cervical HIV-DNA pVL

There appears to be a slow decrease in Log Cervical HIV-DNA pVL at each quarter after cART initiation date, with the range of values becoming smaller over time since cART was first started (figure 28).

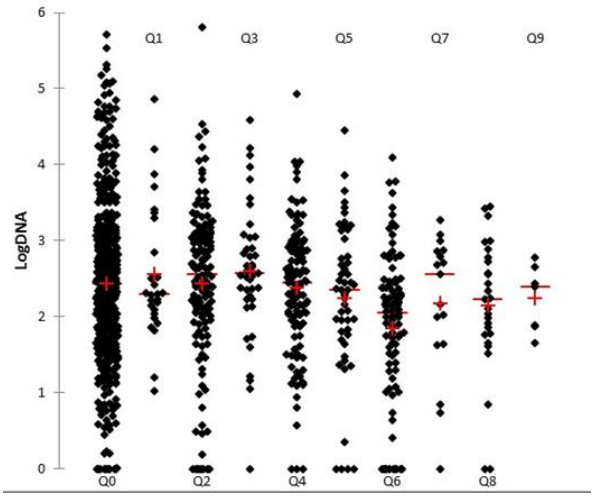


Figure 28 Cervical HIV-DNA pVL at each quarter after cART first started.

There was a loss of % extreme HIV-DNA pVL observations (log HIV-DNA >3.0) through quarter 1-6, with a decrease from approximately 30% to 10% (figure 29). The response appears slower than observed for HIV-RNA determination results (figure 27).

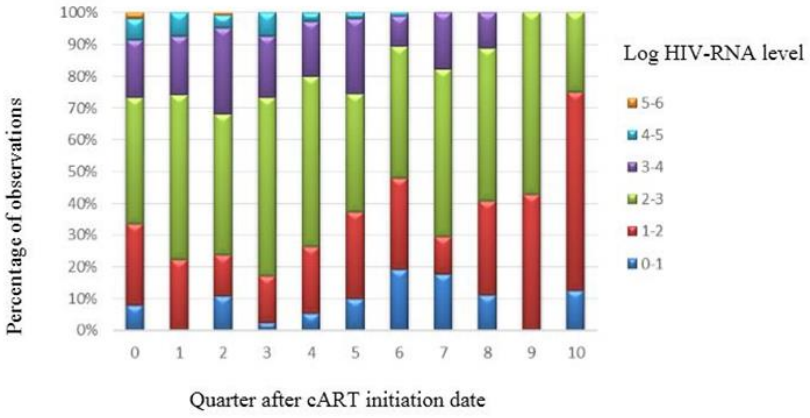


Figure 29 Distribution of Log Cervical HIV-DNA pVL at observation points for quarters 1-10 since cART was first started.

As to whether observations were done when the study subject was receiving or not *on that day*, there appears to be a dramatic difference in plasma HIV-RNA level. In contrast, there is no difference in the

cervical HIV-DNA level when comparing the observations done *on cART* with *not on ART*. The log HIV-RNA and log HIV-DNA was **4.31±1.05** and **2.43± 1.10** for observations done when not receiving cART, and **1.96±0.78** and **2.29±0.99** when receiving cART (figure 30).

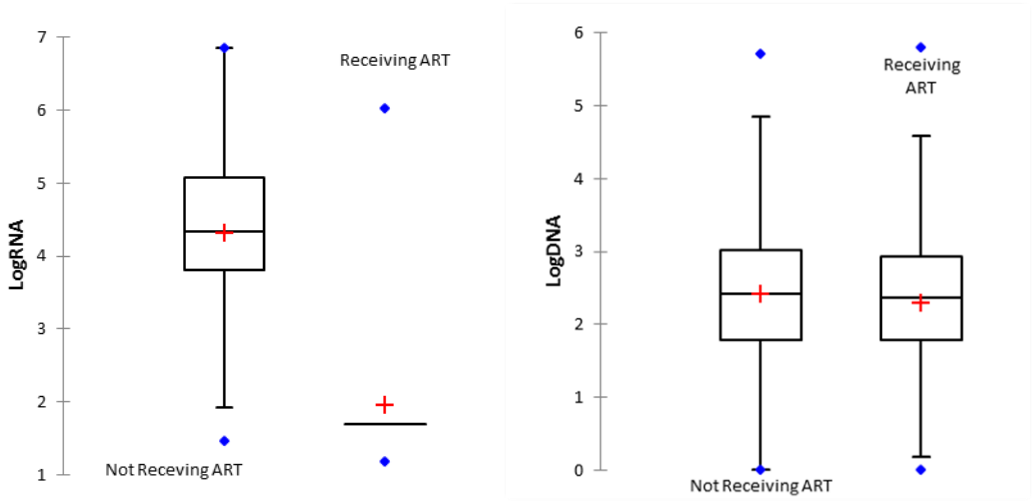
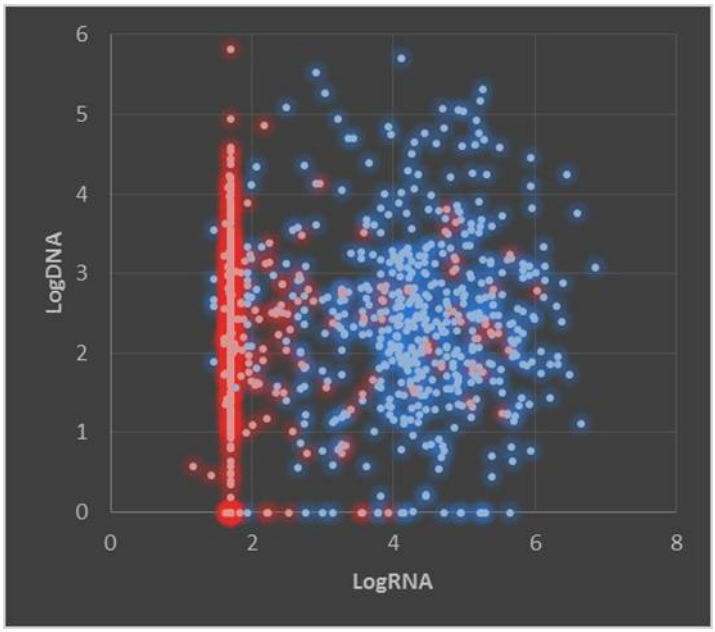


Figure 30 Effect of cART on log plasma HIV-RNA levels vs cervical HIV-DNA pVL

Correlation of Cervical HIV-DNA pVL with plasma HIV-RNA level

When using a scatterplot to compare the relationship of log cervical HIV-DNA pVLwith plasma log HIV-RNA levels at each observation, there is a wide cluster of blue points of which the majority fall within a range of log HIV-RNA 4-5, representing observations taken during ART naive time. There appears to be no monotonic relationship between the values and we therefore did not calculate a Spearman Rho value. The range of log HIV-DNA pVL for patients with plasma HIV-RNA<50 copies/ml (log=1.79) is large, indicating that a low log HIV-RNA level may coincide with from low to high cervical HIV-DNA pVL. There were twenty (20) observations with no HIV-DNA pVL detected (Figure 31). These samples were representative from different plate runs and all had tested positive on our CCR5-DNA positive control gene. Although we were uncertain as to why at these observations showed no HIV presence in spite of detectable plasma-RNA levels, we considered it possible that the samples represented an HIV subtype not detected by our HIV-DNA probe. However, after scrutinizing the samples that had cervical HIV-DNA=0 but plasma HIV-RNA> log 2, only two subjects* (no 9 and 14) had more than one sample with no HIV-DNA detected. Also, although twelve of the negative HIV-DNA pVL observations had correspondingly low plasma-RNA levels of under 20,000 copies/ml, five had higher levels. The cytopathology results were also not uniform (i.e. normal or abnormal for all). We therefore could find no pattern to explain our negative cervical HIV-DNA pVL results in these 20 observations, other than to conclude that they are very few of the total observations of 1036 (table 32).



Blue: Not receiving cART **Red:** Receiving ART

Figure 31 Correlation of visit log plasma HIV-RNA with log cervical HIV-DNA

Figure 32 Subjects with negative HIV-DNA pVL observations and corresponding detectable plasma HIV-RNA levels

Study Subject	Age	ART on study	RNA pre-ART	On ART	Days since cART started	CD4	RNA	Log RNA	DNA	Cytopathology
1	48	yes	775990	yes	491	465	159	2.20	0	Normal
2	40	yes	100000	yes	168	668	166	2.22	0	Normal
3	65	yes	846899	yes	538	147	327	2.51	0	Normal
4	21	no	573	no	0	813	573	2.76	0	LSIL
5	44	no	981	no	0	372	981	2.99	0	Normal
6	24	no	1402	no	0	785	1402	3.15	0	Normal
7	27	yes	75000	yes	175	238	3428	3.54	0	LSIL
8	41	yes	5006	yes	532	212	3659	3.56	0	Normal
9*	27	no	6100	no	0	230	6100	3.79	0	LSIL
10	30	yes	26551	yes	575	305	8411	3.92	0	Normal
11	29	no	13000	no	0	370	13000	4.11	0	Normal
12	21	no	14312	no	0	536	14312	4.16	0	ASCUS
13	45	yes	51719	no	0	169	51719	4.71	0	Normal
14*	22	no	87688	no	0	464	87688	4.94	0	HSIL
15	35	yes	170000	no	0	235	170000	5.23	0	LSIL
16	33	yes	173653	no	0	177	173653	5.24	0	Normal
17	44	yes	200000	no	168	321	200000	5.30	0	Normal
18	37	yes	339457	no	642	79	434828	5.64	0	HSIL

(had two observations)*

Association of plasma HIV-RNA level with Cervical HIV-DNA Proviral Load

During the unadjusted GEE population-averaged model analysis of the cervical log HIV-DNA pVL of >1.0, >2.0 and >3.0, there was no association with the log plasma HIV-RNA level. After adjusting for the other covariates, only the *time since cART was first started* had a significant effect, which appeared stronger for higher HIVDNA pVL (table 27-29).

Table 27 Association of plasma HIV-RNA level with Cervical HIV-DNA pVL >1.0/copies/10^6 cells

Variable	OR (95%CI)	p-value
Unadjusted		
Log plasma HIV-RNA level	1·10 (0·95-1·27)	0·22
Adjusted		
Log plasma HIV-RNA level	0·95 (0·77-1·32)	0·06
CD4 count	0·99 (0·99-1·00)	0·15
cART Status	1·33 (0·54-3·27)	0·54
Months since cART first started	0·96 (0·92-0·99)	0·04
Months Since Excision Treatment	1·00 (0·97-1·03)	0·82

Table 28 Association of plasma HIV-RNA level with Cervical HIV-DNA pVL >2.0/copies/10^6 cells

Unadjusted		
Log plasma HIV-RNA level	0·95 (0·87-1·04)	0·24
Adjusted		
Log plasma HIV-RNA level	0·90 (0·77-1·05)	0·19
CD4 count	0·99 (0·99-1·00)	0·52
cART Status	1·51 (0·88-2·60)	0·13
Months since cART first started	0·95 (0·93-0·98)	<0·001
Months Since Excision Treatment	1·00 (0·97-1·02)	0·97

Table 29 Association of plasma HIV-RNA level with Cervical HIV-DNA pVL>3.0/copies/10^6 cells

Unadjusted		
Log plasma HIV-RNA level	1·05 (0·95-1·15)	0·33
Adjusted		
Log plasma HIV-RNA level	0·96 (0·81-1·13)	0·61
CD4 count	0·99 (0·99-1·00)	0·40
cART Status	1·50 (0·84-2·70)	0·17
Months since cART first started	0·94 (0·91-0·97)	<0·001
Months Since Excision Treatment	1·98 (0·96-1·01)	0·21

Association ART Status with Cervical HIV-DNA Proviral Load

Again applying GEE analysis, now with cART status as the main predictor, time that had passed since cART was first started was the strongest predictor of risk, with a 5% reduction in risk for the cervical log HIV-DNA level to be >1.0, >2.0 or >3.0 for each month that had elapsed. Unadjusted, there was no association of cART status with cervical HIV-DNA pVL (table 30-32).

Table 30 Association of ART Status with Cervical HIV-DNA pVL>1.0/copies/10^6 cells

Variable	OR (95%CI)	p-value
Unadjusted		
cART Status	0·77 (0·50-1·19)	0·24
Adjusted		
cART Status	1·33 (0·54-1·27)	0·54
CD4 count at visit	0·99 (0·99-1·00)	0·15
Log plasma HIV-RNA level	1·01 (0·77-1·32)	0·95
Months since cART first started	0·96 (0·92-0·99)	0·04
Months Since Excision Treatment	1·00 (0·97-1·04)	0·82

Table 31 Association of ART Status with Cervical HIV-DNA pVL >2.0/copies/10^6 cells

Unadjusted		
cART Status	1·10 (0·85-1·43)	0·45
Adjusted		
cART Status	1·51 (0·88-2·60)	0·13
CD4 count at visit	0·99 (0·99-1·00)	0·52
Log plasma HIV-RNA level	0·90 (0·77-1·05)	0·19
Months since cART first started	0·95 (0·93-0·98)	<0·001
Months Since Excision Treatment	1·00 (0·98-1·02)	0·97

Table 32 Association of ART Status with Cervical HIV-DNA pVL>3.0/copies/10^6 cells

Unadjusted		
cART Status	0·83 (0·63-1·10)	0·20
Adjusted		
cART Status	1·50 (0·84-2·70)	0·17
CD4 count at visit	0·99 (0·99-1·00)	0·40
Log plasma HIV-RNA level	0·96 (0·81-1·13)	0·61
Months since cART first started	0·94 (0·91-0·97)	<0·001
Months Since Excision Treatment	0·98 (0·96-1·01)	0·21

Association Time since ART Started with Cervical HIV-DNA Proviral Load

GEE analysis was repeated, now with *Time Since cART was first started* as main predictor, and it was associated with a 4%, 5% and 6% reduction in risk for the cervical HIV-DNA pVL >1.0, >2.0 and >3,0, respectively, for each month that had elapsed since the ART initiation date. In the adjusted models, no other covariate had a significant influence (table 33-35).

Table 33 Association of Months since cART started with Cervical HIV-DNA pVL>1.0/copies/10^6 cells

Variable	OR (95%CI)	p-value
Unadjusted		
Months since cART first started	0·97 (0·95-0·99)	0·02
Adjusted		
Months since cART first started	0·96 (0·92-0·99)	0·04
CD4 count at visit	0·99 (0·99-1·00)	0·15
Log plasma HIV-RNA level	1·01 (0·77-1·32)	0·95
cART Status	1·33 (0·54-3·23)	0·54
Months Since Excision Treatment	1·00 (0·97-1·04)	0·82

Table 34 Association of Months since cART started with Cervical HIV-DNA pVL >2.0/copies/10^6 cells

Unadjusted		
Months since cART first started	0·98 (0·97-0·99)	0·04
Adjusted		
Months since cART first started	0·95 (0·93-0·98)	<0·001
CD4 count at visit	0·99 (0·99-1·00)	0·52
Log plasma HIV-RNA level	0·90 (0·77-1·05)	0·19
cART Status	1·51 (0·88-2·60)	0·13
Months Since Excision Treatment	1·00 (0·98-1·02)	0·97

Table 35 Association of Months since cART started with Cervical HIV-DNA pVL >3.0/copies/10^6 cells

Unadjusted		
Months since cART first started	0·96 (0·94-0·98)	<0·001
Adjusted		
Months since cART first started	0·94 (0·91-0·97)	<0·001
CD4 count at visit	0·99 (0·99-1·00)	0·40
Log plasma HIV-RNA level	0·96 (0·81-1·13)	0·61
cART Status	1·50 (0·84-2·70)	0·17
Months Since Excision Treatment	0·98 (0·96-1·01)	0·21

Association of Cervical HIV-DNA Proviral Load with HPV Detection

During the unadjusted GEE regression analysis of the association of the cervical log HIV-DNA pVL with HPV detection, there was an increased risk for the detection of any HPV subtype with higher HIV-DNA pVL. The unadjusted association was also significant for the detection of HPV 16 and/or HPV 18, but less so for any HR HPV. The association remained significant when adjusting for the other covariates, but not for HR HPV. There was also a significant association with the time that had elapsed since cART was first started. For HR HPV and HPV 16/18, excision treatment was associated with reduced risk of detection. The plasma HIV-RNA level was not a predictor of risk (table 36-38).

Table 36 Association of Cervical HIV-DNA pVL with Detection of Any HPV Type

Variable	OR (95%CI)	p-value
Unadjusted		
Log Cervical HIV-DNA pVL	1·42 (1·16-1·71)	<0·001
Adjusted		
Log Cervical HIV-DNA pVL	1·30 (1·08-1·57)	0·007
CD4 count at visit	0·99 (0·99-1·00)	0·19
Log plasma HIV-RNA level	1·05 (0·80-1·38)	0·71
cART Status	0·49 (0·20-1·19)	0·11
Months Since ART First Started	0·95 (0·93-0·98)	0·003
Months Since Excision Treatment	0·98 (0·96-1·01)	0·21

Table 37 Association of Cervical HIV-DNA pVL with Detection of any HR HPV Type

Unadjusted		
Log Cervical HIV-DNA pVL	1·17 (1·03-1·33)	0·02
Adjusted		
Log Cervical HIV-DNA pVL	1·07 (0·93-1·22)	0·34
CD4 count at visit	0·99 (0·99-1·00)	0·13
Log plasma HIV-RNA level	0·99 (0·83-1·20)	0·99
cART Status	0·69 (0·38-1·26)	0·22
Months Since ART First Started	0·95 (0·93-0·97)	<0·001
Months Since Excision Treatment	0·98 (0·96-1·00)	0·08

Table 38 Association of Cervical HIV-DNA pVL with Detection of HPV 16 and/or HPV 18

Unadjusted		
Log Cervical HIV-DNA pVL	1·23 (1·10-1·39)	<0·001
Adjusted		
Log Cervical HIV-DNA pVL	1·19 (1·05-1·33)	0·005
CD4 count at visit	1·00 (0·99-1·00)	0·13
Log plasma HIV-RNA level	1·10 (0·93-1·31)	0·25
cART Status	0·95 (0·54-1·67)	0·86
Months Since ART First Started	0·95 (0·93-0·98)	0·002
Months Since Excision Treatment	0·96 (0·93-0·99)	0·01

Association of Multiple HPV Detection with Cervical HIV-DNA Proviral Load

Applying Gaussian GEE regression, there was a significant positive association between the number of HPV types detected and HIV-DNA pVL. This remained significant when adjusting for other covariates. There was a highly significant negative association of time that had elapsed since cART initiation and the cervical HIV-DNA pVL detected (table 39).

Table 39 Association of Number of HPV types with Cervical HIV-DNA pVL

Variable	Coeff (95%CI)	p-value
Unadjusted		
Number of HPV Types Detected	0·04 (0·02-0·06)	<0·001
Adjusted		
Number of HPV Types Detected	1·03 (1·01-1·05)	0·01
CD4 count at visit	0·99 (0·99-1·00)	0·24
Log plasma HIV-RNA level	0·96 (0·89-1·04)	0·30
cART Status	1·09 (0·85-1·41)	0·50
Months Since ART First Started	0·98 (0·96-0·98)	<0·001
Months Since Excision Treatment	0·99 (0·99-1·01)	0·64

Discussion

We have previously found that cART reduces risk for cervical HPV detection by almost 50%, but that it occurs in a *time-dependent* manner, and that this effect possibly precedes CD4 recovery or may even not be reflected by the CD4 count in the blood. We also failed to show an adjusted association between the plasma HIV-RNA levels with the risk for cervical HPV detection, where the time since cART was started was always a stronger predictor of risk. We were therefore performed an ad hoc sub-analysis to investigate if there exists an association of *cervical* HIV- pVL with the risk of HPV detection. From our prospective study of the effect of cART on HPV detection described in Chapter 4, we used stored DNA samples and performed real-time PCR to determine the level of HIV-DNA copies per million cells present in each cervical smear. We found that, in contrast to plasma HIV-RNA levels, increased cervical HIV-DNA pVL is associated with an increased risk in HPV detection. Of note, this association remained significant when adjusted for the time that had elapsed since cART was first started.

As this was an exploratory study into a field where little evidence exists as to the events on a cervical level in the time shortly after cART is initiated, we did preliminary descriptive investigative analyses, before moving on to reporting regression results. When we looked at the distribution of plasma HIV-RNA observations, while still ignoring subject relationships, we saw that receiving cART led to a rapid decrease in values that was sustained throughout the observation period. In contrast, cervical HIV-DNA levels showed no significant difference if the observation was taken at a study visit whilst receiving cART or not. The change in observed levels is small and slow, with loss only of the highest HIV-DNA pVL measurements, here noted as a log HIV-DNA >3.0, during the first 18 months after initiating cART.

When exploring the relationship of plasma HIV-RNA levels with cervical HIV-DNA pVL, we found a wide range of cervical HIV-DNA pVL for women who are receiving cART and have plasma HIV-RNA levels <50 copies (log HIV-RNA=1.79). This contrasts with previous reports: initiation of cART was found to not only cause a rapid decrease of plasma HIV-RNA levels, but also to a dramatic drop, within days (159), in HIV-RNA levels in cervicovaginal

samples (160, 161), with good correlation between plasma HIV-RNA level and cervical HIV-DNA pVL (162, 173).

We applied GEE regression analyses to our longitudinal observation sets to include intra-participant relationships in our results. We found that there is no association between plasma HIV-RNA levels and cervical HIV-DNA pVL. Instead, *time since cART started* was associated strongly with a reduction in risk for the cervical HIV-DNA pVL to be >1.0, and it became stronger for log HIV-DNA >2.0 and >3.0. This confirmed our suspicion that cervical HIV-DNA burden decreases more slowly than the plasma HIV-RNA load.

We repeated the analyses with *cART status* and *time since cART was started* as primary predictors separately. Again, it confirmed our suspicion that cervical HIV-DNA pVL was not significantly determined by whether a patient was receiving cART at the observation point, but how much time had elapsed since she started receiving it.

Finally, we used regression to investigate the association of cervical HIV-DNA pVL with HPV detection. There was an increased risk of detection of any HPV type, as well as HPV16 and/or HPV18, with higher HIV-DNA pVL from the cervix. This remained significant when adjusting for the time that had passed since cART was started. However, the effect of HIV-DNA pVL was not as strongly associated with HR-HPV, but time since cART was started still was a strong predictor of risk. Interestingly, excision treatment also became a significant predictor for the HR HPV and HPV 16/18, which relates to these subtypes as being more prevalent in high-grade CIN and the need for excision biopsy treatment.

As explained in Chapter 4, we chose not to investigate HPV viral load measurements or HPV mRNA expression. These have been done before, and persistent high HPV viral loads and HPV mRNA were linked to the severity of CIN and development of cancer (83). In this sub-analysis, we wanted to look at the interaction of *the amount of HIV infection* in the cervix *on the risk for HPV infection*, as the focus of this research was to investigate the behaviour of HPV associated disease in HIV-infected women.

We could now show that the slow decrease in cervical HIV-shedding mirrors the reduction in HPV infection experienced during the time of cART. This is in contrast to the faster HIV-RNA reductions observed in blood. This is an early indication that the cervical milieu

behaves as a separate anatomical HIV compartment. This slow decrease in cervical HIV-DNA pVL may still be mainly due to a continued seeding of HIV to the cervical compartment in response to the presence of HPV. However, there exists the possibility that ongoing viral replication is occurring which is largely isolated from the blood compartment, especially since some antiretroviral agents have very low penetration in cervical tissues. Viral sequencing studies to compare the evolution of the virus between cervical and blood samples are therefore planned. Sequencing may also assist us in gaining further understanding into the reason for the negative HIV-DNA pVL results in the twenty observations.

Chapter 6: Final Conclusions

Cervical intraepithelial dysplastic lesions are caused by infection with HPV. *Persistence* of HPV infection, particularly with oncogenic HPV types, is linked to an increased risk for development of cancer. Women who are co-infected with HPV and HIV have higher persistence of cervical HPV, which would make them at higher risk for the development of cancer of the cervix than their HIV-uninfected counterparts. It has been postulated that the high mortality due to AIDS initially prevented a noticeable increase of cervical cancer in Africa. It was also feared that the rollout of HIV treatment programmes could subsequently cause a re-emergence of cervical cancer due to increased longevity in women living with HIV-infection. Indeed, during the time that we have been engaged in this research, some evidence has emerged as to the increased risk of HIV infected women for development of invasive cervical cancer. This increase was observed in younger women (<40 years) and women with more severe immunosuppression (178-180). In the larger of the investigative studies, 132 women with cervical cancer and 120 with normal cytology were included, and of these women, 89.4% and 31.1% respectively, tested HPV HR positive. The authors report that HIV infection was associated with cervical cancer in the women who were HPV high-risk positive (OR 4.5, 95% CI 1.5-13.6) (181). However, a rise in cervical cancer cases amongst HIV-infected women has not yet been observed in developed countries, and this is why the question first arose if immune recovery as seen in HIV treatment programs has been responsible for the protection against development of the said cancer.

HIV infection is a largely manageable disease. Many Sub-Saharan countries, where the prevalence of both HIV and HPV infection is endemic, have well-established antiretroviral treatment programs. Certainly, we have found that women who are on antiretroviral treatment have a lower risk of progression and reduced persistence of dysplastic lesions. cART also improves excision treatment outcome by reducing post-excision recurrence. This has been confirmed by other authors. The question remained, as we know that HPV causes cervical disease, why have previous studies that measured directly the effect of cART on HPV infection been so conflicting?

We developed two theories that could explain the differences. Firstly, the few studies that had been done covered more than two decades of technical development and epidemiological changes. Very few patients were enrolled to the earlier studies, or too few of the study

subjects actually received cART. With the advancement of detection methods to include more HPV types, we postulated that it could also have been possible that not all types were influenced equally by the immune restoration offered by cART. Interestingly, outcomes had been simplified over time to cope with the expanding spectrum of HPV detection capability, and women were observed as having "any HR type present", without distinguishing between individual subtype effects.

Secondly, in the analysis of outcomes, investigators remained loyal to terminology adopted from research methodology in the field of Cervical Dysplasia, with the outcomes being persistence, clearance, recurrence and incident detection. Not only do these artificial outcomes ignore the previous history during assumptions or generalizations made at time zero, but the effects of changes in other factors that could influence the outcomes are ignored. Logistic regression, setting binary outcomes at a chosen interval, or survival analysis testing time to a specified end point, were performed. The weaknesses of applying such methods are that most data sets or observation points in a patient's follow-up time are ignored and that a left-sided truncation of time is afforded. Also, women who were lost to follow-up, or were followed for shorter periods of time, had to be excluded. This led to loss of statistical power and created bias in ignoring the women likely to have more advanced immunosuppression.

This is again evident in a very recent paper published at the time that this dissertation is submitted. Although longitudinal data was collected on 326 HIV-infected women, HPV types were grouped as oncogenic type, HPV16 or HPV type other than HPV16/18. The authors used a 2-state Markow model, a type of survival analysis, to assess the effect of HAART, CD4 and other covariates on acquisition and clearance of HPV infection. HAART was identified as yes/no, but the time that it had been administered was not taken into account. Also, sexual activity was identified as life-time partners, which is a time-*independent* variable. Nevertheless, they did find that HAART increased the likelihood of clearance of oncogenic types other than HPV16/18 (HR 2.2 p=0.01) (182).

In contrast, we chose to maximize statistical power by using all available data sets in a clustered or repeated measures design. Each woman served as her own control, was not excluded due to earlier drop-out, and each covariate incorporated as a time-dependent variable. Also, we abandoned the artificial end points (e.g. persistence), rather restricting HPV test results to what they were designed to reflect: the detection of HPV infection at a

certain time point. We were able to show that cART reduced the chance of detecting any HPV type on a cervical smear by almost 50%. Moreover, the effect was time-dependent, in that the time since cART was started was the strongest predictor of risk, even stronger than the CD4 recovery measured in blood. According to our knowledge, this was the first expression, as an odds ratio, of reduced risk of cervical HPV infection associated with initiation of antiretroviral treatment. Moreover, we could show that almost all HPV subtypes were affected at least as strongly by the initiation of cART than our chosen base type, HPV16. We attributed prior conflicting study results to research methodology, and not to HPV subtype differences, as cause for these differing reports.

Paradoxically, even though the time that elapsed since cART was first started was the strongest predictor of HPV detection, we found that *plasma* RNA levels were not significantly associated with the HPV detection risk. This was unexpected, as we know that cART reduces plasma HIV-RNA levels. We therefore performed a sub-analysis to investigate the role of amount of *cervical* HIV infection in the risk for HPV detection. Higher cervical HIV-DNA pVL was significantly associated with the increased HPV detection risk. This cannot simply be viewed as a causative relationship, however. The higher cervical HIV-DNA pVL could also be due concomitant processes in the cervix. We suggest that the cervix is behaving more like a separate anatomical compartment, where the inflammatory environment of HPV-infected mucosa is populated by HIV-vulnerable cells. We are concerned about the effect of possible continuing HIV replication in the cervix contributing to viral evolution and efavirenz resistance. Therefore, we plan to use more sophisticated methods to explore the mechanism by which cART reduces cervical HPV infection, including cellular immune markers and HIV sequencing.

To the Clinician, our research already has delivered had some important messages. During the years we have been actively involved in studying cervical dysplasia and the effect of cART on cervical HPV infection, we have observed the improved Pap screening efforts. Indeed, it has been integrated into the HIV management protocol as a standard screening visit procedure, regardless of the woman's age. Referral protocols have been improved, both from our IDC and Colposcopy Clinic. These efforts will be maintained. Importantly, HIV testing has become a standard screening test at the Colposcopy Clinic.

For the Gynaecologist, there are specific lessons learned. Women living with HIV–infection have much higher failure of excision treatment of HSIL. Moreover, initiating cART before excision treatment of HSIL will improve the outcome and lower the recurrence. However, the importance of excision treatment of HSIL, and not just providing cART, was again demonstrated and it remains indicated in women who are receiving cART.

For the HIV Clinician, apart from the awareness of the importance of screening for cervical disease and providing cART, we need to engage in further study of the impact of cervical HIV infection on the viral evolution and resistance development.

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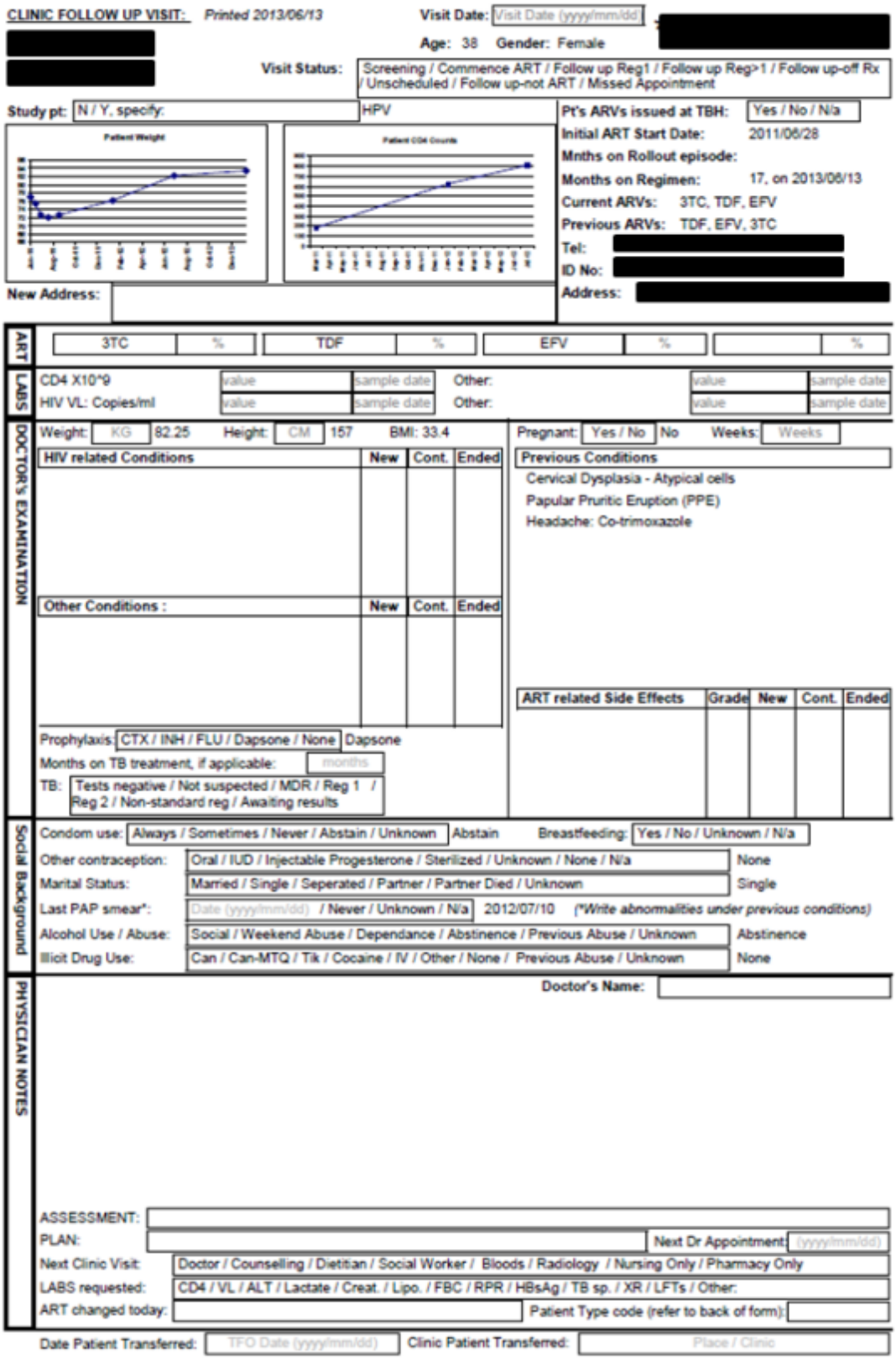
Appendices

Appendix 1: IDC Data Sheet



CLINIC FOLLOW UP VISIT: *Printed 2013/06/13*

Visit Date: Visit Date (yyyy/mm/dd)

[redacted]

[redacted]

Age: 38 Gender: Female

[redacted]

Visit Status: Screening / Commence ART / Follow up Reg1 / Follow up Reg>1 / Follow up-off Rx / Unscheduled / Follow up-not ART / Missed Appointment

Study pt: N / Y, specify: HPV

Patient Weight

Patient CD4 Counts

Pt's ARVs issued at TBH: Yes / No / N/a

Initial ART Start Date: 2011/06/28

Mnths on Rollout episode:

Months on Regimen: 17, on 2013/06/13

Current ARVs: 3TC, TDF, EFV

Previous ARVs: TDF, EFV, 3TC

Tel: [redacted]

ID No: [redacted]

Address: [redacted]

New Address:

ART

3TC	%	TDF	%	EFV	%		%

LABS

CD4 X10^9	value	sample date	Other:		value	sample date
HIV VL: Copies/ml	value	sample date	Other:		value	sample date

DOCTOR'S EXAMINATION

Weight: KG 82.25 Height: CM 157 BMI: 33.4

Pregnant: Yes / No No Weeks: Weeks

HIV related Conditions	New	Cont.	Ended

Other Conditions :	New	Cont.	Ended

Previous Conditions

Cervical Dysplasia - Atypical cells

Papular Pruritic Eruption (PPE)

Headache: Co-trimoxazole

ART related Side Effects	Grade	New	Cont.	Ended

Prophylaxis: CTX / INH / FLU / Dapsone / None Dapsone

Months on TB treatment, if applicable: months

TB: Tests negative / Not suspected / MDR / Reg 1 / Reg 2 / Non-standard reg / Awaiting results

Social Background

Condom use: Always / Sometimes / Never / Abstain / Unknown Abstain Breastfeeding: Yes / No / Unknown / N/a

Other contraception: Oral / IUD / Injectable Progesterone / Sterilized / Unknown / None / N/a None

Marital Status: Married / Single / Seperated / Partner / Partner Died / Unknown Single

Last PAP smear*: Date (yyyy/mm/dd) / Never / Unknown / N/a 2012/07/10 *(*Write abnormalities under previous conditions)*

Alcohol Use / Abuse: Social / Weekend Abuse / Dependance / Abstinence / Previous Abuse / Unknown Abstinence

Illicit Drug Use: Can / Can-MTQ / Tik / Cocaine / IV / Other / None / Previous Abuse / Unknown None

PHYSICIAN NOTES

Doctor's Name:

ASSESSMENT:

PLAN: Next Dr Appointment: (yyyy/mm/dd)

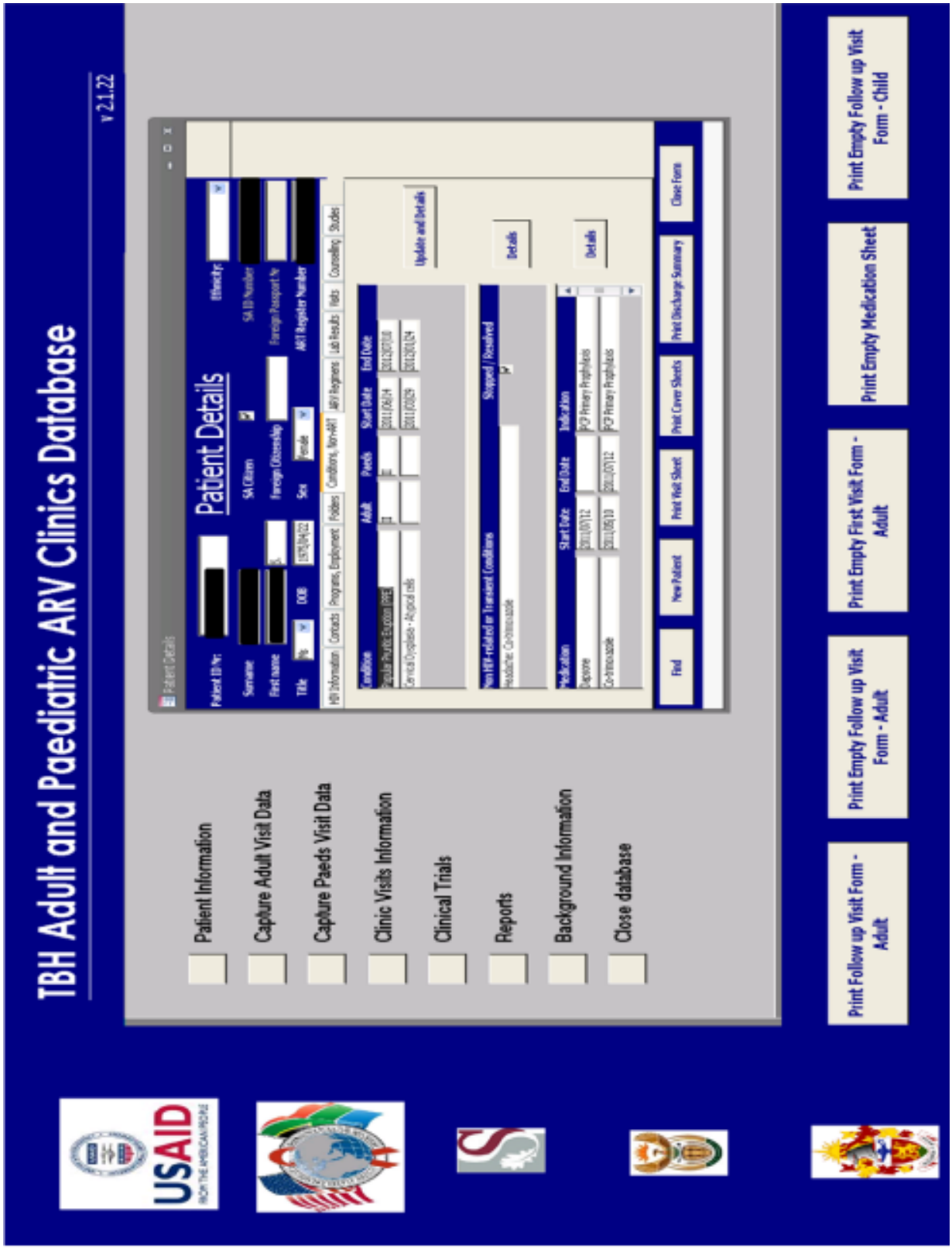
Next Clinic Visit: Doctor / Counselling / Dietitian / Social Worker / Bloods / Radiology / Nursing Only / Pharmacy Only

LABS requested: CD4 / VL / ALT / Lactate / Creat. / Lipo. / FBC / RPR / HBsAg / TB sp. / XR / LFTs / Other:

ART changed today: Patient Type code (refer to back of form):

Date Patient Transferred: TFO Date (yyyy/mm/dd) Clinic Patient Transferred: Place / Clinic

Appendix 2: IDC Capture page, HIV-related diseases



Appendix 3: IDC Transfer Letter

Patient Summary

Printed on: 2013/06/12

Infectious Diseases Clinic
Tygerberg Hospital C8A East
Tel: (021) 9385229

Patient XXXXXX
Patient Number yyyyyyyyy
DOB 1975/04/22
Gender Female

Rollout History:

Adult TBH ARV Rollout
Transferred 2011/09/29
Initiation for Rollout 2011/06/28

History of Conditions:

Condition	ICD 10	Start Date	End Date	Outcome
Papular Pruritic Eruption (PPE)	L30.9	2011/06/14	2012/07/10	
Cervical Dysplasia - Atypical cells		2011/03/29	2012/01/24	

History of ARV's:

From 2011/06/28 to 2011/09/29
Lamivudine mg daily
Efavirenz mg daily
Tenofovir mg daily
Side Effect/s: -

From 2012/01/24 to
Efavirenz mg daily
Tenofovir mg daily
Lamivudine mg daily
Side Effect/s: -

Non - ART Medicines:

Medication	Indication
Dapsone	PCP Primary Prophylaxis

CD4 Counts:

2012/07/10 : 807
2012/01/24 : 620
2011/03/22 : 181

HBsAg results:

2011/06/14 : Negative

HIV Viral Loads:

2012/07/10 : 740
2012/01/24 : 0
2011/06/14 : 156300

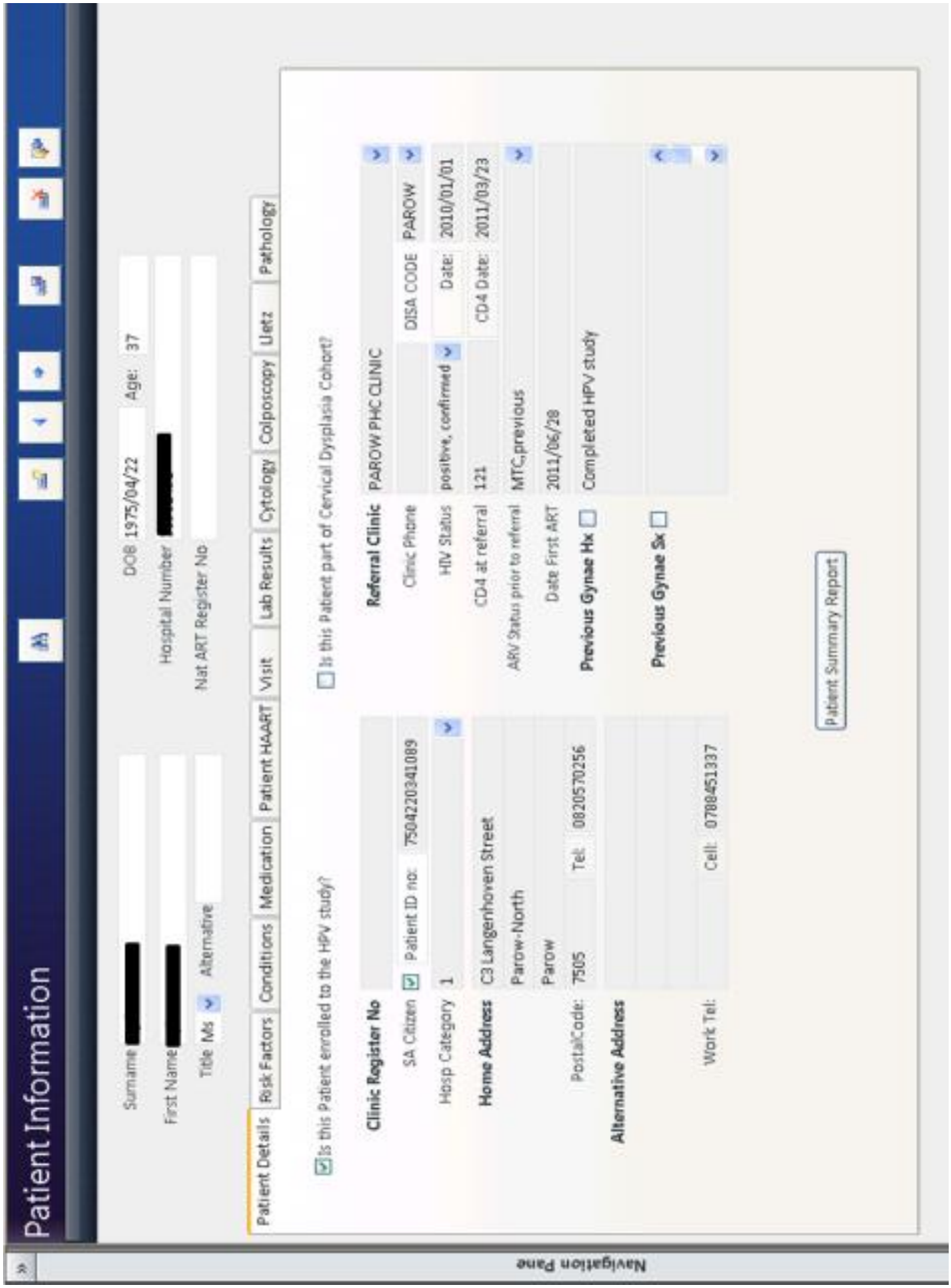
RPR and FTA-abs results:

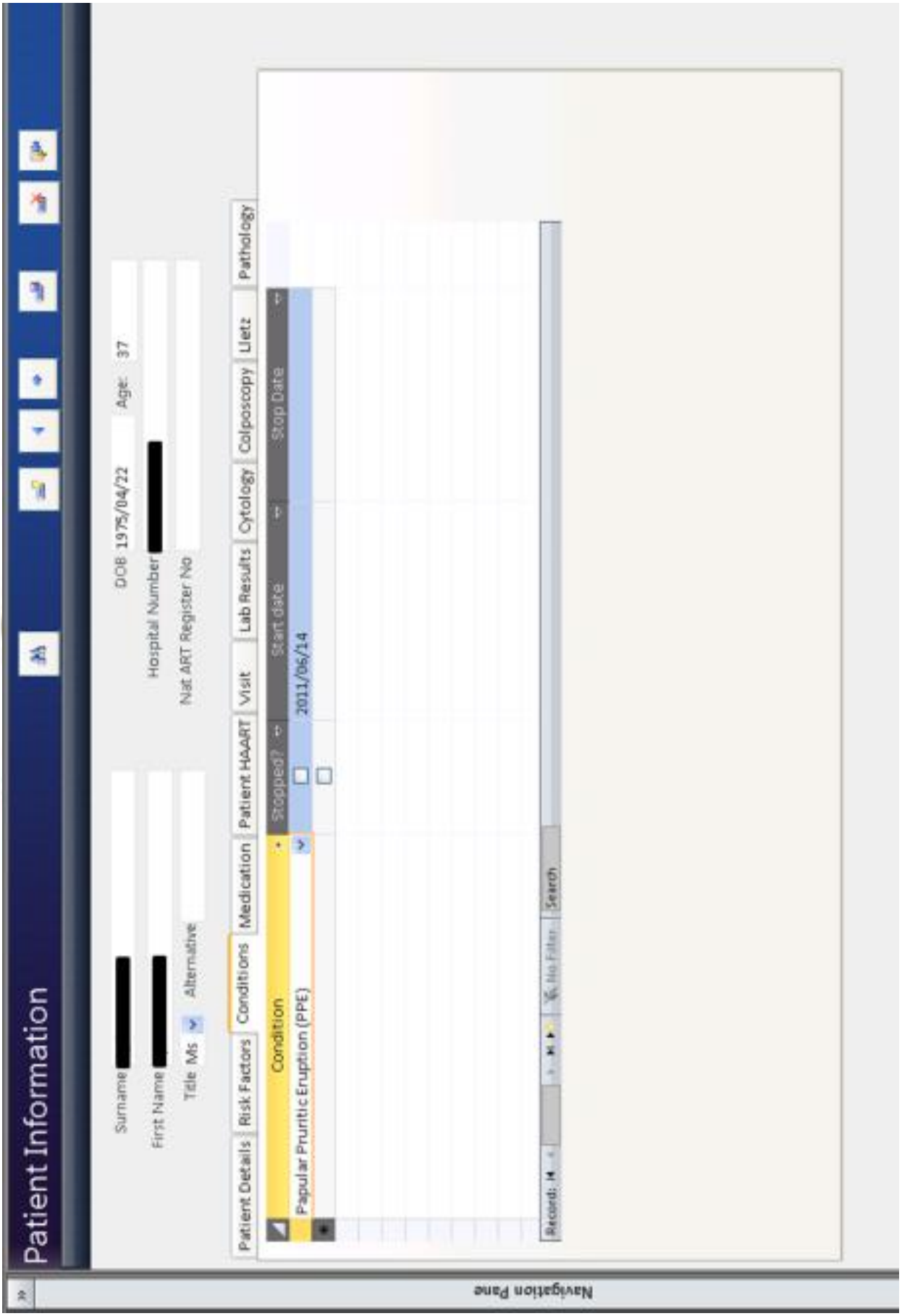
2011/06/14 : RPR Negative
2011/03/22 : RPR Negative

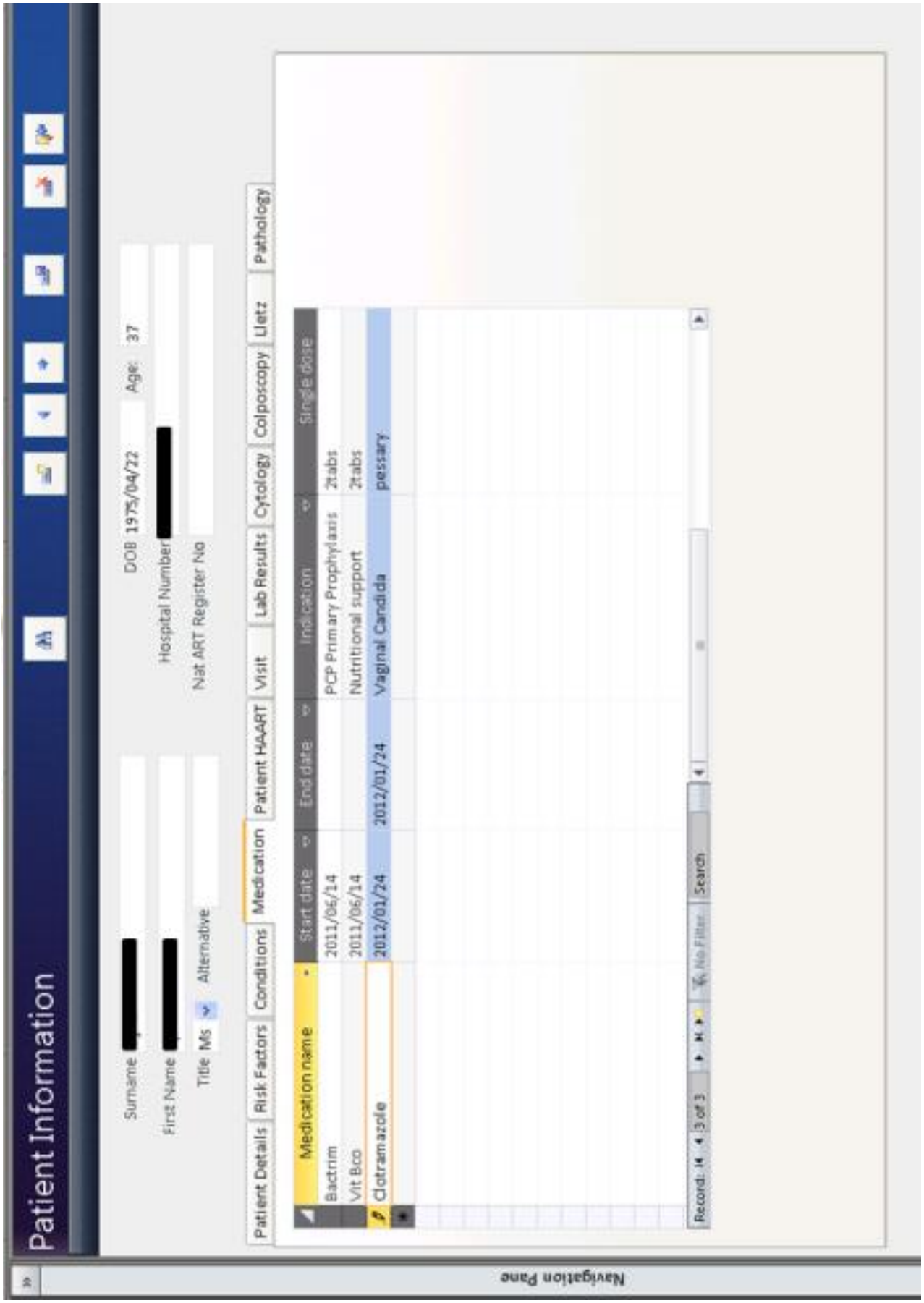
Signature

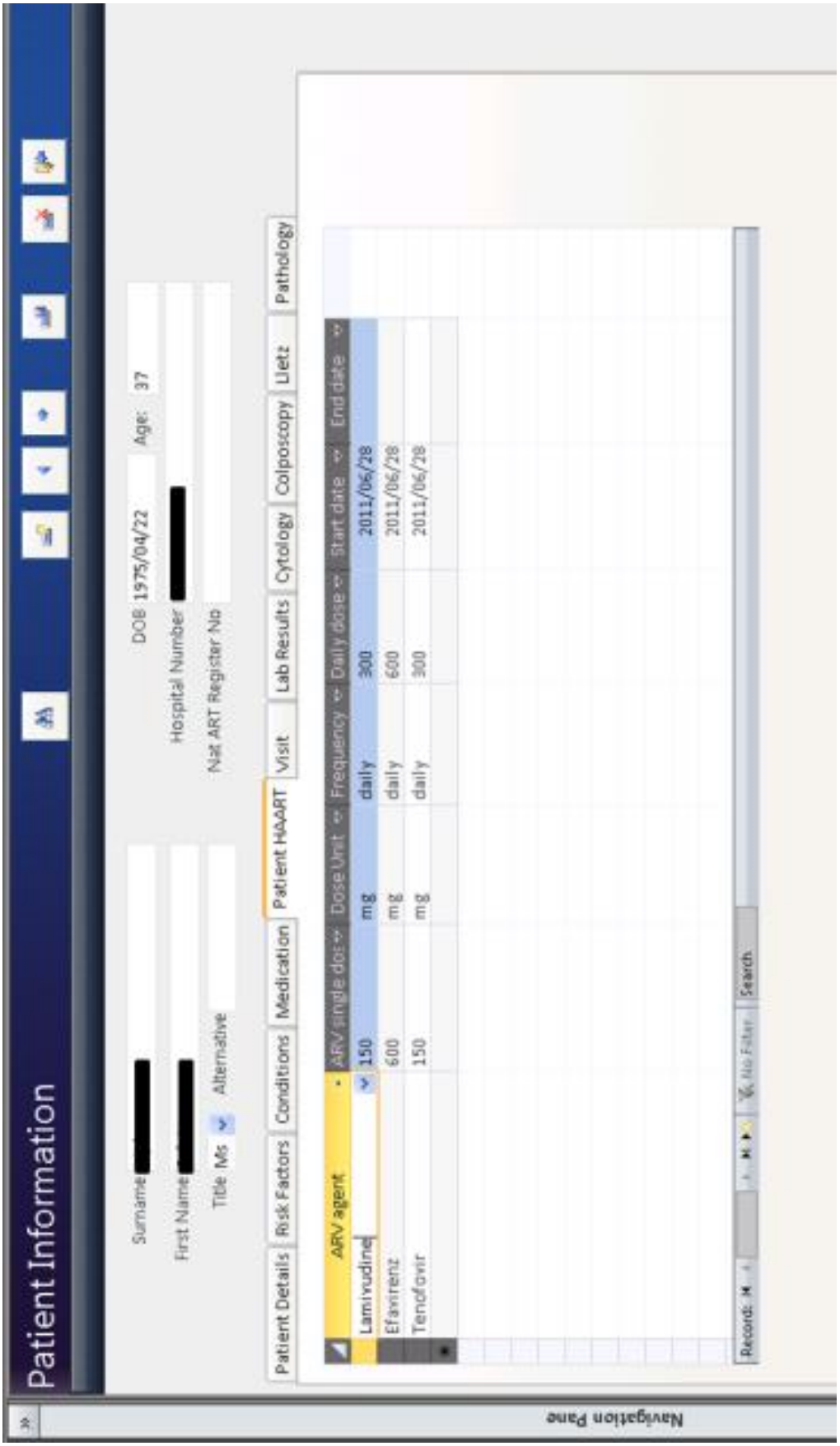
Date signed

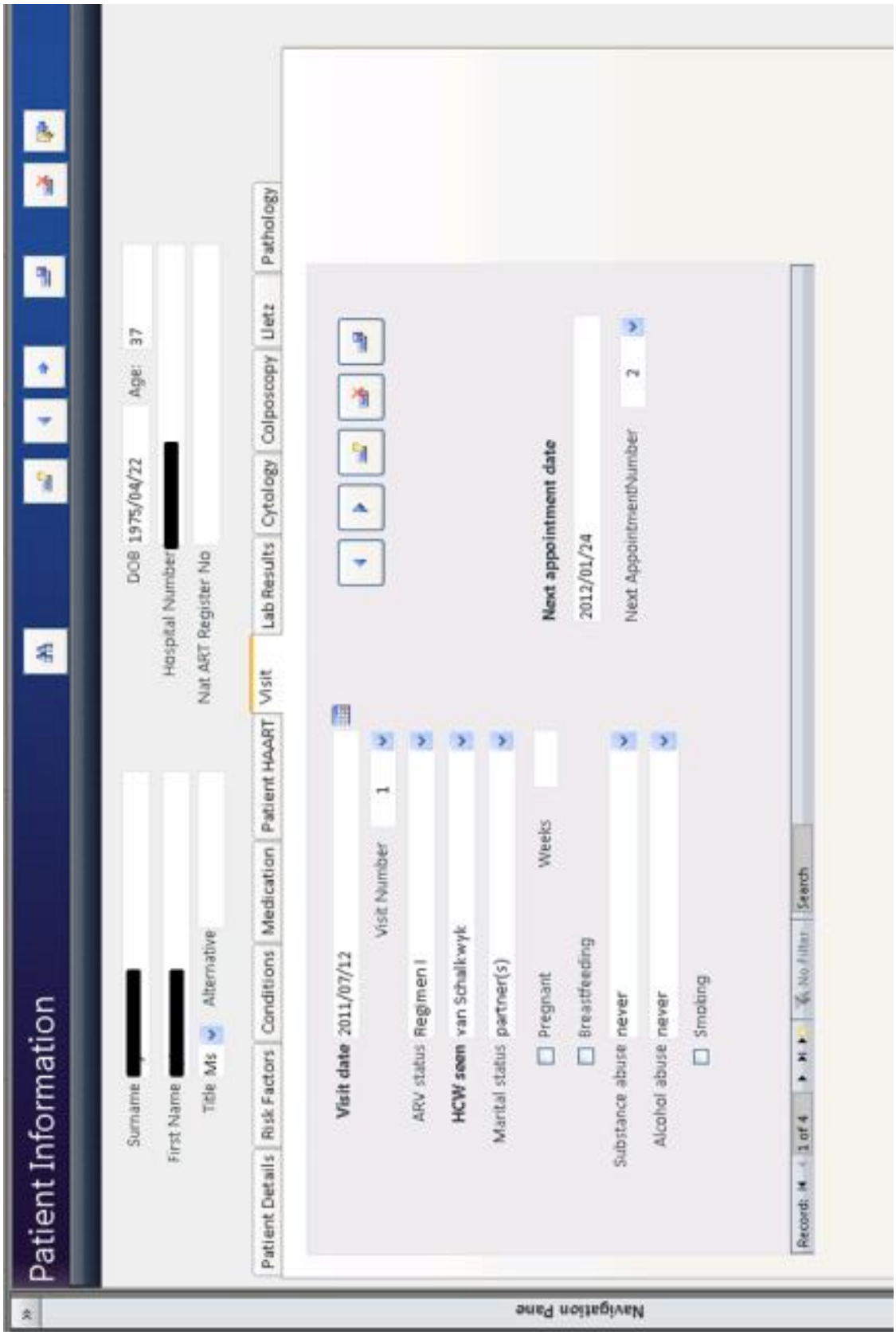
Appendix 4: COLPOS database

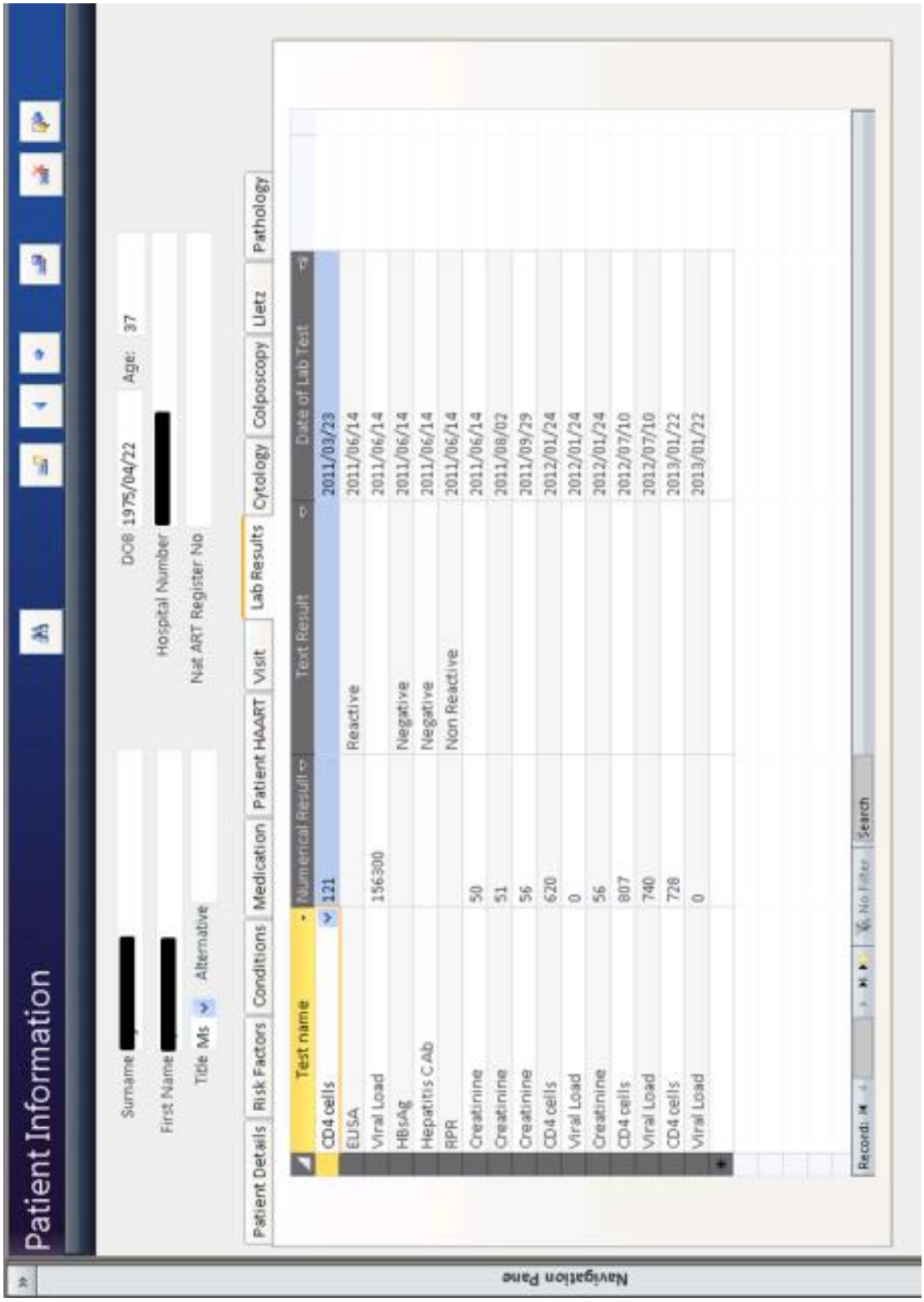


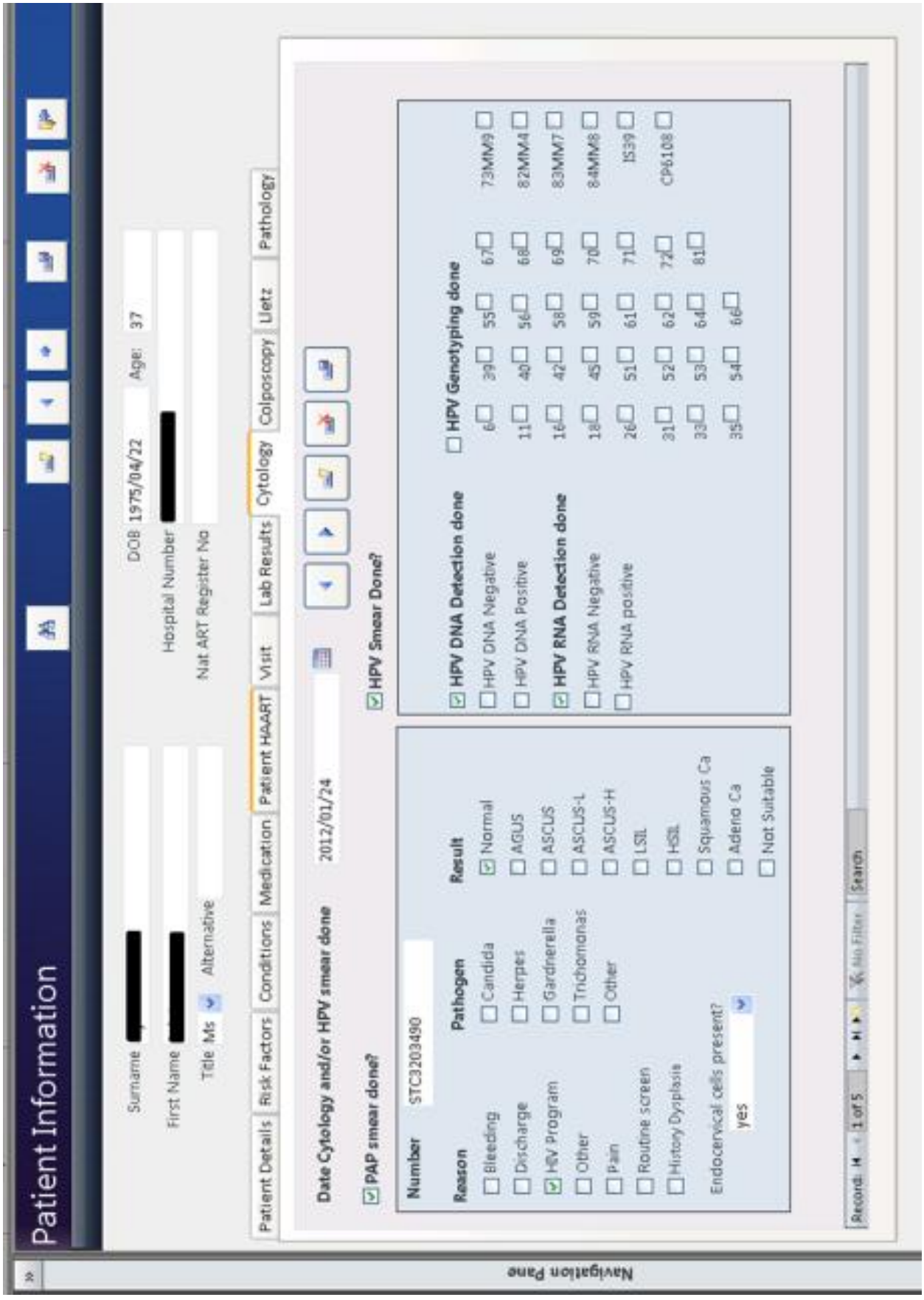


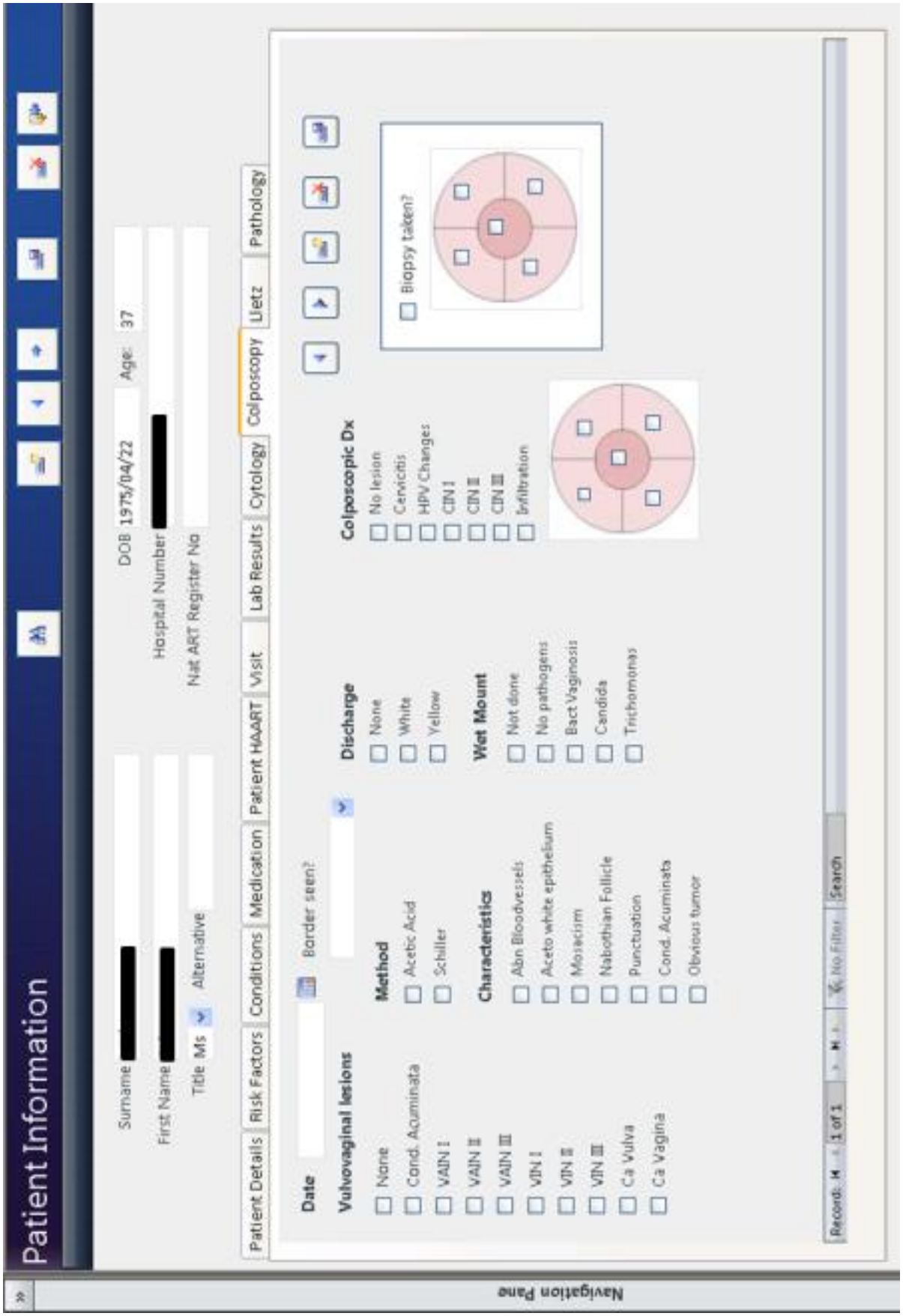


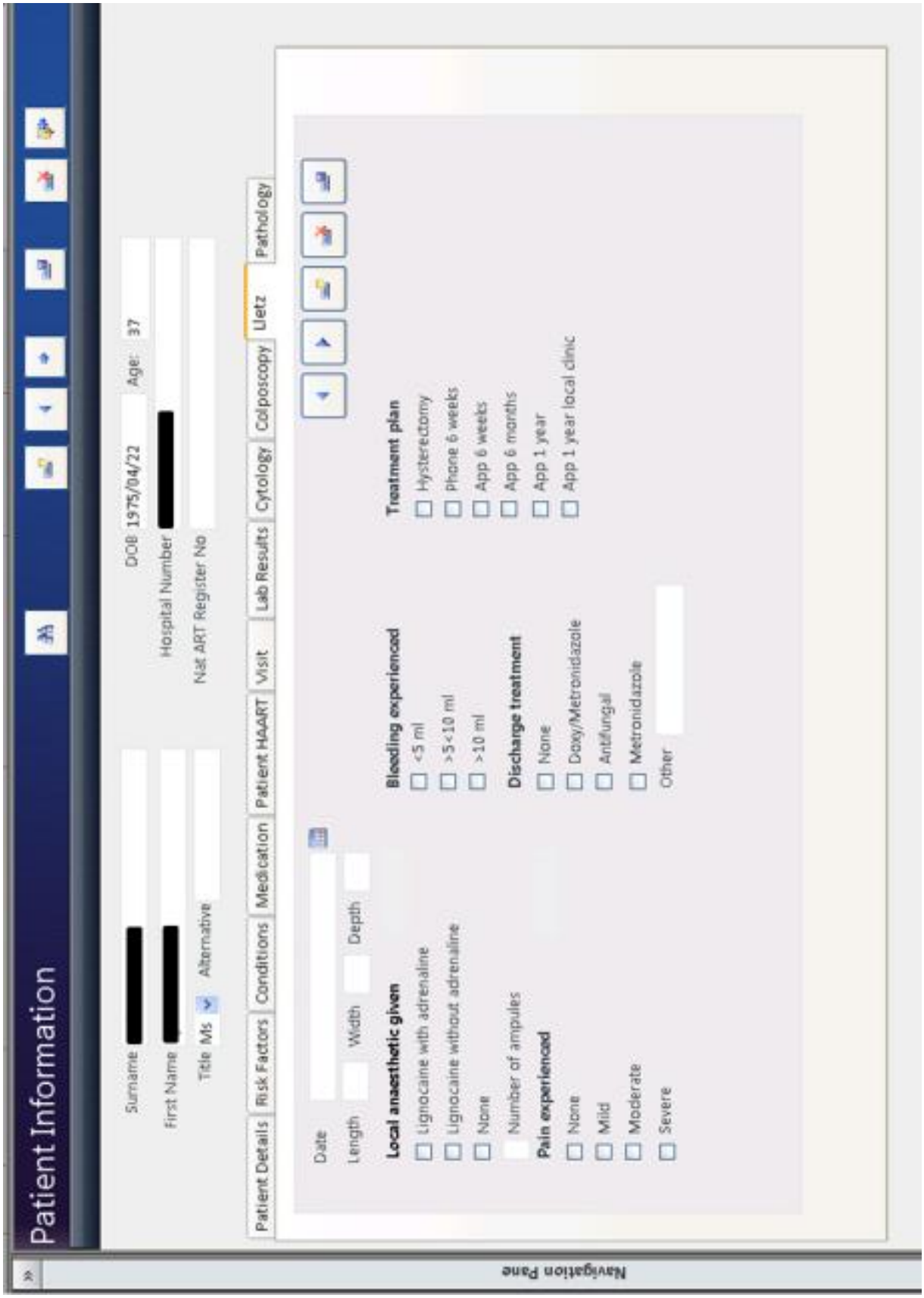


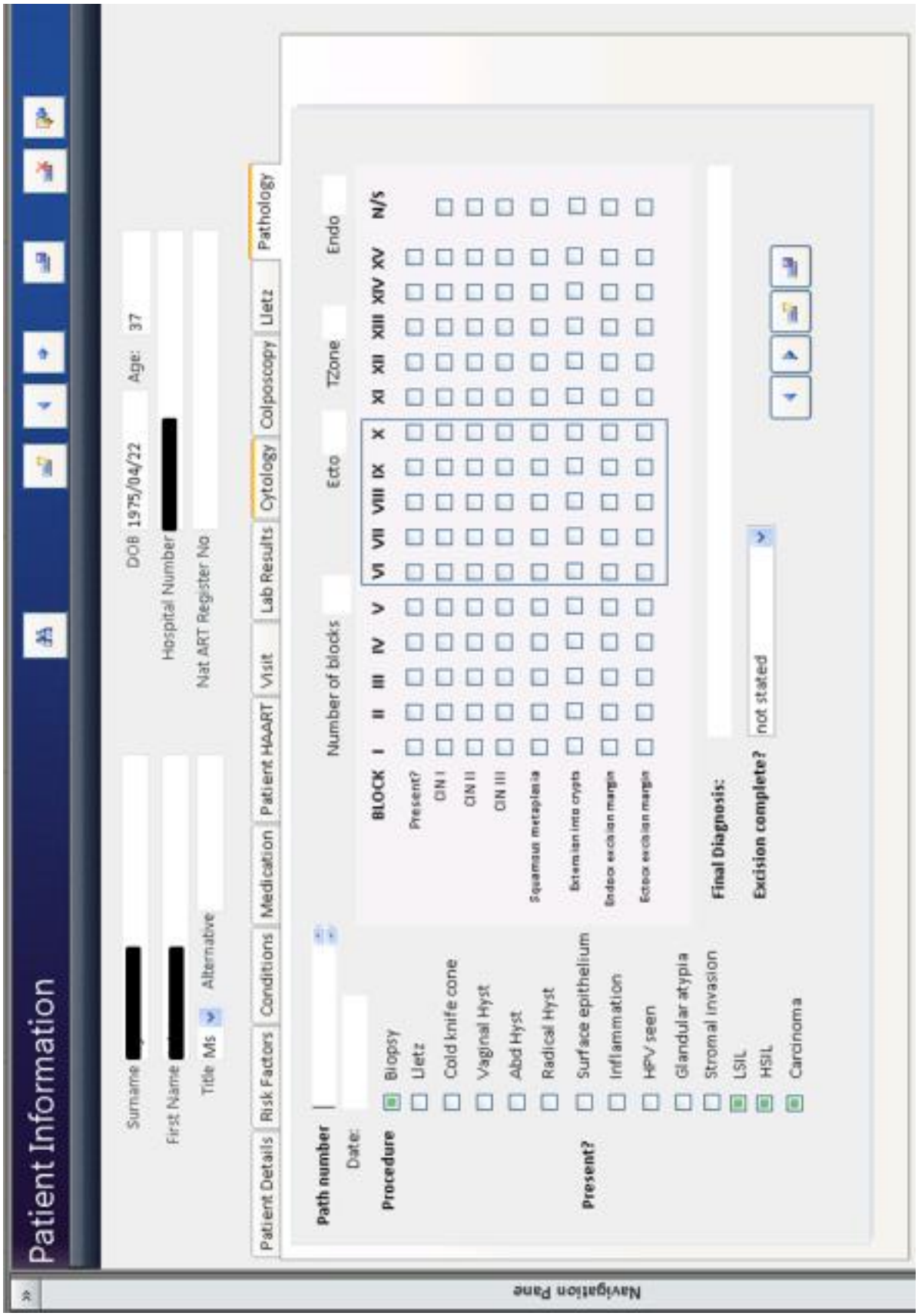












Appendix 5: COLPOS Patient Summary

Patient Summary

Infectious Diseases Clinic
Tygerberg Hospital C8A East
Tel: (021) 9385229
Date: 12-Jun-13

Patient Number: yyyyyyyyy
DOB: 1975/04/22
Patient: XXXXXXXX
Tel: 0000000000
Address: ZZZZZZZZZZ

Cytology History:

Date:	Path No:	Infection:	Result:
2011/03/29	STC3118914		ASCUS, LSIL
2011/07/12	STC3138707		ASCUS, LSIL
2012/01/24	STC3203490		Normal
2012/07/10	STC3238593	Other	Normal

Pathology History:

Data not specified or not applicable

History of Conditions:

Condition:	Start date:	End date:
Papular Pruritic Eruption (PPE)		2011/06/14

History of Medications:

Medication:	Dosage:	Frequency:	Start date:	End date:
Efavirenz	600 mg	daily	2011/06/28	
Lamivudine	150 mg	daily	2011/06/28	
Tenofovir	150 mg	daily	2011/06/28	
Bactrim	2tabs	daily	2011/06/14	
Vit Bco	2tabs	daily	2011/06/14	
Clotramazole	pessary	STAT	2012/01/24	2012/01/24

Lab Tests:

Test name:	Date:	Result:
CD4 cells	2011/03/23	121
ELISA	2011/06/14	Reactive
RPR	2011/06/14	Non Reactive
Hepatitis C Ab	2011/06/14	Negative
HBsAg	2011/06/14	Negative
Creatinine	2011/06/14	50
Viral Load	2011/06/14	156300
Creatinine	2011/08/02	51
Creatinine	2011/09/29	56
CD4 cells	2012/01/24	620
Creatinine	2012/01/24	56
Viral Load	2012/01/24	0
CD4 cells	2012/07/10	807
Viral Load	2012/07/10	740
CD4 cells	2013/01/22	728
Viral Load	2013/01/22	0

Next Appointment date:2013/01/22

---------- ----------
Signature Date signed

Appendix 6: Participant Information Leaflet and Consent Form

Title of research project:

A Prospective Descriptive study of the Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Cytological Abnormalities and in HIV Infected Women

Principal Investigator:	**Address:**	**Contact number:**
Dr MD Zeier	Room 77 Infectious Diseases Clinic C8A East Tygerberg Hospital Parow 7505	Tel: +27-21-9385230 Cell: +27827846605 Fax: +27-86-5032614

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study will be conducted at the colposcopy clinic on the third floor and the Infectious Diseases Clinic on the eighth floor at Tygerberg Hospital. There are no other sites. There will be approximately one hundred and fifty participants recruited (this number may vary according to the findings of the study). The study will continue for approximately two years.

There is evidence that HIV positive women with HPV infection (the virus that causes warts) may be more likely to develop cancer of the Cervix (mouth of the womb). Therefore we advise all women with HIV infection to undergo regularly testing for early lesions of this cancer. This is called Pap smear testing. More sensitive tests are now available where we can also test for this HPV directly, in the same way we would normally do a PAP smear. We are trying to find out if providing HIV women with antiretroviral treatment protects them from the HPV infection or helps them to get rid of it more quickly.

Why have you been invited to participate?

You have been identified as being a carrier of the Human Immunodeficiency Virus, or as it is usually called, as being HIV positive. HIV infection causes weakening of the immune system over some years, with the result that your body may lose its ability to protect you against certain infections,

such as infection with certain viruses. It may be that the HPV may cause you to develop early forms of cancer of the mouth of the womb. Normally, the only way we can find out if you already have this infection, is to do a Pap smear to see if the virus has caused these lesions to develop. In this study, we can do other more sensitive tests for the HPV virus that causes cancer of the cervix. We will follow normal procedure and refer you for antiretroviral therapy (this is used to limit the activity of the HIV) to the Infectious Diseases Clinic in Tygerberg Hospital, or if you have already been seen there, refer you for Pap smear and HPV testing.

What will your responsibilities be?
You will have to undergo a Pap smear screening every six months, which will include the HPV tests, every six months, for a period of 18 months.

Will you benefit from taking part in this research?
You will benefit from this study because we will be able to offer you an additional test for HPV infection. Because there is conflicting evidence if antiretroviral therapy may help you to get rid of HPV infection, it is not yet known whether treatment with ARV will protect you from developing cervical cancer.

Are there in risks involved in your taking part in this research?
There is minimal risk involved. You may developed some bleeding or infection after the HPV smear is taken, in the same way this may happen after a Pap smear.

If you do not agree to take part, what alternatives do you have?
If you do not take part in the study you will have access to standard management of abnormal Pap smear results which will include referral to the colposcopy clinic and removal of these lesions as prescribed by standard management protocols, but this will not include the HPV test. The HIV infection and treatment will be provided regardless if you take part or not, according to the Government Roll-out Programme of antiretroviral treatment.

Who will have access to your medical records?
All information will remain strictly confidential and will be protected. The members of the research team on the study are all also members of the staff at the Infectious Diseases Clinic and the Colposcopy Clinics at Tygerberg Hospital and they will be the only people that have access to the information. During the data analysis phase, all participants will remain anonymous and then there will be no way of identifying the participants. The anonymous information from this study will be used to compile a document which will be published.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?
There will be no injury as the additional test, the HPV tests, are done in the same manner as a routine Pap smear and will be done during the same procedure.

Will you be paid to take part in this study and are there any costs involved?
No, you will not be paid to take part in the study. Your will be reimbursed for the cost of your transport to the hospital for each visit, to assist you with attending your outpatient clinic for the

specific study-related appointments. The funding for the study is provided by the HIV Research Unit of the Stellenbosch University as well as PEPFAR (US **President's Emergency Plan for AIDS Relief**).

Is there anything else that you should know or do?

You can contact Dr MD Zeier at tel. (021) 9385230/0827846605 if you have any further queries or encounter any problems.
You can contact the Committee for Human Research at (021) 9389207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …… agree to take part in a research study entitled:

A Prospective Descriptive study of the Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Cytological Abnormalities in HIV Infected Women.

I declare that:
I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
I have had a chance to ask questions and all my questions have been adequately answered.
I understand that taking part in this study is **voluntary** and I have not been pressurized to take part.
I may choose to leave the study at any time and will not be penalized or prejudiced in any way.
I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) ..... on (date) ……..…… 20…..

..... *.....*
Signature of participant *Signature of witness*

Declaration by investigator

I *(name)* …… declare that:

I explained the information in this document to …….
I encouraged her to ask questions and took adequate time to answer them.
I am satisfied that she adequately understands all aspects of the research, as discussed above
I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below).*

Signed at (place) ..... on (date) ……. 20.

..... *.....*
Signature of investigator *Signature of witness*

Declaration by translator

I *(name)* …… declare that:

I assisted the investigator (*name*) …… to explain the information in this document to (*name of participant*) ……. using the language medium of Afrikaans/Xhosa.
We encouraged her to ask questions and took adequate time to answer them.
I conveyed a factually correct version of what was related to me.
I am satisfied that the participant fully understands the content of this informed consent document and has had all her questions satisfactorily answered.

Signed at (place) ..... on (date) ……. 2010.

..... *.....*
Signature of translator *Signature of witness*

Appendix 4: Participant Information Leaflet and Consent Form, Addendum 7 June 2011

Title of research project:
A Prospective Descriptive study of the Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Cytological Abnormalities and in HIV Infected Women

Principal Investigator:	**Address:**	**Contact number:**
Dr MD Zeier	Room 77 Infectious Diseases Clinic C8A East Tygerberg Hospital Parow 7505	Tel: +27-21-9385230 Cell: +27827846605 Fax: +27-86-5032614

You completed 18 months enrolment to this research project. Please take some time to read the information presented here, which will explain the details of this addendum. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this addendum all about?
This addendum does not replace or change any part of the original consent form you have signed. We would like to offer you to continue participation in this project by one more year (two more visits) if you have not yet cleared the HPV viral infection on your Pap smears, i.e. your last test was still positive. At the two extra visits, we will do the same procedures as with all other follow-up visits: a Pap smear, a HPV test on that smear and a CD4 count (if not done at your local clinic).
We also want to ask you permission to do a special test on the samples we collected at the first visit. This is similar to the HPV test, where we characterized the HPV virus as being one of the oncogenic (cancer) types or not, but we also want to test which type of HIV you have in your blood and in the Pap smear. We will not do any tests on your own (human) DNA. Please indicate on the signature page if you agree to this test.

Is there anything else that you should know or do?

You can contact Dr MD Zeier at tel. (021) 9385230/0827846605 if you have any further queries or encounter any problems.

You can contact the Committee for Human Research at (021) 9389207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …… agree to take part in a research study entitled:

A Prospective Descriptive study of the Effect of Highly Active Antiretroviral Therapy on Human Papilloma Virus Infection and Cervical Cytological Abnormalities in HIV Infected Women.

I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is **voluntary** and I have not been pressurized to take part.

I may choose to leave the study at any time and will not be penalized or prejudiced in any way.

I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

☐ I agree or ☐ I DO NOT agree

to have a test done on the sample taken at the first visit to have a test done on the samples taken at the first visit which will determine the type of HIV I have.

☐ I want to ☐ I DO NOT want to

continue participation in the study for a further year (two visits).

Signed at (place) ..... on (date) …… 201…..

Signature of participant.....Signature of witness.....

Declaration by investigator

I *(name)* …… declare that:
I explained the information in this document to ……..
I encouraged her to ask questions and took adequate time to answer them.
I am satisfied that she adequately understands all aspects of the research, as discussed above
I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below).*

Signed at (place) ..... on (date) ……..…… 201

..... *.....*
Signature of investigator *Signature of witness*

Declaration by translator

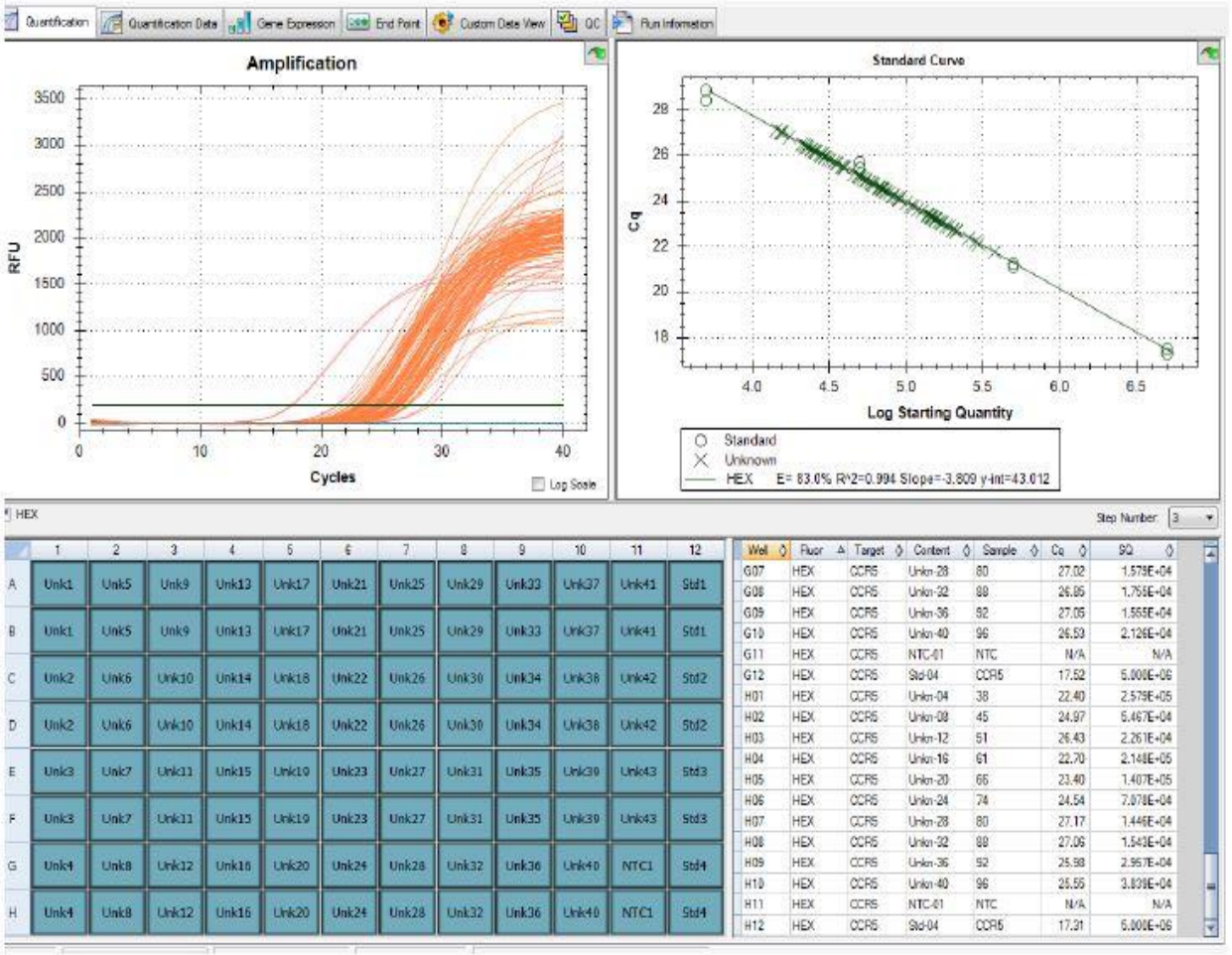
I *(name)* …… declare that:

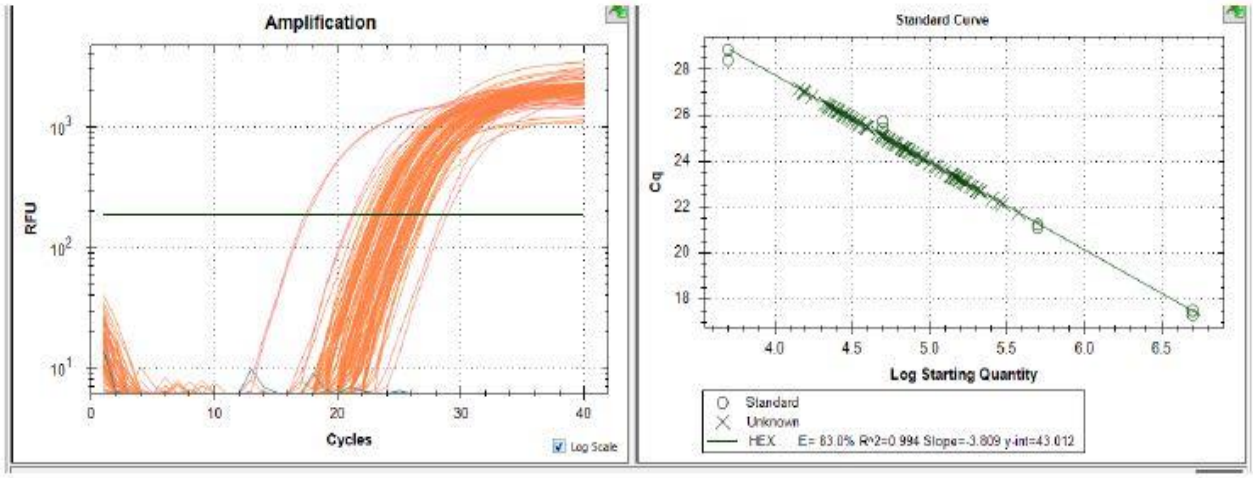
I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.
We encouraged her to ask questions and took adequate time to answer them.
I conveyed a factually correct version of what was related to me.
I am satisfied that the participant fully understands the content of this informed consent document and has had all her questions satisfactorily answered.

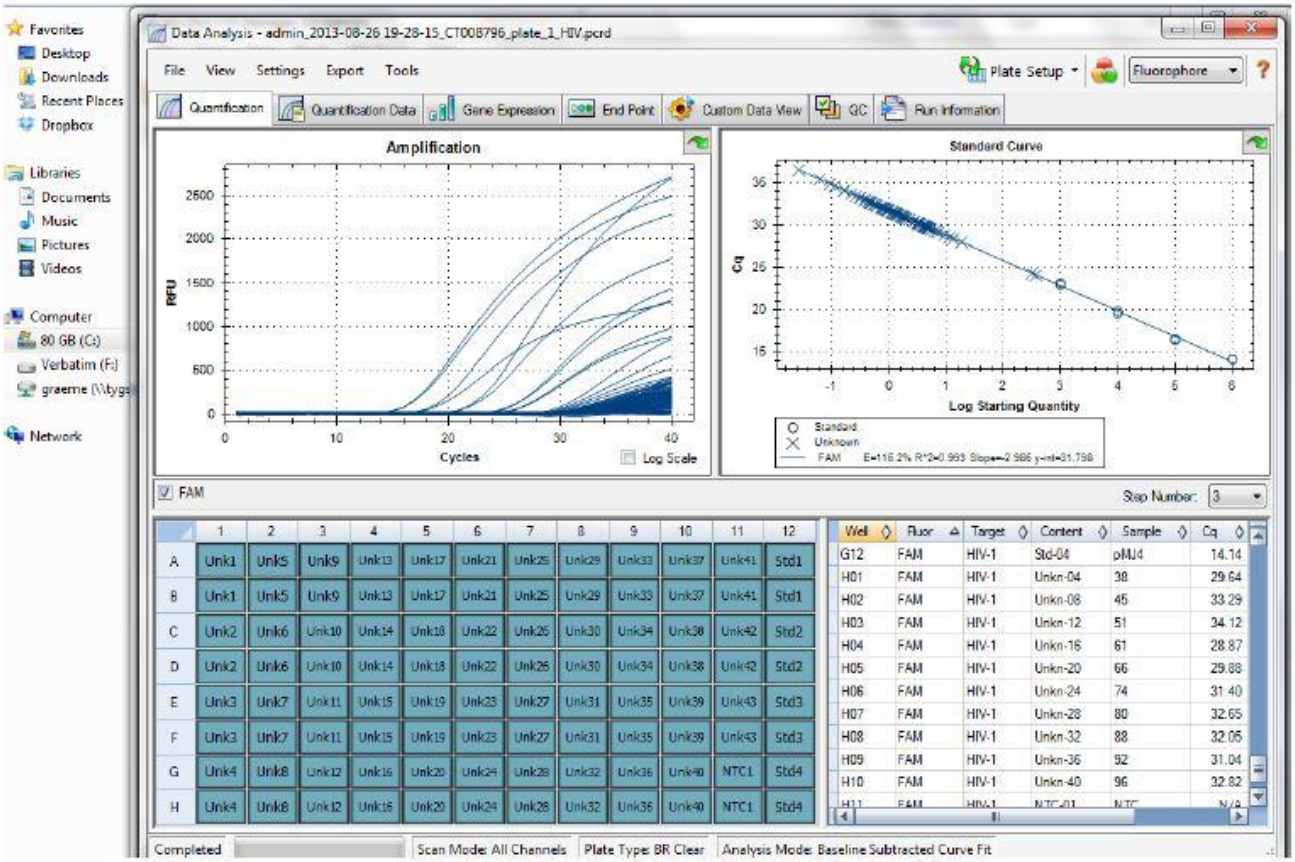
Signed at (place) ..... on (date) ……..…… 201

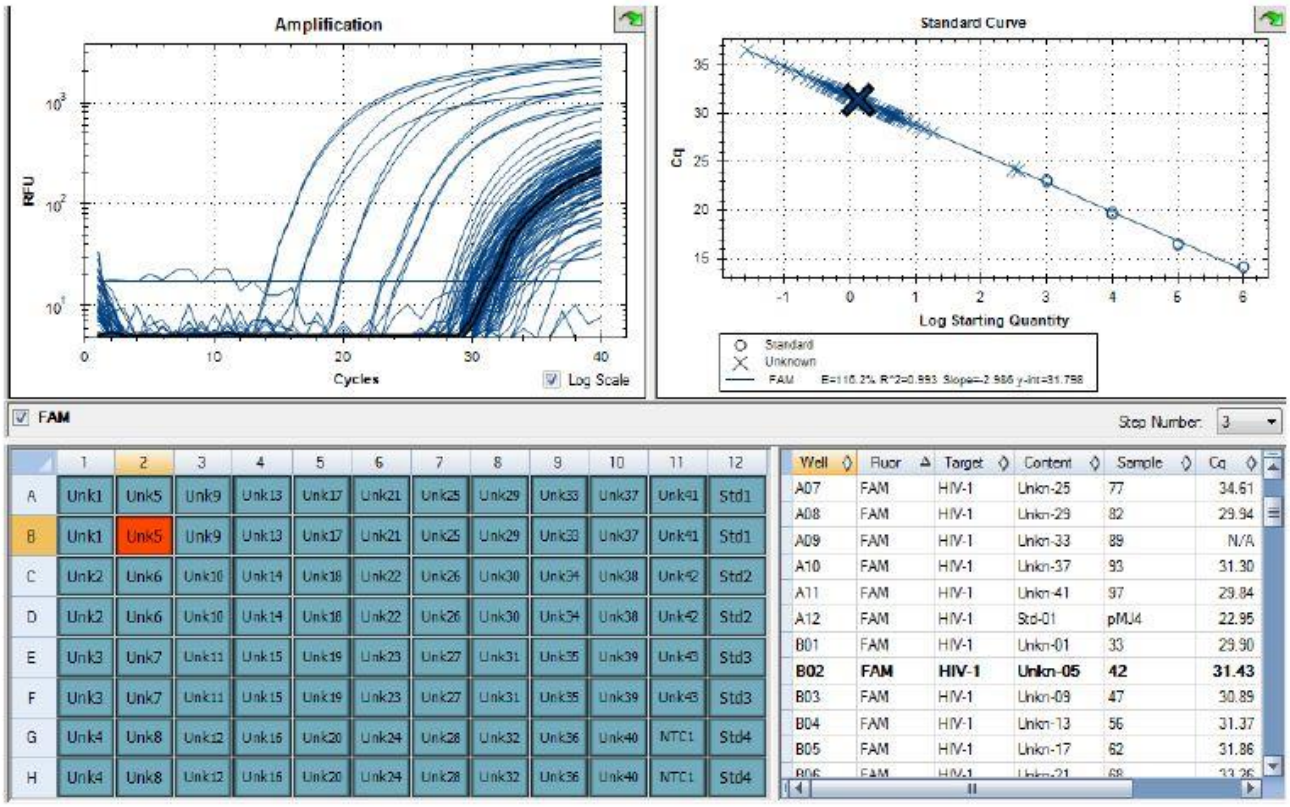
..... *.....*
Signature of translator *Signature of witness*

Appendix 5: CCR5 and HIV-DNA standardization Curves





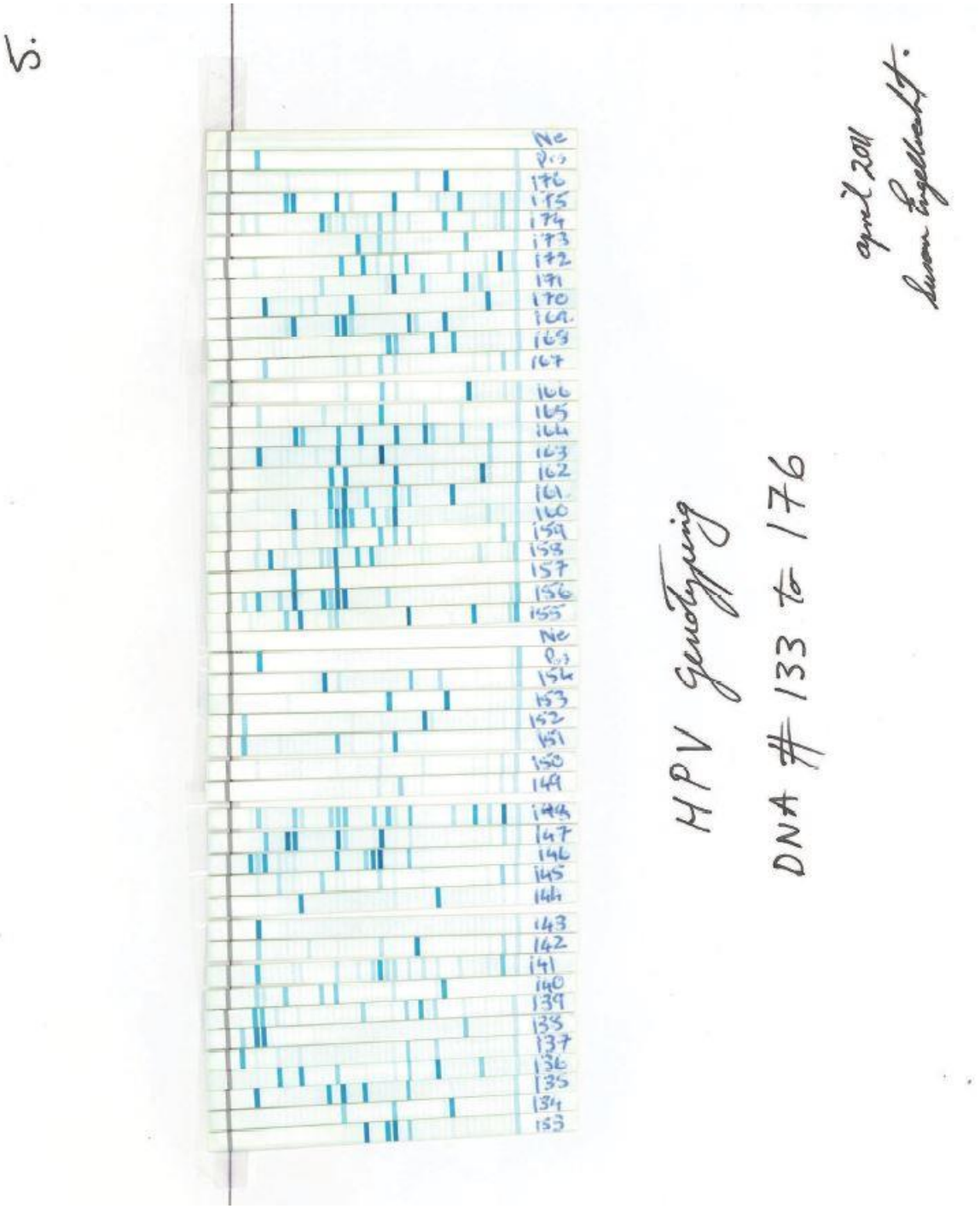




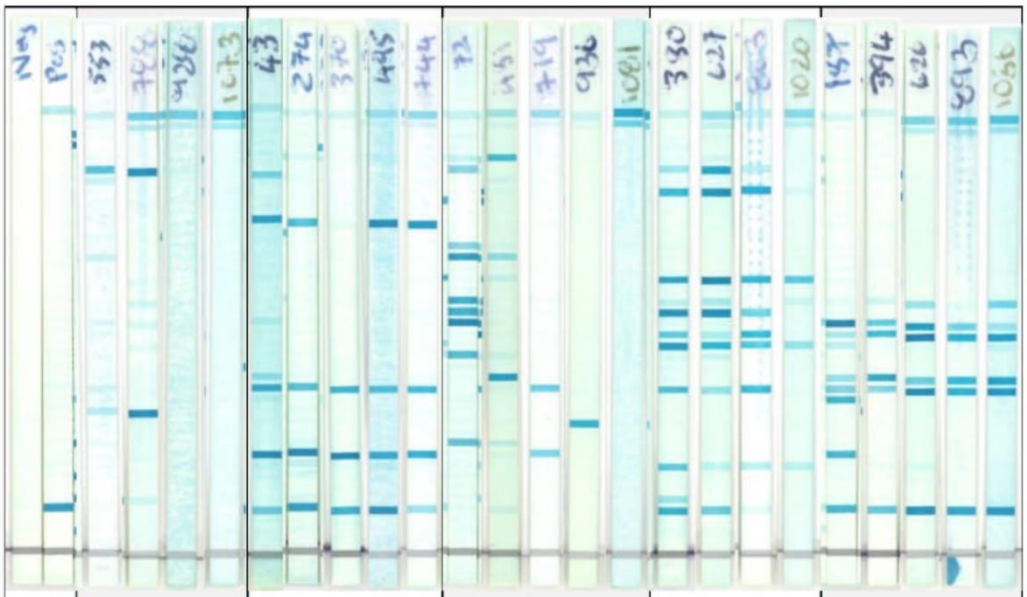
	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk1	Unk5	Unk9	Unk13	Unk17	Unk21	Unk25	Unk29	Unk33	Unk37	Unk41	Std1
B	Unk1	Unk5	Unk9	Unk13	Unk17	Unk21	Unk25	Unk29	Unk33	Unk37	Unk41	Std1
C	Unk2	Unk6	Unk10	Unk14	Unk18	Unk22	Unk26	Unk30	Unk34	Unk38	Unk42	Std2
D	Unk2	Unk6	Unk10	Unk14	Unk18	Unk22	Unk26	Unk30	Unk34	Unk38	Unk42	Std2
E	Unk3	Unk7	Unk11	Unk15	Unk19	Unk23	Unk27	Unk31	Unk35	Unk39	Unk43	Std3
F	Unk3	Unk7	Unk11	Unk15	Unk19	Unk23	Unk27	Unk31	Unk35	Unk39	Unk43	Std3
G	Unk4	Unk8	Unk12	Unk16	Unk20	Unk24	Unk28	Unk32	Unk36	Unk40	NTC1	Std4
H	Unk4	Unk8	Unk12	Unk16	Unk20	Unk24	Unk28	Unk32	Unk36	Unk40	NTC1	Std4

Well	Fluor	Target	Content	Sample	Cq
A07	FAM	HIV-1	Unkn-25	77	34.61
A08	FAM	HIV-1	Unkn-29	82	29.94
A09	FAM	HIV-1	Unkn-33	89	N/A
A10	FAM	HIV-1	Unkn-37	93	31.30
A11	FAM	HIV-1	Unkn-41	97	29.84
A12	FAM	HIV-1	Std-01	pMJ4	22.95
B01	FAM	HIV-1	Unkn-01	33	29.90
B02	**FAM**	**HIV-1**	**Unkn-05**	**42**	**31.43**
B03	FAM	HIV-1	Unkn-09	47	30.89
B04	FAM	HIV-1	Unkn-13	56	31.37
B05	FAM	HIV-1	Unkn-17	62	31.86

Appendix 6: Sample HPV-DNA Results



Appendix 7: Samples of Patient HPV-DNA Results

Subject ID	Specimen ID	ART	ART Days	CD4	CytoPath	Post Excision	Days since Excision	Sex	HPV Test Result Strips
Controls	-	-	-	-	-	-	-	-	
7833	553	No	-	167	Normal	No	-	active	
	788	Yes	182	309	Normal	No	-	active	
	928	Yes	320	334	Normal	No	-	active	
	1073	Yes	516	236	Normal	No	-	active	
7948	43	No	-	438	LSIL	No	-	active	
	274	No	-	407	LSIL	No	-	active	
	370	No	-	451	LSIL	No	-	active	
	495	No	-	494	Normal	No	-	active	
	744	No	-	521	LSIL	No	-	active	
7803	72	No	-	322	LSIL	No	-	active	
	481	Yes	14	121	ASCUS	No	-	active	
	719	Yes	210	620	Normal	No	-	abstinence	
	936	Yes	378	807	Normal	No	-	abstinence	
	1081	Yes	574	728	Normal	No	-	abstinence	
7749	380	No	-	182	LSIL	No	-	active	
	627	Yes	143	348	LSIL	No	-	active	
	863	Yes	311	547	LSIL	No	-	active	
	1020	Yes	479	479	LSIL	No	-	active	
7646	187	No	-	750	HSIL	No	-	abstinence	
	394	No	-	784	Normal	Yes	182	active	
	626	No	-	949	LSIL	Yes	362	active	
	893	No	-	889	Normal	Yes	553	active	
	1036	No	-	628	Normal	Yes	719	active	