Natural History of Human Papillomavirus Infections and Other Sexually Transmitted Infections in Rwanda-Immunological Aspects of the Uterine Cervix

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Cover illustration

Microscopic image from immunostaining with Toll like receptor 6 antibody of the rat uterine cervix, viewed at x200 magnification

Illustrated by Marie Francoise Mukanyangezi

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”Through persistence, self-knowledge, prayers, commitment, optimism, a resolute trust in God and the building of your own personal moral strength, you can enjoy the blessings of a deeper faith and face the difficulties of life with courage and confidence.”

- Norman Vincent Peale

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Abstract

Objective: Cervical cancer stands for the predominant cause of cancer death among Rwandan women. Chronic Human Papillomavirus (HPV) infection constitutes the main risk factor. We here assessed the prevalence and incidence of high-risk (HR)- and low-risk (LR)-HPVs, low-grade and high-grade squamous intraepithelial lesions (LSIL and HSIL) and cancer and associated risk factors in 400 HIV- and HIV+ Rwandan women. Whether HPV testing could serve as a screening method for detecting HSIL was analyzed. We also assessed prevalence and curing rates of different sexually transmitted infections (STIs) and sexual behaviour. Advanced cervical cancer is often treated with radiotherapy. In an animal model for radiation cervicitis we wanted to assess how the normal uterine cervix responds to ionization radiation and whether hyperbaric oxygen therapy (HBOT) may reverse these responses.

Methods: Women were interviewed, screened for STIs (baseline and 9 months) and underwent cervical sampling for cytology and a test for 37 HPV strains. Cytological samples were taken again 9, 18 and 24 months later in 100 HIV- and 137 HIV+ women. We explored whether the single nucleotide polymorphism (SNP) rs1297860 in IL28B correlates with

susceptibility to HPV infection and persistence as well as development of SILs. In the preclinical studies, rats underwent cervical irradiation and were either exposed to HBOT or no intervention. Immunological and oxidative responses induced by radiation were assessed and whether HBOT was able to reverse these responses.

Results: HPV16 and HPV52 were the most common HPV strains. The sensitivity was 78% and the specificity 87% to detect HSIL with HPV screening. Chronic and incident HR-HPV infections occurred more frequently in HIV+ women than in HIV- women. HSIL or cancer was diagnosed in 38% of HIV+ women with persistent HR-HPV infections. The C/T and T/T genotypes of the IL28B SNP rs12979860 were more common in the group of women contracting HPV compared with women not contracting HPV. STIs were common in Rwandan women and the use of condoms was not affected by present STIs. TLR5, TRIF, NF-κB, oxidative stress (8-OHdG) and antioxidant enzymes (SOD-1 and catalase) were up regulated, while cytokines were down-regulated 14 days after cervical irradiation. Changes in 8-OHdG and catalase were normalized after HBOT.

Conclusions: HPVs and STIs are common among Rwandan women. HPV screening may be of particular importance if provided for risk patients such as HIV+ women that develop more often persistent HPV infections and HSIL. Ionizing radiation induces oxidative stress and immune responses in the cervix that may be reversed by HBOT.

Keywords: Human papillomavirus, cervical cancer, squamous intraepithelial lesion, screening, IL28B SNP rs12979860, Rwanda, radiotherapy, hyperbaric oxygen therapy

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: Cervixcancer utgör för den vanligaste cancerrelaterade dödsorsaken i Rwanda. Detta orsakas av en hög förekomst av humant papillomavirus (HPV) som är den viktigaste riskfaktorn för cancerformen. Vi undersökte prevalensen och incidens av hög-risk (HR)- och låg-risk (LR)-HPV, låggradiga och höggradiga skivepitellesioner (LSIL respektive HSIL) och cancer och kopplade riskfaktorer hos 400 HIV- och HIV+ rwandiska kvinnor. Vi undersökte också om HPV test kan utgöra en screeningmetod för att detektera HSIL. Vidare studerade vi prevalens av sexuellt överförda infektioner (STI) och sexuellt riskbeteende. Lokalt avancerad cervixcancer behandlas ofta med strålbehandling men är associerad med strålrelaterade biverkningar. I en djurmodell för strålcervicit undersökte vi hur strålning påverkar den normala cervixslemhinnan och om hyperbar oxygenterapi (HBOT) kan reversera förändringar inducerade av strålning.

Metoder: Kvinnor intervjuades och screenades för STI vid baseline och vid 9 månader. Vid baseline, 9, 18 och 24 månader togs prov från cervix för cytologi och för screening av 37 HPV typer. Vi undersökte om single nucleotide polymorphism (SNP) rs1297860 i IL28B korrelerar med ökad risk för att infekteras av HPV och utveckla HPV persistens och SIL. Vi strålade cervix hos råttor och 14 dagar senare blev en grupp behandlad med HBOT och en grupp fick ingen HBOT. Immunologiska och oxidativa responser inducerade av strålbehandling undersöktes åren och om HBOT kunde reversera detta undersöktes 28 dagar efter strålning.

Resultat: HPV16 och HPV52 var de vanligaste HPV typerna och HPV screening gav en sensitivitet på 78 % och en specificitet på 87 % för att detektera HSIL. Kronisk och incident HR-HPV infektion var vanligare hos HIV+ kvinnor än HIV- kvinnor. HSIL eller cancer vid cytologi förekom hos 38 % av HIV+ kvinnor som utvecklat persistent HR-HPV infektion. C/T och T/T genotyperna av IL28B SNP rs12979860 var vanligare hos kvinnor som smittats av HPV än kvinnor som inte smittats av HPV. STI var vanliga bland rwandiska kvinnor. De flesta HIV+ kvinnor uppgav användning av kondom vid samlag men HIV- kvinnor använde kondom i låg grad trots vetskap om STI. TLR5, TRIF, NF-κB, oxidativ stress (8-OHdG) och antioxidativa enzymer (SOD-1 och catalase) uppreglerades medan cytokiner nedreglerades i cervix 14 dagar efter strålning. Förändringar i 8-OHdG och catalase normaliserades efter HBOT.

Konklusion: HPV och STI är vanliga bland kvinnor i Rwanda. HPV screening kan vara av stort värde för riskpatienter såsom kvinnor med HIV som oftare utvecklar persistenta HPV infektioner och HSIL. Strålning inducerar oxidativ stress och immunologisk respons i cervix som kan reverseras med HBOT.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Mukanyangezi MF, Sengpiel V, Manzi O, Tobin, G, Rulisa, S, Bienvenu, E and Giglio D. Screening for human papillomavirus, cervical cytological abnormalities and associated risk factors in HIV-positive and HIV-negative women in Rwanda. *HIV Medicine*. 2018:19(2): 152-66.

II. Mukanyangezi MF, Rugwizangoga B, Manzi O, Rulisa S, Hellstrand K, Tobin G, Martner A, Bienvenu E and Giglio D. Persistence Rate of Cervical Human Papillomavirus Infections and Abnormal Cytology in Rwanda. Submitted, 2018.

III. Mukanyangezi MF, Manzi O, Tobin G, Rulisa S, Bienvenu E and Giglio D. Sexual Risk Behaviour in a Cohort of HIV Negative and HIV Positive Rwandan Women. In press in *Epidemiology and Infection*. 2018.

IV. Mukanyangezi MF, Podmolíková L, Tobin G and Giglio D. Radiation Induces Changes in Toll-Like Receptors of the Uterine Cervix of the Rat. Submitted, 2018.

V. Mukanyangezi MF, Dahlqvist A, Oscarsson N, Winder M, Seeman-Lodding H and Giglio D. Hyperbaric Oxygen Therapy Reverses Changes Induced by Irradiation of the Uterine Cervix of the Rat. Manuscript. 2018.

CONTENT

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome	ECL	Enhanced chemiluminescence
AIS	Adenocarcinoma in situ	EPO	Erythropoietin
APCs	Antigenic-presenting cells	G	Group
ART	Antiretroviral therapy	G-CSF	Granulocyte colony-stimulating factor
CD4	Cluster of differentiation	GM	Granulocyte-macrophage
CIN	Cervical intraepithelial neoplasia	GP	General primer
CIS	Carcinoma in situ	GRO/KC	Human growth-regulated oncogene / Keratinocyte chemoattractant
CTL	Cytotoxic T lymphocyte	GS	Goat serum
DCs	Dendritic cells	HDI	Human development index
FGT	Female genital tract	IARC	International Agency for Research on Cancer
HBOT	Hyperbaric oxygen therapy	ICH	International conference of harmonization
HBsAg	HBV surface antigen	IFNL	Interferon lambda
HBV	Hepatitis B	IHC	Immunohistochemistry
HCV	Hepatitis C	IUD	Intrauterine device
HIV	Human immunodeficiency virus	KDa	Kilodalton
HO-1	Heme oxygenase 1	L	Late
HPV	Human papillomavirus	LDS	Lithium dodecyl sulfate
HR	High risk	MCP-1	Monocyte chemotactic protein 1
HSIL	High-grade squamous intraepithelial lesion	MIP-1α	Macrophage inflammatory protein
IFN-γ	Interferon gamma	mRNA	Messenger ribonucleic acid
IL	Interleukin	MyD88	Myeloid differentiation primary response 88
KCs	Keratinocytes	NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
LCs	Langerhans cells	Nrf2	Nuclear factor erythroid 2-like 2
LR	Low risk	ORs	Odd ratios
LSIL	Low-grade squamous intraepithelial lesion	ORFs	Open reading frames
M	Macrophage	PAMPs	Pathogen associated molecular patterns
MHC	Major histocompatibility complex	PBS	Phosphate buffered saline
NK	Natural killer	PHR	Possibly high risk
RANTES	Regulated on activated normal T-cell expressed and secreted	PRRs	Pattern recognition receptors
ROS	Reactive oxygen species	RP	Ribosomal protein
SNPs	Single nucleotide polymorphisms	RT-PCR	Real time polymerase chain reactions

SSA	Sub-Saharan Africa	RTIs	Reproductive tract infections
STIs	Sexually transmitted infections	SCBT	Santa Cruza Biothechnology
(H+L)	(Heavy and light chains)	SCC	Squamous cervical cancer
HRP	horseradish peroxidase		
8-HdG	8-hydroxy-2'-deoxyguanosine	SDS	Safety data sheet
ABI	Applied Biosystem	SIL	Squamous intraepithelial lesions
ACG	Atypical glandular cells	SOD	Superoxide dismutase
AORs	Adjusted (ORs)	TBS-T	Tris -buffered saline containing Tween 20
ASC-H	Atypical squamous cells can not exclude high grade lesions	TGFβ1	Transforming growth factor β
ASCUS	Atypical squamous cells of undetermined significance	Th	T help lymphocytes
CCR	Chemokine receptor	TLR	Toll-like receptor
CFRs	Case report forms	TNF-α	Tumour necrosis factor alpha
CHUB	Centre Hospitalier Universitaire de Butare	Treg.	Regulatory T cell
CHUK	Centre Hospitalier Universitaire de Kigali	TRIF	TIR-domain-containing adaptor inducing IFN-b
CIs	Confidence intervals	TYMS	Thymidylate synthetase
CXCL1	Chemokine (C-X-C motif) ligand 1	TZ	Transformation zone
DAMPs	Damaged -associated molecular patterns molecules	VEGF	Vascular endothelial growth factor
DAPI	4', 6-diamidino-2-phenylindole	VIA	Visual inspection with acetic acid
dsDNA	Double stranded deoxyribonucleic acid	WB	Western blot
E	Early		

1 GENERAL INTRODUCTION

1.1 Introduction

Rwanda is a landlocked small country in Central/Eastern Africa with 26.000 km^2 of area and a population of around 12 million. The life expectancy of men is 66 years and of women 70 years [1]. Rwanda, one of the poorest countries in the world is the country with the highest enrolment in health insurance in Sub-Saharan Africa [2]. It was also the first country in Africa to implement the cervical cancer vaccine with high coverage [3]. However like many other developing countries, Rwanda is in a phase of epidemiological transition. While communicable diseases remain the major causes of morbidity and mortality in the, the increasing incidence of non-communicable diseases *i.e* cancer and hypertension, results in a double burden of diseases [4].

This chapter discusses the background literature reviewed for this study. It describes the general characteristics of human papillomavirus (HPV) and its interaction with the immune system of the uterine cervix. The mechanisms involved in the development of HPV-induced cervical cancer, the current state on cervical cancer prevention strategies and the need to develop newer technologies better adapted to low-income countries are reviewed. Finally, the aims of the project showing the rational for this study and its contribution to the research are presented.

1.2 Epidemiology of HPV infections and cervical cancer

It is well known that chronic HPV infection is a risk factor to almost all cervical cancer cases [5]. Worldwide, 4.5% of all new cancer cases are associated with HPV infection [6]. Among these cases, cervical cancer accounts for 83% and women in less developed countries are the most affected [7]. According to the recent reports, worldwide cervical cancer ranks fourth for both incidence and mortality [8]. Each year more than half million women aged 15-44 years are diagnosed with the disease and more than a quarter of a million of die worldwide [5]. HPV16 and HPV18 infections are responsible for 70% of all cervical cancer cases worldwide [5, 9].

1.2.1 Cervical cancer in sub-Saharan Africa

Studies show that countries in sub-Saharan Africa (SSA) experience 85% of the total cervical cancer burden in the world [10]. In SSA, cervical cancer is the second most frequent cause of female cancer and the leading cause of female cancer deaths in women aged 15–44 years [11]. In 2017, there were 93,225 new cases of cervical cancer and 57,381 deaths were reported worldwide [12]. The annual age-standardized incidence and mortality rates are highest in East Africa (Malawi, Zimbabwe, and Uganda). These countries also have the highest HPV prevalence in the general population, i.e., (20.5% in the general population compared with globally 4.1% [11]. The age-standardized mortality rate for cervical cancer in East Africa was 28 cases per 100 000 women compared with only 3 cases per 100 000 women in North Africa [11] It is expected that due to aging population and growth of the population in SSA, but also to the lack of access to appropriate prevention services and concomitant human immunodeficiency virus (HIV/acquired immunodeficiency syndrome (AIDS) epidemic, cervical cancer incidence and mortality rates in SSA will rise over the next 20 years [9, 13-15].

1.2.2 Type specific HPVs prevalence differs between Western countries and SSA

In contrast to Western countries, in SSA, HPV16 and HPV18 contribute to only 60-65% of all cervical cancer cases [13]. HPV35, 45, 52, 56 and HPV58 are significantly more common in SSA than in Western countries [9, 13]. This contribution of HPV16 and HPV18 to the prevalence of cervical infection did not changed in 60 years [16]. Similarly to the Western countries however, the combined relative contribution of HPV16, 18, 31, 33, 35, 45, 52, and HPV58 to the total burden of cervical cancer was estimated at 91% [17] and the non-oncogenic strains HPV6 and HPV11 are accountable for about 90% of genital warts. Few studies have been conducted on the prevalence of HPV in Rwanda. In HIV positive Rwandan women with normal cytology, HPV16, HPV35, HPV52 and HPV58 were found to be the most prevalent strains, while HPV16, HPV33 and HPV35 and HPV58 were found to be most prevalent among women with high-grade squamous intraepithelial lesion (HSIL or cancer [18]. In 2016, when we were conducting our HPV prevalence study (paper I), a large-scaled study in Rwanda showed that the most prevalent types among women with normal cytology were HPV 16, HPV52 and HPV35, whereas HPV16, HPV58 and HPV18 were the most prevalent among women with HSIL or cancer [19].

1.2.3 Cervical cancer in Rwanda

Cervical cancer is the most common cancer-related deaths cause in Rwandan women. It is responsible for more than 1000 new cases diagnosed each year and it is classified as the leading cause of deaths among women of 15-44 years old [20]. However, the real number of cervical cancer cases may be much more in Rwanda due to poor access to health care in the country. Among HIV positive women in Rwanda, 46% are positive for carcinogenic HPV subtypes [18]. Studies in Rwanda show that 9% of HIV+/HPV+ women are diagnosed with cervical intraepithelial neoplasia (CIN) grade 3 [18, 21, 22].

1.3 Human papillomavirus

1.3.1 General characteristics

HPV is a small, non-enveloped, double-stranded deoxynucleic acid (dsDNA) virus that belongs to the Papillomaviridae family [23]. There are five major known HPV genera: (1) α-papillomavirus, (2) β-papillomavirus, (3) ϒ-papillomavirus, (4) mu-papillomavirus and (5) nu-papillomavirus [23]. The oncogenic mucosal HPV types in the α-papillomavirus genus are a major cause of cervical cancer [24]. The viral genome can be divided into the early (E) and late (L) regions, containing open reading frames (ORFs) coding for viral proteins (Fig.1).

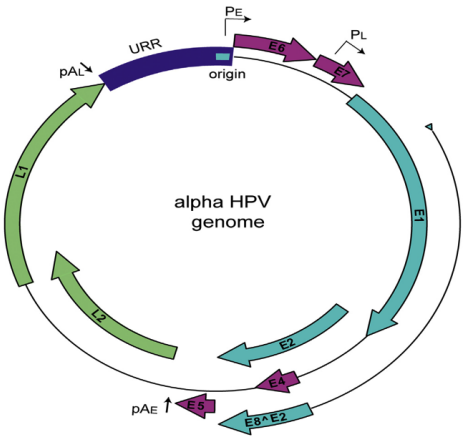

***Figure 1**. HPV genome. Alpha-HPVs have a circular dsDNA genome of approximately 8000 base pairs [25]*

The E region encodes proteins (E1, E2, E4, E5, E6 and E7) involved in control of transcription, viral DNA and cell replication. The L region contains the genes that encode the viral capsid

proteins (L1 and L2). The classification of HPV is based on the nucleotide sequences of ORF. HPV infects epithelial cells and its replication cycle is closely linked to epithelial cell differentiation [26, 27]. In dividing basal epithelial cells, dsDNA episomal genome of the HPV enters the nuclei [28]. Upon basal cell division, an infected daughter cell begins the process of keratinocyte differentiation that activates a strongly organized pattern of viral gene expression to achieve a productive infection [29]. HPV proteins disturb different cellular processes in the epithelial cell. Two of the most important features are that E7 binds to and degrades the tumor suppressor Rb, while E6 binds to and inactivates the tumor suppressor p53 [30]. This leads to disruption and prolongs the normal cell cycle of the host cell, suppresses apoptosis and disposes the cell to neoplastic transformation [31].

1.3.2 Classification of HPVs

HPV strains can be divided into mucosotropic types, which are mainly found on the mucous epithelium of the oropharynx and anogenital tract, and cutaneous types, which predominantly infect the skin [32]. Both types can be grouped in high risk (HR)-HPVs or oncogenic HPVs and low risk (LR)-HPVs or non-oncogenic HPVs [33]. In 2003, in a pooled data of 11 case-control studies from eleven countries by using GP5+/6+ primers, using GP5+/6+ primers, assessed HPV DNA assessed in 1918 women with cervical cancer and 1928 control women. HPV DNA was detected in 96.6% of cancer patients and in 15.6% of controls [33]. The authors classified 15 HPV strains as HR-HPVs (16,18,31,33, 35, 39, 45, 51, 52, 56, 58,59,68, 73 and 82), 3 HPV types as probable HR-HPVs (26, 53, 66) and 12 HPV as LR-HPVs (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP108) [33].

In 2008, three categories were suggested for the 51 established genital HPV types. Those categories are: (1) mild or possibly non-carcinogenic HPV category, including all the genital HPVs from G1 to G5 (G1: HPV61 and 61, 62,72, 81, 83, 84, 86, 87, 89, 102; G2: HPV71, 90 and 106; G3: HPV7, 40,43,79 and 91; G4: HPV6, 11, 13, 44 and 74; G5: HPV32 and 42), (2) moderate or possibly carcinogenic HPV category, from G6 to G9 (G6: HPV18, 39, 45, 59, 68, 70, 85 and 97; G7: HPV30, 53, 56, 66; G8: HPV 26, 51, 69 and 82; G9: HPV 34, 73) and (3) severe or carcinogenic HPV category and from group G10 (G10: HPV16, 31, 33, 35, 52, 58 and 67) [34]. In 2009, the International Agency for Research on Cancer (IARC) classified 18 HPV mucocutanous belonging to the α-genus, as HR-HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) or probably HR-HPVs (HPV26, 53, 66, 68, 73, 82), while other 12 HPV types were classified as LR-HPVs (HPV6, 11, 40, 42, 43, 44, 54, 55, 61, 72, 81, 89). Twenty-five HPVs were classified as of undetermined risk (HPV2, 3, 7, 10, 27, 28, 29, 30, 32, 34, 55, 57, 62, 67, 69, 71, 74, 77, 83, 84, 85, 86, 87, 90 and 91) [35].

1.3.3 Natural history of HPV infection

Several studies have described the natural history of HPV infection and its association with the development of cervical cancer [36-43]. Here we will use a schematic model of the natural history of HPV infection and cervical cancer as presented by Patti E. Grant and Rachel L. Winer (2017) (Fig.2). We will also present some unresolved issues, which may impede the development of new tools for improving the management and prevention of HPV and early detection and treatment of HPV-associated cancer [44].

The natural history of cervical HPV cancer starts by HR-HPV infection via sexual exposure in a newly sexually active adolescent or a young adult women who are at the highest risk of HPV acquisition [45] . Studies show a peak for HPV infection prevalence around 20-25 years of age [46, 47]. This specific age is characterized with high sexual activity [45]. Other observations also support that the number of sexual partners and a young age at sexual debut strongly correlate with HPV infection [48]. The contribution of other viral related factors such as viral load and coinfection with other HR-HPV are still under discussion [49].

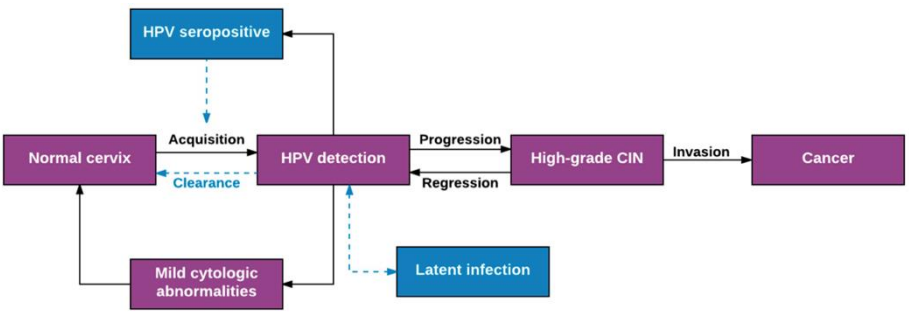

Figure 2. *Schematic model of the population-level natural history of human papillomavirus infection and cervical cancer: Purple boxes indicate well-accepted natural history model parameters; blue boxes represent uncertainties [50].*

The majority (≈90%) of newly acquired HPV infections become cleared within 1-2 years [51-54]. This is what we normally call “viral clearance”. During the productive HPV infection, low-grade squamous intraepithelial lesion (LSIL) /CIN1 may be clinically detected but usually they are transients and cleared within 1-2 years [46, 55, 56]. Latent infections by HPV are also common [57]. Serum antibodies are generated in around 60% of the time following the HPV infection [58]. The minority of HPV infections is persistent and detectable over 12 months

and this long persistence increases the risk of carcinogenesis [46, 55, 56]. The virus clearance after long persistent infections are uncommon [50]. However several questions persist around the standard time for the infection to be qualified as persistent [49]. It is also difficult to distinguish a cleared infection to a latent, non-detectable infection [59]. Another issues resides in the definition of a new infection following a so-called clearance [50]. Some detected HPV infections could be the result of reactivation of a latent infection and not the result of contracting a new infection by sexual activity [60]. Women may also develop a reinfection not related to sexual exposure but due to an autoinfection from e.g. the anal area [61]. Most studies of the natural history of cervical HPV cancer failed to identify factors associated with clearance of HPV and regression of precancerous lesions [47].

1.3.4 Clinical characteristics of HPV infection

HPV infections are among the most common sexually transmitted infections (STIs). The majority of women acquire cervical HPV infection soon after the onset of sexual activity [62]. However, notwithstanding the fact that around 80% of sexually active women will face HPV at some point in their lives, the majority will experience natural elimination of HPV infection because of an intact immune system. HPV16 infection is eradicated within a two year-period in the majority of women [12, 63]. Persistent infection with a HR-HPV puts women at high risk of developing precursors of cervical cancer or carcinoma itself. Clinically, HPV infections manifest in one of three possible results, depending largely on which HPV type is involved.

(i) The first results in latent or inactive infection, in which only few people know that they are infected, since visible symptoms are rarely produced and the infected area remains cytologically normal [64]. This infection is only detected by HPV DNA detection methods. In the general population this is present in 2-44% of women and it can also be detected among women who are not yet sexually active [65].

(ii) The second results in subclinical infection with minimal clinical manifestations [66] which usually are diagnosed by colposcopy or cytology or histology [67]. These lesions represent 60% of all external anogenital HPV infections and 95% of all cervical HPV infection [68] and CIN is the most common manifestation of HPV in the cervix [69].

(iii) The third results is an active infection (clinical form) [70]. It is associated with HR-HPV types in which the virus causes changes in infected cells, which may result in penile, urethral, bladder, vaginal, vulvar, or cervical intraepithelial neoplasia[70]. HR-HPV types include types associated with HSIL/CIN3 and cervical cancer [71].

1.3.5 Determinants of HPV-related cervical cancer

Chronic HPV infection does not constitute the only contributing factor to development of cervical cancer. These other variables are considered as determinants of HPV infection acquisition and persistence [72-77]. The importance of these HPV cofactors is justified in planning for cervical cancer preventive strategies, which will be discussed in the last part of this chapter. The following part summarizes what is already known and what is still to be clarified about relevant determinants of cervical cancer categorized under two categories: host and viral factors (Fig. 3).

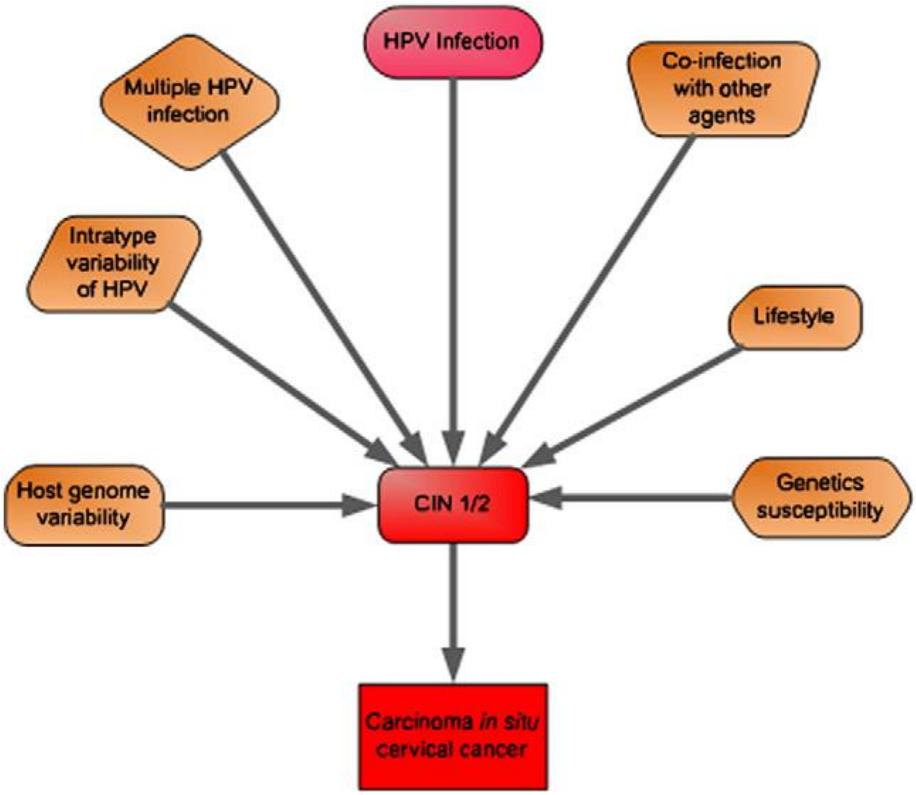

Figure 3. *Several genetics and environment factors involved in susceptibility to cervical cancer [78]*

Viral factors

a) HPV infection and HPV types

As mentioned, the most common HPV types causing cervical cancer are HPV16 and HPV18 [16, 79]. CIN2/3 lesions associated with HPV16 are less likely to resolve spontaneously than those caused by other HPV strains [80, 81]. However, there is geographical variability in the distribution of other oncogenic HPV types [82].The reason for the variability is not yet identified but it has been speculation on the ethnogeographical differences contribution [83, 84].

b) Multiple HPV type infections

Infection by multiple HPV types has been suspected to constitute a risk factor for cervical lesions to progress to cancer. Helen Trottier et al., (2006) found that infection with multiple types HPV seemed to act synergistically in carcinogenesis [85]. Wentzenzen N. et al., (2009) and Salzar KL et al., (2015) did not found any interaction/synergism in multiple HPV infections [86]. According to Cuschieri K.S and collaborators (2010), multiple HR-HPVs are not more common in HSIL than in LSIL [87]. Their finding was supported by the IARC, in concluding that women infected by multiple HPV types are not at higher risk of cervical cancer than those infected with a single HPV type [88].

c) HPV viral load

Studies show that the development of cervical cancer precursors is associated with an elevated HR-HPV viral load [89-92]. Surprisingly, a study conducted on 2080 women followed for 10 years concluded that high HR-HPV viral load was not associated with the risk for development of CIN3+ [93]. Furthermore, Cheung Jo L.K.et al., 2009 in their study did not find any correlation between HPV18 load and an increase in cervical lesions severity [94].

Host factors

a) Sexual behaviour

Most of HPV infections are acquired through sexual contact and acquisition is strongly associated with the number of sexual partners [95, 96]. Sexual behaviour in women and their sexual partners such as having multiple sexual partners [97] and vaginal intercourse at early age are known to put women at a higher risk of contracting HPV infection [98]. Several studies have found that young age at sexual debut as an important risk factor for HPV infection and for cervical cancer development [99-101]. For example, a large meta-analysis

study combined data from 10,773 women with invasive cervical carcinoma, 4,688 with CIN3/carcinoma in situ (CIS) and 29,164 women without cervical carcinoma (21 studies), to assess association between lifetime sexual partners or age at first intercourse and cervical carcinoma. As results, the analysis demonstrated the risk of developing either invasive cervical carcinoma or CIN3 to increase with the increase in number of lifetime sexual partners and the early age at first intercourse [102].

b) Parity

High parity has been shown to be associated with an increased risk of cervical cancer [84, 98, 103]. From a population-based study conducted among HPV infected and non-infected women, Hildesheim A et al., (2001) found multiparty (≥ 3 pregnancies) and smoking as risk factors for HSIL and cancer [104].

c) Poverty

Studies show that poverty is associated with a low age for beginning sexual activity [82]. Poverty is the strongest determinant of the incidence and of mortality of cervical cancer [105]. Globally, it has been shown that human development index (HDI) and poverty rate explain about 52% of the variability in cervical cancer [106-108]. In addition, factors such as lack of education, an employment, low socioeconomic level, rural residence and insufficient access to the health care were found to be associated with cervical cancer mortality [109]. In fact all those factors are related to the poverty [110].

d) Marital status

Widows and separated women in Africa are at a higher risk for contracting HPV infection than married women due to risky sexual behaviour such as an increased number of sexual partners [111-115]. Unmarried women are less likely to seek medical attention for their health problem due to financial issues or due to the lack of family and social support [98, 116].

e) HIV infection

Concomitant HIV infection has been shown to be an important risk factor for HPV infection [14, 15, 117, 118]. HIV infected women develop more often persistent HPV infection. Clifford GM et al., (2016) confirmed those results among HIV positive African women [119]. Furthermore, cervical cancer is considered to be an AIDS-defining illness [120, 121]. In a study from South Africa, it was demonstrated that women infected with HIV-1 presented with late

stage of cervical cancer at ages 15 years younger than HIV-negative women [122]. Studies suggest that there are synergistic interactions between HIV and HPV infections [14].

f) **HPV coinfection with STIs or other reproductive tract infections (RTIs) other than HIV**

STIs other than HIV constitute also a risk factor for incident and persistent HPV infections [123-125]. The change in vaginal microbiota has been shown to play a role in the acquisition and persistence of HPV and in the subsequent development and progression of CIN [126, 127]. In addition, it is suggested that persistent infections caused by STIs increase the access of HPV into the deeper cervical tissue and cause cervical cell abnormalities [128, 129]. HPV-infected women who have concurrent infections with chlamydia [130, 131], gonorrhea, cytomegalovirus [132] or Herpes Simplex Virus type-2 (HSV-2) [131, 133, 134] are at a greater risk for HPV persistence and development of cervical cancer.

g) Smoking

Smoking has been demonstrated to constitute a risk factor for cervical cancer in several studies [84, 135-137]. Smoking was the most significant environmental risk factor for cervical cancer in Sweden [138]. A pooled analysis of IARC multi-centric case-control studies confirmed smoking to increase the risk of cervical cancer among HPV positive women [139]. The presence of nitrosamine, a carcinogenic component in tobacco smoking [140], in human cervical mucus further strengthened the association between cervical cancer and tobacco smoking [141].

d) Reproductive factors

Long-term use of oral contraceptive for more than five years has been suggested to negatively affect the immune modulation effect of the body, which can lead to neoplastic changes in the cervical epithelium [84, 135, 142]. Contrary to this observation, a recent study on a cohort of 16-17 year-old women found the use of oral contraceptives to protect against cervical abnormalities [143]. The use of intrauterine device (IUD) may also constitute a protective cofactor against cervical carcinogenesis [79]. One theory put forward was that IUD protects against cervical carcinogenesis by triggering the cellular immunity [79].

e) Host genetic factors

Genetic factors in the host play an important role in the susceptibility to contract HPV infection and to develop cervical cancer. These factors may also regulate the rate of disease progression [144]. Variations in different immune response genes lead to subtle changes in the antiviral and anti-tumour immune responses, which eventually lead to differences in the

risk of developing cervical cancer [145]. Single nucleotide polymorphisms (SNPs) in immune-regulatory genes may be associated with the risk of developing cervical cancer. SNP in the promoter of tumor necrosis factor-α (TNF-α) was associated with the risk to develop cervical cancer in a cohort of Argentinian women [146]. SNPs at rs361525, rs1800629, and rs1799964 of the TNF-α promoter were also associated with an increased risk of cervical cancer in Chinese women [147]. SNPs in IL4, IL6, IL10 and transforming growth factor β (TGFβ1) have also been shown to be associated with the development of cervical cancer [148]. The toll-like receptor (TLR) 9 2848 G/A polymorphism was associated with the risk to develop cervical cancer in Chinese women [149]. Although the African population is characterized by a high heterogeneity, only few studies have been conducted in Africa on whether the immune system contributes to the susceptibility to contract HPV and develop cervical cancer. SNP in the chemokine receptor 2 (CCR2) showed that South African women with CCR2-64I variant had a higher risk to develop cervical cancer [150]. In Nigeria, SNPs at rs2305809 in ribosomal protein (RP) S19 and rs2342700 in thymidylase synthetase enzymes (TYMS) under allelic model were highly associated with prevalent HR-HPV [151]. In Rwanda there is a scarcity in the literature on cervical cancer and few available studies are limited on small groups of women. Thus, the generalizability of these results is not possible.

1.4 HPV infection immunology

1.4.1 Immune system defense against HPV induced cervical lesions

The immune system protects the body from possibly harmful substances by recognizing and responding to antigens. Antigens are usually proteins on the surface of cells, viruses, fungi, or bacteria. Non-living substances such as toxins, chemicals, drugs, and foreign particles can also be antigens. The immune system recognizes and destroys or tries to destroy antigens that it recognizes as harmful.

Human cervix description and immune system

The cervix is the lower end of the uterus and both are parts of female genital tract (FGT; Fig. 4). In the adult, the ectocervical and endocervical region comprise 1/3 of the length of the uterus. The ectocervix is lined by a stratified squamous mucosa containing abundant glycogen. The underlying fibrovascular connective tissue of the lamina propria merges with smooth muscle bundles [152, 153]. At the cervical os, the squamous epithelium changes to a

tall columnar mucinous epithelium. This squamocolumnar junction is called the transformation zone (TZ).

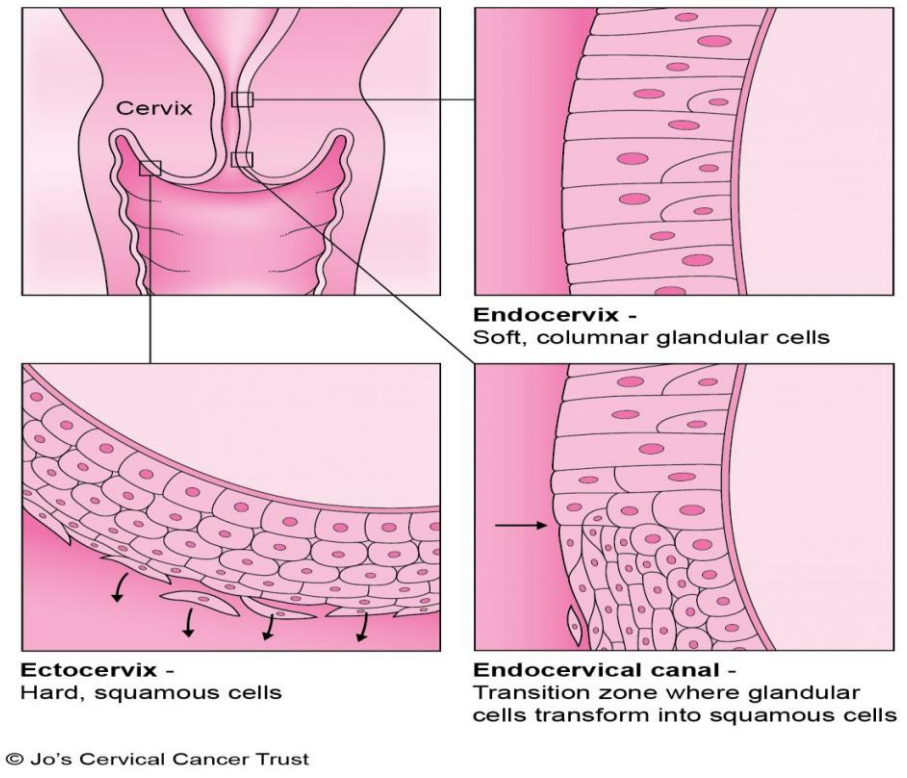

***Figure 4**. Human female cervix histology [154]*

Under the columnar mucosa in the lamina propria, there are prominent branching tubular glands also lined by a columnar mucosa producing mucin [7]. These are endocervical glands that extend into the lower uterine segment along the endocervical canal. The nature of the epithelial lining of the cervix varies according to location, with both columnar and squamous epithelia present at different locations within the cervix [65]. Most of the ectocervix consists of stratified squamous epithelium similar to that found in the vagina. The parts of the cervix closer to the uterus are covered by epithelia consisting of simple columnar epithelium, or rectangular column-like cells, which secrete mucus [65]. This glandular epithelium covers a varying portion of the ectocervix, as well as lining the endocervical canal [152]. The mucosal tissue of the cervix is a key factor for its immunity [155].

Concepts of the innate and acquired immunity

a) Innate immunity

1. Cells of the innate immunity

The innate immunity comprises the anatomic (skin and mucous membranes) and physiological barriers (temperature, pH, lysozymes and circulating factors such as interferon (IFN) and complements). It also comprises the inflammatory (cellular and chemical mediator of the inflammatory response) and phagocytic barriers (granulocytes, peripheral monocytes, tissue macrophages and dendritic cells). Macrophages and dendritic cells present antigens to be recognized by lymphocytes and therefore called antigenic-presenting cells (APCs) [156, 157]. They also present foreign materials to the cells of the immune system and regulate the immune response [158, 159]. In addition, the innate immune system comprises natural killer (NK) cells, mast cells and the complement system [160].

2. Pattern recognition receptors and toll-like receptors

Pattern recognition receptors (PRRs) are a family of receptors that recognize highly conserved antigenic structure, termed pathogen associated molecular patterns (PAMPs) [157, 161]. PAMPs are shared by large groups of pathogens. PRRs are secreted or expressed at the surface of the cells [162-164]. PRRs also recognize molecules that are released from damaged or necrotic host cells. Those molecules are called damaged-associated molecular patterns (DAMPs) [165]. TLRs constitute an important family of the PRR, mainly expressed on phagocytes and T-regulatory cells. Ten members of the TLR family have been identified in humans (TLR1, 2, 3, 4, 5, 6, 7, 8, 9,10) and each recognizes a small range of conserved pathogens molecules [166, 167]. The binding of PAMPs to TLRs induces the production of proinflammatory cytokines and the up-regulation of surface co-stimulatory molecules [168]. Recent studies have identified intermolecular signaling pathways specific for individual TLRs that lead to the release of cytokines specific for particular PAMPs [169]. The ability of individual TLRs to discriminate among different PAMPs is an important determinant of the unique gene expression profile activated in the host by different invading pathogens or environment danger signaling [170]. Thus, TLRs confers a certain degree of specificity for the innate immune response. Moreover, TLR-mediated recognition represents a cross-talk between the innate and the acquired immune system [171].Our focus on the innate immune system has been specifically on TLRs and their downstream molecules (Fig 5).

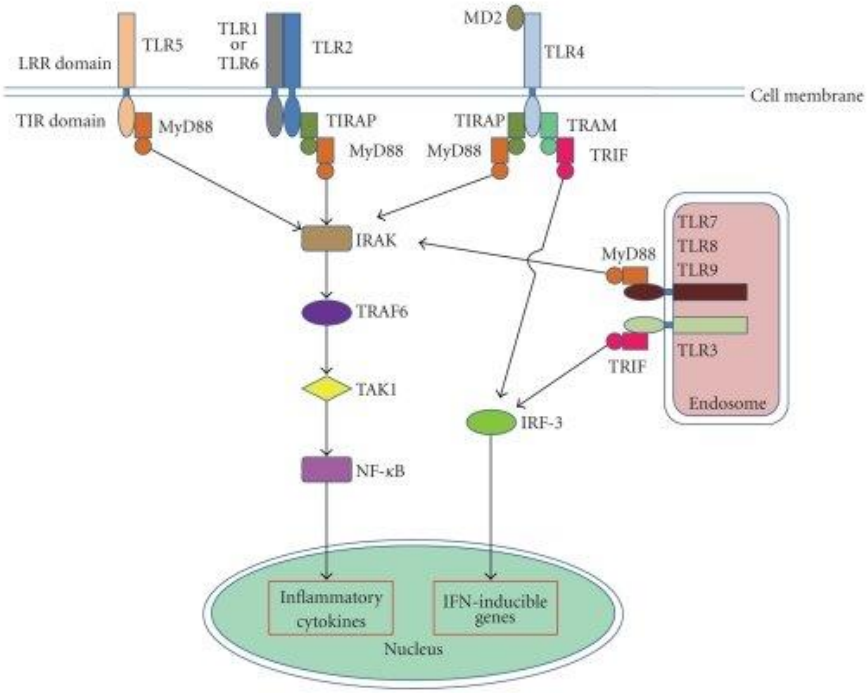

Figure 5. *Simplified diagram of TLR signaling pathways [172]*

Cytokines

Cytokines are small-secreted proteins released by cells and have a specific effect on the interactions and communications between cells [173]. Many cell populations produce cytokines, but the predominant producers are CD4 helper T-cells and monocytes/macrophages. [174]. Cytokines produced by lymphocytes are called lymphokines while those produced by monocytes are called monokines. Other groups of cytokines include IFNs and chemokines [174]. Chemokines are cytokines with chemotactic properties that attract leucocytes to the infection site [175]. The general term interleukins (ILs) is used to define cytokines produced by leucocytes and acting on other leucocytes. They have been numbered in the order in which they were identified, thus the first interleukin identified was named IL-1.

b) Specific acquired immunity

1. Lymphocytes

Lymphocytes are the predominant cells involved in acquired immunity, which includes humoral and cell-mediated responses. Soluble antibodies presented in the serum mediate humoral response, while cell-mediated responses result from the interaction between different types of cells in the immune system [176]. This distinction correlates, respectively, with the existence of two types of lymphocytes: B-cells and T-cells [177]. Most of the T-cells belong to one of the two-sub populations distinguished by the presence on their surface of one of two glycoproteins, designated CD4 and CD8. The majority of CD4 T-lymphocytes are helper T-lymphocytes (Th) whereas the majority of CD8 T-lymphocytes are cytotoxic T-lymphocytes (CTL) [178].

2. T-helper lymphocytes

There are at least two subsets of helper T-cells (Th1 and Th2). While the Th1 subset produces large amount of cytokines that promote cell-mediated immune responses, the Th2 subset produces an environment favoring humoral immunity by providing B-cell help for antibody production [179]. The Th1 response is characterized by production of IFN-α, IFN-β and IFN-γ and IL-2. The characteristic of the Th2 response includes production of IL-4, IL-5, IL-9, IL-10 and IL-13 [179]. The immune regulation involves homeostasis between Th1 and Th2 activity directing different immune response pathways [180].

The immune system of the cervix

Different regions of the cervix display different concentrations of immune cells. The ectocervix and the transformation zone are more biased towards cytotoxic T-lymphocyte response whereas the endocervical tissue mediates humoral responses due to a high amount of plasma cells [181, 182]. These plasma immune cells express a variety of TLRs, allowing them to recognize the different repertoire of a wide range of PAMPs [183-185]. The menstrual cycle also affects the immune system of the cervix and it has been suggested that female sex hormones are primary responsible for the antibody concentration in the cervix [186-188]. To ensure a healthy microenvironment while still allowing the mucosal tissue to fulfill its role as a barrier, the mucosal immune system has developed a remarkable balance. In this regard the FGT has to be tolerant to sperms and the implementation of the fetus to ensure pregnancy, while on the other hand it combats STIs targeting the FGT [189].

Cervical immune response to HPV

The squamous epithelium of the cervix is subdivided into two distinct regions, the epidermis and the dermis, separated by the epidermal basement membrane. The squamous epithelium of the cervix is populated with an array of immune cells [190, 191]. Keratinocytes (KCs) constitute the majority of the cells of the squamous epidermis while the majority of immune cells are located in the dermis [192]. Even if KCs are not considered as classical immune cells, they have some immune functions and can act as APCs. They can also secrete proinflammatory cytokines and chemokines. Langerhans cells (LCs) together with dendritic cells (DCs) constitute the majority of the immune cells in the dermis [193]. HPV infects only the epidermal cells of the cervical mucosa, without penetrating into the dermal tissue. Even if infections may persist for months or years, in the majority of cases, immunity-based regression of HPV lesions does finally occur [194-196]. The dsDNA and L1-L2 capsid components of the HPV virions are potential PAMPs that can trigger TLRs [183]. Studies showed that up-regulation of TL3, TLR7, TLR8 and TLR9 occurred in women where HPV16 infections were cleared [197]. Other studies have shown that up-regulation of TLR9 is associated with HPV persistence [198]. In cervical cancer, a down-regulation of TLR1, TLR3 and TLR5 and up-regulation of TLR2 compared to the controls was demonstrated [199]. In addition, studies suggest that a Th1 immune response favors clearance of cervical HPV infection [200], while a Th2 immune response favors progression to cervical cancer [201, 202].

1.4.2 Mechanism involved in HPV escape from the immune system and development of cervical cancer

Perturbation of antigen processing and presenting

In general, the immune system is able to destroy HPV and eliminate infected cervical epithelial cells. However, but about 10% of HPV infections leads to cervical lesions due to a failure of the immune response [203]. Host defense against viral infections is a collaboration between the innate immunity and the adaptive immunity [190]. HPV possesses mechanisms to evade the host's immune response and it is thought that the virus manipulates various molecular and cellular pathways in the host cell to evade host immune surveillance and antiviral immune responses [190].

KCs are the major cell type in the epidermis and have some immune functions such as secreting cytokines [192]. The compromised innate immune defense in KCs is an important

reason for the evasion of the HR-HPV infection from the immune response, eventually resulting in a persistent HPV infection and the development of precancerous lesions. The infectious cycle of HPV is itself an immune evasion mechanism inhibiting host detection of the virus [25, 204]. The first mechanism that HPV uses resides in the fact that it only causes neoplasia to a particular site where vulnerable KC cells are found [190, 205]. In addition the virus targets KC cells, constituting sentinels of the host defense [205, 206]. The E5 HPV oncoprotein plays a key role in cell growth and impairs several signal transduction pathways of the host [207]. It has been shown that the antigen processing by major histocompatibility complex (MHC) I [190] constitutes the major immune mechanism disrupted by HPV. In this process, HPV disrupts MHC I expression in a variant-selective manner to protect infected cell against NK and CTL cytotoxicity [30, 190].

The detection by the innate immune response of PAMPs is an important feature of the immune system [205]. To avoid this detection, HPV infection targets only epithelial cells and viral replication happens outside the basement membrane and distant from the resident dermal immune effectors [205]. In addition, in the normal case the viral oncoproteins E5, E6 and E7 are detected by the immune system [30], however, for most of HR-HPVs the expression of these oncoproteins happens in KC of the upper layer of the epithelium where the immune system has limited access [205]. HPV is also a purely intraepithelial pathogen and its life cycle is coupled to the cycle of differentiating KC [25, 204]. The lack of damage to host cells ensures a minimal immune response. Little or no release of proinflammatory cytokines is induced in HPV infection [31]. Studies show that oncoproteins E6 and E7 interfere with the expression of proinflammatory cytokines and chemokines [25, 204]. E6 and E7 are also known to directly interfere with TLR9 mediating pathways [29] and reduce the expression of proinflammatory cytokines.

Interaction with host Th1/Th2 phenotypes

HPV has been shown to induce a shift from a Th1 (known as cellular response) to a Th2 response (known as humoral response) [200, 208]. Furthermore, type I IFNs are produced by most of cells in response to viral infection in both infected and neighbor cells besides participating in the activation of adaptive responses by the innate immunity [209]. E6 and E7 genes may interact directly in altering the immune response against infected cells by suppressing IFN expression and signaling pathways [30]**.** Moreover, HR-HPV infection has also been shown to induce regulatory T cell (Treg) [208]. This HPV-mediated immune suppression during virus persistence may contribute to tumor cell evasion of antitumor immune

responses expression [30] . E5 may be involved in the inhibition of cytotoxic T cell function locally [25, 204].

1.5 Prevention of HPV infection and cervical cancer

Cervical cancer is particularly amenable to prevention as it has a long preclinical phase and the natural history of cervical carcinogenesis is well researched [44, 81, 208]. Cervical cancer prevention requires multidimensional approach involving primary, secondary and tertiary prevention [210]. Primary prevention aims to reduce the incidence of a disease within a population. It involves interventions that are applied before there is any evidence of disease. The aim of secondary prevention is to detect a disease in its earliest stages of development, before symptoms appear, and to stop its progression with lighter treatment methods leading to a greater chance of recovery. Tertiary prevention primarily aims to prevent or control the morbidity caused by cancer therapy, but it also encompasses the prevention of cancer recurrence.

1.5.1 Primary prevention

In terms of cervical cancer, primary prevention includes population education about cervical cancer and HPV vaccination.

Mass education

Although HPV is the most prevalent sexually transmitted infection in worldwide, public knowledge and awareness about cancer continue to be at poor level [211, 212]. A study was designed to examine the knowledge and beliefs about HPV among college students in Vietnam compared to college students in the US. On average, both Vietnamese and US participants could correctly answer less than half of the survey questions regarding HPV knowledge [213] . Studies of university students and employees in England report that many women underestimate the likelihood of receiving abnormal Pap test results [214, 215]. In addition, one of the main reasons for the high cancer mortality in sub-Saharan Africa is poor public knowledge and awareness about cancer. Poor cancer awareness and knowledge among primary health-care providers in sub-Saharan Africa has also been documented [216, 217], which negatively affects accurate diagnosis at the primary care level and causes delays in referrals to specialists, and late diagnosis [218]. Cancer awareness is important to improve risk reduction behaviours, promote timely cancer screening for early detection, and ultimately reduce the cancer burden in sub-Saharan Africa [211, 219, 220].

Vaccination

Vaccines against cervical cancer can be divided into two groups, the prophylactic and the therapeutic vaccines.

a) Prophylactic vaccines

Prophylactic vaccines contain HPV L1 self-assembling virus-like particles that induce strong neutralizing antibody responses against HPV infections. These antibodies are thought to block the HPV virions before they gain access to the proliferating basal cell layer of the epithelial surface through micro-abrasions [221]. There are currently three prophylactic HPV vaccines [222], *i.e.*, a bivalent vaccine targeting HPV16 and HPV18 (Cervarix®), a quadrivalent vaccine (Gardasil®) targeting HPV16 and HPV18 and HPV6 and HPV11 that cause genital warts and a nonavalent vaccine (Gardasil 9®) which has been licensed recently in the USA, Europe, and other high income countries .It targets also additional oncogenic HPV serotypes: HPV31, 33, 45, 52 and HPV58 [223]. There is good evidence that prophylactic HPV vaccines are immunogenic and effective against targeted-type HPV infections and cervical lesions when administered prior to HPV infection [224-226]. In addition, evidence supports that HPV vaccines are safe and there is good evidence for some cross-protection against non-targeted types occurring following the administration of HPV vaccines [227]. By the year 2014, 58 countries introduced HPV vaccination in their national immunization program [228]. Countries that implemented HPV vaccination before 2010 have already experienced a decrease in population prevalence in targeted HPV genotypes [229, 230]. Studies show a reduction in the prevalence of abnormal cervical cytology due to HPV vaccination [231, 232]. Importantly, after more than 100 million doses given worldwide, HPV vaccination has demonstrated an excellent safety profile [233]. Rwanda was the first African country to implement a national vaccination program against HPV [3]. In 2011, over 92,000 girls in primary school grade six were vaccinated with the quadrivalent vaccine, Gardasil® [3]. The three-dose vaccination coverage was estimated at 93% in the target population [3]. During the period 2012 and 2013, despite being heavily criticized [234, 235], a catch-up vaccination program targeting girls 15 years old was also initiated [236].

Therapeutic vaccines

Therapeutic vaccines targeting E6 and E7 along with broadly targeting immunotherapies or peptides are in clinical development. The rationale of these vaccines is to avoid the need for surgical procedures by developing immune responses against HPV [237]. Clinical trials have been moderately successful in eliciting cell-mediated immune responses to E6 and E7 in

patients; however, clinical responses have not been consistent [238-240].

1.5.2 Secondary prevention

Secondary prevention includes screening asymptomatic patients (primary screening) or screen positive patients to detect precancerous lesions (triage) before they turn into cancer [241]. The primary goal of cervical cancer screening is the accurate detection and timely treatment of intraepithelial precursor lesions of the cervix at a population level for the purpose of cervical cancer prevention rather than cancer control [242]. Therefore the long process of carcinogenic transformation from HPV infection to invasive cancer provides ample opportunities to detect the disease at a stage when treatment is highly effective [241]. High-quality screening programs can lower the incidence of cervical cancer by up to 80% [243]. The introduction of regular cervical cancer screening program is anticipated to lead to a fall in the incidence of invasive cervical cancer cases and deaths [242]. The mean age at the detection of CIN 2 is 35 and that of CIN 3 is 40 years. Hence, the possibility of detecting high-grade lesions is highest if the women are screened between 35 and 45 years of age [244]. Invasive cancers are rare before the age of 30 years, and screening women too young leads to the detection and unnecessary treatment of spontaneously regressing low-grade lesions [245]. Based on such evidence, the World Health Organization (WHO) recommended initiation of screening at the age of 30 years in developing countries [246]. All sexually active HIV-infected women however, should be screened for cervical cancer immediately after their HIV status is known because of the aggressive nature of cervical neoplastic process in HIV positive women [242, 247].

A number of different methods are available for cervical cancer screening. In some screening programs, cytology (both conventional and liquid-based) is the primary screening mode. In others, cytology is combined with HPV DNA testing (co-testing). Some countries and regions are moving toward or have adopted primary HPV DNA testing or visual inspection with acetic acid (VIA) [248]. Other modalities such as direct visual inspection (DVI), visual inspection using acetic acid and magnification (VIAM); visual inspection using Lugol's iodine (VILI) can be used for further evaluation of abnormal results [249].

Conventional Cytology

The primary method for detection of cervical cancer is still the Papanicolaou-stained (Pap) smear. The Pap smear is a screening tool that detects changes in cells of the transformation zone of the cervix [250]. Liquid-based cytology is an improved form of cytology. It has practical advantages since cytology and HPV testing can be done on the same sample. The

reporting system of Pap smear results has evolved and been refined over time. The current reporting system is the Bethesda System, 2014 [251]. In this system, the smear is reported as negative for intraepithelial lesion or malignancy; atypical squamous cells of undetermined significance (ASCUS); atypical squamous cells, cannot exclude high grade lesion (ASC-H); LSIL; HSIL; squamous cell carcinoma; atypical glandular cells (ACG); adenocarcinoma in situ (AIS); or adenocarcinoma. Other reporting systems exist (Fig. 6).

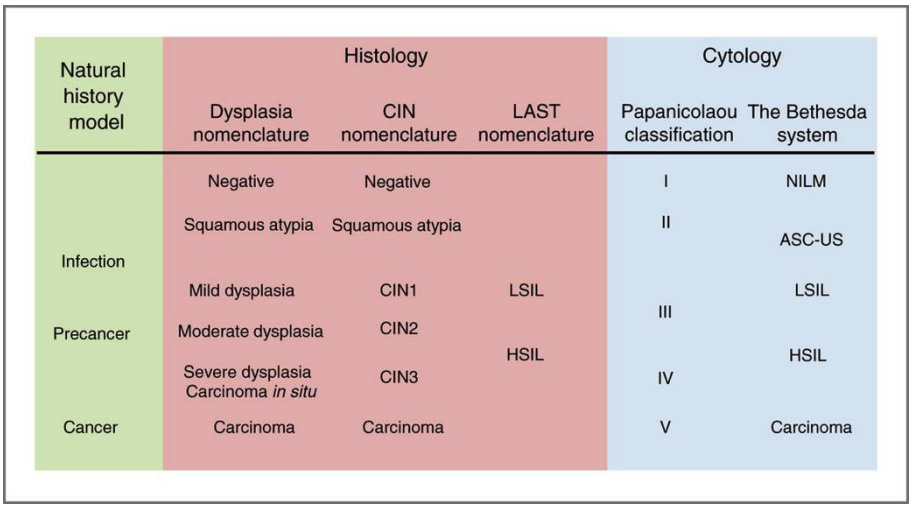

Figure 6.*Terminology of cervical diseases categories [252]. The figure shows histological and cytological terminologies of cervical disease categories. CIN [253]; LAST [244, 254]; NILM, negative for intraepithelial lesion or malignancy [255].*

HPV test

The commercially available HPV tests detect either the viral DNA or the messenger ribonucleic acid (mRNA) of the E6 and E7 oncoproteins of the most oncogenic HPV types. The introduction of HPV testing in primary cervical cancer screening was initiated as a response to the low sensitivity of cytology test [256]. In fact many clinical trial and comparatives studies conducted in Europe confirmed high sensitivity of HPV testing in detecting high-grade lesion than cytology (95% versus 55%), and found that it had a slightly lower specificity (94% versus 97%) [257, 258]. In India, Sankaranarayanan et al., (2009) showed a significant reduction in cervical cancer mortality associated with a single HPV test in comparison to a single cytology test [259]. This observation was supported by other studies [260-262]. Because of the inferior

specificity of HPV tests, a second test may be done on the HPV-positive women (triaging test) to identify those who are at higher risk of progressing to the disease and thereby only referring those positive on both tests to colposcopy. Combining the high sensitivity of HPV DNA test and the high specificity of cytology can increase the screening interval for testing in women negative by both methods [249]. Recently, HPV testing with cytology was introduced as an alternative to cytology screening in most of the countries following a Food and Drug Administration (FDA) approved use of HPV test in association with cytology [258, 263]. Cytology as a primary screening method is less sensitive than HPV testing [264]. However combining the high sensitivity from HPV DNA testing with the high specificity of cytology can increase the screening interval for women being negative for both methods [265].

Visual inspection with acetic acid (VIA) testing

The principle of VIA test bases on the fact that the neoplastic lesions of the cervix become white after application of 3-5% acetic acid for 1 min and the density and characteristics of aceto-whitening depend on the severity of the lesion. However, the VIA test is associated with a high level of subjectivity [266] and it has a low sensitivity especially in postmenopausal women [267].

In Rwanda, there is no systematic screening program implemented yet but at occasion, VIA and colposcopy services are currently provided at four referral hospitals and four district hospitals. Only two pathologists located in Kigali and Butare service the entire country [236, 268]. In addition many women consult in late stages and for numerous reasons [269]

1.6 Radiotherapy-induced adverse effects in normal tissue

While earlier stages of cervical cancer are treated with surgery, advanced stages are often treated with radiotherapy alone or in combination with chemotherapy. Despite advances in dose planning in radiotherapy, healthy tissue surrounding the tumour is still included in the radiation field and radiation-induced injuries may arise [270]. Cervical cancer patients treated by radiotherapy may be affected by acute and late radiation-related toxicity in 51% and 14% of cases, respectively [271]. Some of consequences of radiotherapy are radiation-induced cystitis, proctitis and ureteral stenosis [272-274]. Radiotherapy may also lead to structural and morphological changes in the FGT affecting sex life negatively [275, 276]. In addition,

cases of cervical necrosis (1.76%) had been reported among cervical cancer patients three to four months following radiotherpy [277].

Radiation induces oxidative stress and immune responses in normal tissue

Radiotherapy leads to an increase in reactive oxygen species (ROS) leading to oxidative stress [278]. Increased ROS may lead to apoptosis due to cytochrome c and caspase activation in mitochondria [279]. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor encoding antioxidant genes including heme oxygenase 1 (HO-1) and protects the cell against oxidative stress induced by radiation [280, 281]. Accumulation of extracellular matrix leading to the development of fibrosis may take place in normal tissue exposed to radiation and oxidative stress [282]. Besides oxidative stress, TGFβ is a key player in the development of fibrosis [283, 284]. Inhibition of TGFβ has been shown to attenuate the development of radiation-induced pulmonary fibrosis [285]. Treatment with antioxidants may also reduce levels of TGFβ and lead to decreased radiation-induced lung injuries [286].

Radiation also activates the innate immune response, which as a consequence lead to the release of cytokines and inflammation [278, 287, 288]. Previous studies show that the immune system is changed following irradiation of the lung [289], the colon [290], and the urinary bladder [291]. TLRs are down regulated in the urinary bladder and in macrophages in response to irradiation [291, 292]. Stimulation with agonists targeting TLR4 and TLR5 may abrogate immunological changes induced by colorectal irradiation of the rat [293].

Radiotherapy induces damage in epithelia, microvasculature and nerves

Radiation may affect crypts and lead to disruption of the barrier function of the colon [294, 295], which in some cases may lead to severe diarrhea [296]. Although Nrf2 in most tissues generates radioresistance [280], Nrf2-knockout mice were shown to be less sensitive to abdominal radiation than wild-type mice [297]. Radiation leads to damage of microvessels in the intestines [298]. Cardiac exposure to radiation may lead to dysfunction of microvascular endothelial cells [299]. In animal models for radiation cystitis, radiation may lead to changes in the neuronal control of the urinary bladder leading to urinary frequency [300, 301], a symptom pathognomonic for radiation cystitis [302]. Moreover, changes have been demonstrated in the density of nerves in the urinary bladder in an animal model for radiation cystitis [303]. Pudendal nerve dysfunction may also develop after brachytherapy against prostate cancer [304].

Radiotherapy-induced adverse effect of the FGT

Cervical cancer patients undergoing radiotherapy develop often radiotherapy-induced adverse effects of the FGT of various degrees of severity [305]. These women often report vaginal dryness, reduced vaginal dimension and dyspareunia [306]. Studies show that vaginal fibrosis and elastosis may develop in women undergoing pelvic radiotherapy [276]. Sexual dysfunction in gynecological cancer survivors may have severe impact on quality of life [307]. There is a scarcity in effective therapeutic alternatives for this patient group and few studies have been conducted in this research field [307].

HBOT in the treatment of adverse effects to radiotherapy

Hyperbaric oxygen therapy (HBOT) is a treatment where patients are exposed to oxygen at pressures higher than atmospheric pressure at sea level [308]. The treatment is the standard of care for decompression sickness [309], however, today other medical conditions in patients undergoing HBOT dominate. HBOT increases the oxygen tension in tissue and is effective in the treatment of chronic ulcers [310, 311]. HBOT stimulates angiogenesis as demonstrated in patients undergoing HBOT due to exposure to irradiation of the buccal mucosa [312]. Animal models also show that HBOT increases the microvessel density of the penumbra after ischemic stroke [313]. Besides chronic ulcers, HBOT is effective against carbon monoxide poisoning [314]. HBOT is an important treatment option also for radiation cystitis and proctitis [272]. The exact mechanisms by which HBOT alleviates symptoms associated with radiation-induced cystitis and proctitis are unknown. In an animal model for radiation cystitis, immunological and oxidative changes were reversed by HBOT [315]. Whether HBOT is also effective in the therapy of radiation-induced adverse effects of the FGT is not known due to a very limited number of studies conducted in this research field. A prospective study in women treated for gynecological malignancy with late adverse tissue injuries, failed to show any alleviating effects in pain after HBOT [316]. Other studies suggest that the treatment is effective in women developing vaginal ulcers and fistula after radiotherapy [317].

2 STATEMENT OF THE PROBLEM AND STUDY JUSTIFICATION

Cervical cancer is a very important health issue in Rwanda contributing to a high morbidity and mortality in the country. The following are some of limitations identified in Rwanda:

A. In Rwanda there is a known scarcity in national epidemiological data on cervical cancer. The only few studies available were conducted in Kigali, the capital and only describe cervical cancer among HIV infected women. This was because not only HIV infection was regarded as a public health problem for women but also because most of the Rwandan health sector partners targeted mainly HIV/AIDS prevention.

B. Rwanda is among few African countries that have introduced large-scaled HPV vaccination programs. Many studies conducted in Africa demonstrated that the currently used vaccines do not target the most common HPV genotypes. Whether the currently used vaccine in Rwanda gives protection against HPV infections in the country has not been studied.

C. The recommendation is that girls should be vaccinated before the onset of their sexual activities; however, no studies have been performed in Rwanda assessing the age of sexual debut among women.

D. No cervical cancer-screening program is provided for the general female population in Rwanda.

E. Cervical cancer patients referred for radiotherapy in Uganda are generally not followed up and there are no studies on the extent of side effects related to radiotherapy among these patients.

F. In combining all these persisting issues for an effective cervical cancer control in Rwanda we conclude that there is a need of a more complete understanding of the natural history of HPV infection and cervical abnormalities.

3 AIMS

3.1 General aims

The overall objectives of the study were to assess the prevalence and the natural history of HPV infections in Rwanda, the contributions of STIs and other risk factors and how the immune system of the cervix responds to inflammatory stimuli.

3.2 Specific aims

3.2.1 Clinical studies (Paper I-III)

- To determine the prevalence of HPV infections and related cytological abnormalities among HIV+ and HIV- Rwandan women.
- To assess whether HPV test could work as a screening method for detecting HSIL and worse cytology.
- To describe the prevalence and risk factors for STIs/RTIs in HIV+ and HIV- Rwandan women.
- To assess the effect of sexual behaviour on the awareness of HIV positivity and present STIs in HIV+ and HIV- Rwandan women.
- To identify risk factors associated with HR-HPV persistence and those associated with cytological abnormalities in HIV+ and HIV- Rwandan women.
- To identify risk factors for LSIL and HSIL/cancer in HIV+ and HIV- Rwandan women.
- To determine whether IL28B SNP rs12979860 correlates with HPV susceptibility and HPV persistence and development of HSIL

3.2.2 Preclinical studies (Paper IV-V)

- To explore the effect of a single high dose of ionizing radiation on cervical immune system in rat model.

- To evaluate the effect of HBOT on rat cervical tissues following high dose of radiation treatment.

4 METHODS

4.1 Clinical studies (paper I-III)

4.1.1 Study design

Paper I was a cross-sectional study on the prevalence of HPV and SIL in Rwandan women. Paper II and paper III were prospective cohort studies following identified HPV positive women and women with abnormal cytology and an age-matched control group from paper I. Women were followed for 9 months (paper II) and for 24 months (paper III). After obtaining the informed consent from all study participants, women meeting inclusion criteria were enrolled to the studies.

4.1.2 Study area

Women participating in the studies were recruited between July 2015 and October 2017. HIV positive women were recruited at the HIV clinic at the University Teaching Hospital of Kigali (CHUK; HIV positive group). Participants were also recruited at the Department of Obstetrics and Gynecology at CHUK and at the University Teaching Hospital of Butare (CHUB). Women were tested for HIV and if being tested negative referred to the HIV negative group and if being tested positive referred to the HIV positive group. While CHUB is the referral hospital for the southern region of Rwanda, CHUK, situated in Kigali in the middle of Rwanda, is the referral hospital for most of the rest of the country.

4.1.3 Study population

All women aged between 17 years and older visiting the gynecology and obstetrics department at CHUB or CHUK and all HIV-infected women followed at HIV clinics at internal medicine at CHUK were invited to take part in the study. Inclusion criteria were the following: women seeking voluntary for cervical cancer screening service, who were 17 years old or older, who were literate in either Kinyarwanda, English or French, who had filled and signed the Informed Consent Form and who did not had plans to relocate in the next two years. Exclusion criteria were present pregnancy, a history of HPV infection and/or cervical cancer and presenting any known concurrent disease likely to limit life expectancy to less than 24 months or presenting any other factor suggesting inability to comply with the study protocol.

4.1.4 Sampling and sample size

A non-proportional quota sampling was applied to enroll participants to our study. For paper I, two hundred women in each one of the two cohorts were recruited to assure analysis even for small subgroups in the population. All participants in different cohorts were continuously recruited until the desired number was reached. At baseline, 119 women tested positive and or with cervical cytology abnormalities (referred as cases), regardless of their HIV status, were included in the study. Then 118 women tested to be HPV negative with normal cervical cytology test and age-matched to cases were purposely selected to constitute the control group.

4.1.5 Study procedures

Procedures at enrolment and follow-up

A nurse or physician at each concerned health facility received women at daily basis and reviewed if the women met the requirements to participate in the study. When inclusion criteria were met, the patient was informed about the study orally and by written information. After the informed consent form was signed, a face-to-face interview was conducted by after what the women were examined by a physician and samples were taken (see below). Women underwent a new interview at 9 months (paper II). Women were called back for cervical samplings 9 months, 18 months and 24 months after inclusion in the study.

4.1.6 Data collection

Interview and analysis of patients' medical records

A standardized questionnaire was administered to participants in the study at baseline and at 9 months. Data collected during interviews covered demographics (age, years of schooling and marital status), reproductive history (number of live births, number of abortions, hormonal contraceptive and intrauterine device use) and sexual activity (age at first intercourse, number of sexual partners). History of sexually transmitted infections (chlamydia, gonorrhea), medical history (cervical cancer screening test, HIV status) and smoking (past or current) were also part of the questionnaire. At 9 months, a short questionnaire covering information on sexual activity, number of new sexual partners and condom use during the last 9 months was used. Data on CD4 counts, viral load and antiretroviral therapy (ART) were found in the medical record of the patients.

Cytology and HPV screening

At each clinical examination, a cytobrush (Hologic, Inc., Marlborough, USA) was inserted through a speculum and rotated in the cervical canal. The collected cells were then agitated into 20 ml of PreservCyt solution (Hologic, Inc.) and stored at room temperature till the transport to Sweden for further processing and analysis. Once in Sweden, 2 ml of each sample were sent to the Laboratory of Infectious Epidemiology, Department of Laboratory Medicine, the Karolinska Institute, Stockholm, Sweden, for HPV DNA and genotyping. HPV DNA analysis was performed by the use of the Multiplex Luminex system (Bio-Rad Laboratories, Inc., Irvine, CA, USA). Forty different HPV strains were analysed organised as follow: low-risk LR-HPV: 6, 11, 30, 40, 42, 43, 54, 61, 67, 73, 74, 81, 82, 83, 86, 87, 89, 90, 91; high-risk HR-HPV: 16, 18, 18v, 31, 33, 35, 35v, 39, 45, 51, 52, 56, 58, 59, and possibly high-risk (PHR)-HPV 26, 53, 66, 68a, 68b, 69, 70. The rest of the specimens were sent to the Cytology and Pathology Department, Sahlgrenska University hospital, Gothenburg, Sweden, for Thinprep Pap test. Liquid-based cytology was prepared and performed according to the manufacturer's instructions on PreservCyt samples, using the fully automatic ThinPrep 5000 System (Hologic, Inc.) followed by Pap staining (Hologic, Inc.). All slides results were read and interpreted by a cytologist at the lab. In case of HSIL or invasive cancer suspicion, a pathologist reviewed slide also. All cytology results were reported according to the Bethesda 2001 system as: Normal, Not Determined, ASCUS, AGUS, ASC-H, LSIL, HSIL, AIS and cancer. SNP analysis was also performed on the cytology solution (see below).

Testing for STI and RTIs

At baseline and at nine months, a vaginal swab for bacterial vaginosis, candidiasis and trichomoniasis was collected. At the same time, a blood sample was also taken for HIV (for non-HIV+ cohorts), *Treponema pallidum* (syphilis), hepatitis B (HBV) at the CHUK and CHUB clinical laboratories by well-trained laboratory technicians.

HIV testing

In brief, serum specimens were first screened by the use of Alere HIV Combo (Alere Medical Co., Ltd, Chiba, Japan), and then reactive samples were confirmed by the second test, HIV ½ Stat- Pak® (Chembio Diagnostic System, Inc. New York, USA). Reactive samples were finally reported as positive.

Syphilis, HBV and HCV testing

Testing for syphilis was conducted using the rapid plasma reagin (RPR) test (Macro-Vue RPR Card Test, BD, USA). The RPR-positive samples were sent to the National Reference Laboratory within 24 hours for confirmation using the Treponema pallidum haemagglutination (TPHA) test, the rapid test to detect syphilis antibodies. A sample that was positive for both tests was recorded as positive and a non-reacting sample to the second test was recorded as negative. The Screening for HBV was performed using the HBsAg Rapid Test Strip (Rapid Labs Ltd, Colchester, United Kingdom) to detect HBV surface antigen (HBsAg). A reacting sample was recorded as positive. HCV testing was performed using the HCV Rapid Test Strip (Rapid Labs Ltd, Colchester, United Kingdom). The test detected HCV antibodies (anti-HCV). A reactive sample was recorded as positive. All rapid tests were performed and interpreted according to the manufacture's recommendations.

Testing for candidiasis, trichomoniasis and bacterial vaginosis

Cervicovaginal samples were cultured on Sabouraud culture medium for detection of candidiasis. The identity of clinical isolates was confirmed by germ tube induction test in serum for *Candida albicans***.** Trichomoniasis was diagnosed by wet mounts evaluation and examined microscopically within 15 min for motile *Trichomonas vaginalis* identification. Bacterial vaginosis was diagnosed by the presence of three of the clinical and microscopic findings on Gram stain, standardized by Nugent scoring method [318].

SNP analysis

From the cervical sample solution, the IFNL3 SNP for *IL28B* rs12979860 polymorphism was determined by using TaqMan SNP Genotyping Assays (Applied Biosystems (ABI), Carlsbad, California, USA). Each genomic DNA (2µl) contained in cervical samples, in PreservCyt transport medium (Hologic Inc.), was amplified with TaqMan genotyping Master mix reagent (ABI) combined with TaqMan SNP genotyping primers and probes (FAM and VIC) (ABI). The real time polymerase chain reaction (RT-PCR) reactions were performed using 7500 Fast RT-PCR Systems and following standard mode of thermal cycling conditions. Automatic call decisions were made with the SDS software for the allelic discrimination.

Data Management

All collected data have been managed in accordance with the International Conference of Harmonization (ICH) guidelines, and data management principles. The data management

routines included procedures for handling Case Report Forms (CRFs), database set-up and management, data entry and verification, data validation, and documentation of the performed activities including information of discrepancies in the process. All the data in the CRFs were subject to both visual checks and computer-driven procedures. Data were stored on a computer with a login that was only accessible for the investigators and personnel within the project. The database and the data entry screens were designed in accordance with the study protocol and the CRF. Data validation and check out were performed after data entry and verification by manual review. Data from patients were coded so participant could not be identified.

4.1.7 Data analysis and statistics

Statistical analysis was performed with the use of IBM SPSS 22 & 25 versions and Graph Pad Prism 6 and 7 versions. Descriptive statistics were done wherever appropriate to describe baseline characteristics of participants. All statistical tests were two-sided and considered significant when p-values were <0.05. Depending on the sample size we used the chi-square test or the Fisher's exact test to compare categorical data. Univariate logistic regression analysis was performed and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Variables with a univariate p-value of <0.05 (paper I) or a p-value of <0.2 (paper II) were entered in the multivariate logistic regression analysis by the enter method and adjusted odds ratios (AORs) were then determined.

In paper II, PHR-HPV and HR-HPV were grouped into one category denoted HR-HPV. Kaplan-Meier curves were created for HR-HPV and LR-HPV persistence and the log-rank test was performed to compare Kaplan-Meier curves. The percentage of incidentally infected (HR-HPV or LR-HPV) women at 9-24 months who were HPV negative at baseline divided with the number of women at risk was calculated to give the cumulative incidence. Incident HPV infections were considered as HPV infections not present at baseline but present at any time point 9-24 months. Positive test results for the same PHR/HR-HPV type at three consecutive time points or two non-consecutive time points separated with a negative test result were considered type-specific HR-HPV persistent infections. Type-specific persistence was also considered in the case of two consecutive HPV positive results followed by missing data. HPV clearance was considered in a situation when a HPV positive test was followed by at least two negative tests or one positive test followed by one negative test followed by missing data or no more sampling occasions. In the statistical analyses, women infected only with LR-HPV(s) were considered LR-HPV infected, while women infected by HR- or PHR-HPV(s) were considered HR-HPV infected. Women infected by LR-HPV(s) and HR/PHR-HPV(s) were

considered HR-HPV infected. Women with unsatisfactory results (not determined) were considered as abnormal in the statistical analyses. In case one cytology sample was evaluated as not determinable and the other samples were evaluated normal the woman was considered to have normal cytology.

In paper III, syphilis, trichomoniasis, hepatitis B and hepatitis C were considered STIs, while bacterial vaginosis and candidiasis were considered RTIs. Since the number of individual RTI and STI cases was low, statistics were performed on the STIs and RTIs group level. PHR and HR-HPV infections were also grouped in one category denoted HR-HPV.

4.2 Preclinical studies (paper IV-V)

4.2.1 Animals

Sprague-Dawley female rats (200–250 g; CD® IGS rat, Charles River, Germany) were housed in a temperature-controlled facility with a day and night cycle, and had free access to food and water *ad libitum*. In paper IV, rats were either irradiated or only sedated (controls) and irradiated rats were either euthanized at 1 day, 3, 7 or 14 days after cervical irradiation. In paper V, rats were assigned to four groups, *i.e.*, irradiated, irradiated + HBOT, HBOT g or control group (neither irradiation nor HBOT). All rats were euthanized 28 days after cervical irradiation (or sedation for control group).

4.2.2 Irradiation of uterine cervix

On the day of cervical irradiation, rats were sedated with pentobarbitone (50 mg/kg) *im* and medetomidine (10 µG/kg) *ip*. Rats were placed in the supine position and the uterine cervix was then irradiated with one fraction of 20 Gy given by a linear accelerator (Varian Medical Systems Inc., Palo Alto; 6 MV). Radiation was given in two side-fields to minimize the exposure of the spinal cord. Rats had food and water *ad libitum* and were sacrificed by an overdose of anesthetics 1, 3, 7 and 14 days (paper IV) or 28 days (paper V) after irradiation of the uterine cervix.

4.2.3 HBOT

HBOT was given in a hyperbaric chamber (GDA Sverige AB, Gothenburg, Sweden). In the chamber, rats were exposed to 100% oxygen pressurized to 200 kPa for 90 minutes twice daily, Monday-Friday for a total of 20 sessions given over two weeks. Control rats, (no irradiation, no HBOT), were sedated on day 1 and then kept in their cages for 28 days. HBO

control rats (no irradiation, HBOT) were sedated and then kept in their cages for 14 days before receiving 20 sessions of HBOT over a period of 14 days. Irradiated rats (radiation, no HBOT) were sedated and irradiated on day 1 and were kept in their cages for 28 days. HBO irradiated rats (radiation, HBOT) were sedated and irradiated on day 1, kept in their cages for 14 days before receiving 20 sessions of HBOT over a period of 14 days.

4.2.4 Collection of cervical specimens

After euthanization of the rat, the uterine cervix was excised and cut into two sagittal parts where one part was frozen to -70 ^{o}C degrees for late protein extraction for western blot and cytokine analysis whereas the other part was put in formalin for paraffin embedding.

4.2.5 Antibodies

List of primary antibodies (immunohistochemistry (IHC)/western blot (WB))

Mouse anti-β-actin (WB 1:2000, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA, USA, sc-47778), mouse anti-catalase (IHC 1:100/WB 1:500, SCBT, sc-271803), mouse anti-8-OHdG (IHC 1:2000, Abcam, ab62623), mouse anti-SOD-2 (IHC 1:50, SCBT, sc-137254), mouse anti–TLR9 (WB 1:500, Sigma Aldrich, WH0054106M3), mouse anti-α-tubulin (WB 1:2500, Abcam, T9026), rabbit anti-MyD88 (IHC 1:100, SCBT, sc-11356, WB 1:200, Abcam ab2064), rabbit anti-NFκβ (WB 1:1000, Abcam, ab86299), rabbit anti-SOD1 (IHC 1:1000/WB 1:50000, SCBT, sc-11407), rabbit anti-SOD-2 (WB 1:1.500, Abcam, GR3318-56), rabbit anti-TLR1 (IHC 1:100/WB 1:250, SCBT, sc-30000), rabbit anti-TLR2 (IHC 1:100/WB 1:500, SCBT, sc-10739), rabbit anti-TLR3 (IHC 1:100/WB 1:500, SCBT, sc-28999), rabbit anti-TLR-4 (IHC 1:100/WB 1:500, SCBT, sc-30002), rabbit anti-TLR5 (IHC 1:100/WB 1:500, SCBT, sc-30003), rabbit anti-TLR6 (IHC 1:100/WB 1:500, SCBT, sc-30001), rabbit anti-TLR7 (IHC 1:100/WB 1:500, SCBT, sc-30004), rabbit anti-TLR8 (IHC 1:100/WB 1:500, SCBT, sc-25467), rabbit anti-TLR9 (IHC 1:100, SCBT, sc-25468) and rabbit anti-TRIF (IHC 1:500/WB 1:1000, Thermo Fisher Scientific, PA5-23467).

List of secondary antibodies

Alexa Fluor 488 goat anti-rabbit IgG (H+L)*2 mg/mL (IHC 1:250, Life Technologies, A11008), goat anti-mouse IgG (H+L)-HRP (WB 1:4000, Thermo Fisher Scientific, SA248187), goat anti-rabbit IgG-HRP (WB 1:5000, Abcam, ab 6721) and Texas red goat anti mouse IgG (H+L) (IHC 1:250, Life Technologies, T862).

4.2.6 Western blotting

Cervix tissue specimens were homogenized using homogenization buffer. Homogenates were then centrifuged at 10000g for 15 min at 4^{o}C. The total protein concentration in the homogenate was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and stored at -20^{o}C until utilization. The protein samples were mixed with NuPAGE LDS Sample Buffer (Life Technologies) and NuPAGE Reducing Agent (Life Technologies) and then boiled for 10 min at 70^{o}C. Protein samples (15µg/well) were loaded on NuPAGE 4-12% Bis-Tris gels (Life Technologies) for electrophoresis under reducing conditions in MOPS buffer (Life technologies Ltd) followed by transferring the proteins onto a nitrocellulose membrane (Life Technologies). The membrane was washed in tris-buffered saline containing 0.3% Tween 20 (TBS-T; Sigma-Aldrich). After washing the membrane, it was blocked in 5% of non-fat milk solution in TBS-T for 1 hour. The membrane was then incubated, overnight, with the primary antibody. On the next day, the membrane was washed in TBS-T and then incubated with the appropriate horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. After washing, the signal was detected using Amersham ECL Plus™ Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK). The film was scanned by Fujifilm Image Reader LAS-1000 Pro v.2.6 (Stockholm, Sweden) and the protein expression was quantified with the Fujifilm Multi Gauge v.3.0 software (Stockholm, Sweden). The membrane was washed in TBS-T, stripped in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL, USA) and incubated with a housekeeping gene for loading control.

4.2.7 Immunohistochemistry

Sections of paraffin-embedded cervical specimens (6 µm) were deparaffinized in xylene, ethanol 99-95% and hydrated in tap water. The antigen retrieval was done by boiling the sections in citric buffer for 30 min. To block for autofluorescence, sections were covered with $CuSO_4$ for 2 hours. Sections were then blocked with 5% goat serum (GS) in phosphate buffer saline solution (PBS) for 1 hour. Sections were incubated overnight at 4^{0}C with the primary antibody diluted in 1% GS, while negative controls were covered by 1% GS solution only. The next day, sections were incubated with the secondary antibody for 1 hour at room temperature. Sections were dehydrated (ethanol 95-99%) and then covered with Prolong® Gold Antifade Reagent with DAPI (Life technologies) and cover glasses .The average pixel intensity of antigen staining was measured in one representative area of cervical epithelium, submucosa, smooth muscle and submucosal blood vessel wall in specimen with Photoshop (version 12.0.4).

4.2.8 Cytokine analysis

Cervical tissues homogenates were analyzed for the expression of cytokines, chemokines and growth factors by using magnetic beads multiplex immunoassays, Bio-Plex Assays (Bio-Rad Laboratories Inc., Irvine, CA, USA). Erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, Gro/KC (chemokine (C-X-C motif) ligand 1; CXCL1), interferon gamma (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, macrophage (M)-CSF, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-3α, regulated on activated, normal T-cell expressed and secreted (RANTES), tumour necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF) were analysed. The concentrations were determined using the Bio-Plex Assay Reader (Bio-Rad). The Bio-Plex Manager software optimizes the standards curves automatically and returned the data as Medium Fluorescence intensity (MFI) and concentrations in pg/ml.

4.3 Ethics

The clinical studies (paper I-III) were approved by the Institutional Review Board at the College of Medicine and Health Sciences, University of Rwanda and by the Ethics Committee at the University of Gothenburg. All women were contacted individually if laboratory analysis indicated a need for any treatment. In addition, participants were always informed about the HPV and cytology tests results and those with HSIL or cancer were referred for colposcopy. The preclinical studies (paper IV-V) were approved by the local ethics committee at the University of Gothenburg.

5 RESULTS AND DISCUSSION

5.1 Paper I-III

5.1.1 Differences in baseline characteristics between HIV+ and HIV- cohort

In paper I, we studied the prevalence of LR- and HR-HPV strains and cytological abnormalities. We wanted to identify risk factors associated with the development of SILs and HR-HPV infection among 206 HIV+, 172 HIV- and 22 women with unknown HIV status in Rwanda. We also wanted to assess whether HPV screening could identify women with SILs. The HIV+ cohort and the HIV- cohort had similarities in baseline characteristics but also significant differences. The educational level was similar, the proportion of who never had smoked, the number of live births and abortions and the proportion who never had used IUD or hormonal contraceptives were similar in the HIV+ and the HIV- cohort. The HIV+ cohort was, however, older (46±8 years *vs.* 40±10 years, respectively), were more often widowed and had had more often a sexual debut before the age of 21 compared with the HIV- cohort. While 61% of

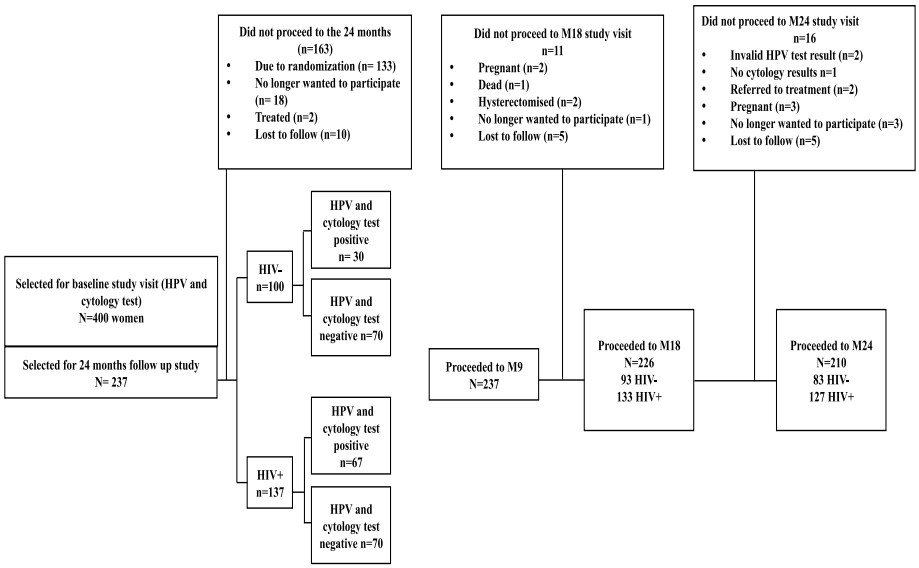

Figure 7*. Study participants' flow chart*

HIV- women reported to have had only one sexual partner, the corresponding figure for HIV+ women was 27%. Almost 90% of HIV+ women were under ART, 96% had a CD4+ count of more than 200 cells/mm^3 and more than 80% had a HIV viral load at less than 400 copies/ml. Of women recruited in at outpatient gynecology clinics 2.9% were diagnosed with HIV, in line with previously reported prevalence figures in Rwanda [319]. The predominant part of all women at the HIV clinic at CHUK was under ART, had normal CD4 count and had low levels of viral load. The percentage of HIV+ patients under ART has increased significantly during the last years and in 2015 the percentage was 79% [320].

In paper II, all women from the 400-patient cohort (cohort A; paper I) with HPV infections and/or with cytological abnormalities and an age-matched control group (137 women with HPV and/or with cervical cytological abnormalities and 100 controls; cohort B) were re-screened for HPVs, cytological abnormalities and STIs/RTIs 9 months later. In paper III, cohort B was screened again at 18 months and 24 months for HPVs and cervical cytological abnormalities. Due to a higher prevalence of HPV in HIV+ women, there were more HIV+ women than HIV- women in cohort B (57.8% and 42.2%, respectively).

5.1.2 HPV strains in HIV+ and HIV- women

Baseline prevalence of being infected by any HPV was higher in HIV+ women than in HIV- women (27.7% and 11.6%, respectively; $p<0.001$; paper I). Baseline prevalence of PHR/HR-HPV infections was 21.4% in the HIV+ group and 8.1% in the HIV- group ($p<0.01$). Among HIV+ women, it was more common to be infected by multiple HPV strains than among HIV- women (9.2% *vs* 1.7%, respectively; $p<0.001$). The most common HR-HPV strains detected were HPV16 (4.3%) and HPV52 (4.3%), which constituted 39.7% of all HPV-infections, while HPV18 was only found in three of 400 participants. The low prevalence of HPV18 is in accordance with previous prevalence figures from Rwanda [321]. In addition, the prevalence of 32-34% in PHR/HR-HPV infections had been reported previously in Rwanda [321, 322], however, our cohorts consisted of an older population of women (89% were ≥35 years old) who were from different regions in Rwanda. This may have contributed to the differences in HPV prevalence.

During the 24-month follow-up (paper II), all analysed HPV strains (prevalent at baseline and incident HPV strains combined) were detected among HIV+ and HIV- women but not HPV30, HPV43, HPV73 and HPV82. Of all 170 detected HPV infections, the most common strains detected were: HPV16 (13.53%), HPV52 (11.76%), HPV58 (5.88%) and HPV66 and HPV70

(4.71% each). Gardasil® gives protection or cross-protection against HR-HPV strains HPV16, HPV18 and HPV31 [323], however, these strains constituted only 25.6% of all detected HR-HPV infections and only 10.2% of all persistent HR-HPV infections. HIV+ women developed chronic HR- and LR-HPV infections (present at baseline and 24 months) in 56% and 33% of cases, respectively, while no HIV- women developed chronic HPV infections ($p<0.001$ for HR-HPV). The cumulative incidence in HIV- and HIV+ women was 16% and 30%, respectively, for developing HR-HPV infections ($p=0.0759$). Among HPV negative women at baseline, HIV positivity enhanced the risk of contracting a HR-HPV infection ($p<0.001$).

The high degree of HPV persistence in HIV+ women has been reported from studies in South Africa and Burkina Faso [324]. The high incidence of HPV infections in HIV+ women is in accordance with previous studies [324-326].

5.1.3 Multiple HPV infections in HIV+ women

In line with previous reports [327], HIV+ women were more commonly infected by multiple HPV strains than HIV- women specifically with multiple HR-HPV infection. Studies suggest that patients with multiple HR-HPV infections are more likely to have chronic LSIL [328]. Multiple HPV strains are also more common in HSIL than in LSIL [329]. Multiple HPV infections have been shown to be associated with larger cervical lesions [330].

5.1.4 Cytological abnormalities common in HIV+ women

At baseline (paper I), the percentage of women being infected by PHR/HR-HPV strains and presenting cytological abnormalities decreased with age both among HIV+ and HIV- women. Cytological abnormalities were observed in 24.3% of HIV+ women and in 9.9% of HIV- women ($p<0.001$). LSIL and HSIL/squamous cervical cancer (SCC) were diagnosed in 3.4% and 5.8%, respectively, of HIV+ women and in 4.7% and 1.2%, respectively, of HIV- women. HIV+ women with chronic HR-HPV infection presented with an abnormal cytology in 86% of cases during the 24-month follow-up ($p<0.05$; paper II). HIV+ women with persistent HR-HPV infections had a 38% risk of being diagnosed with HSIL or worse cytology at any time point 0-24 months, however, at the last sampling occasion (normally at 24 months) only 8.7% (2/23) were diagnosed with HSIL. Among HIV- and HIV+ women negative for HPV at baseline, only 3% were diagnosed with HSIL during the 24-months´ follow-up.

5.1.5 HPV screening to detect HSIL/cancer

Rwanda is a country where healthcare resources are scarce and the number of physicians working in the country is low. Therefore, there is a need to find ways of screening out women at risk of HSIL and cervical cancer without overloading the healthcare system. Paper I shows that the sensitivity and specificity to detect HSIL or worse with HPV screening were 0.78 and 0.87, respectively, which are relatively high values. Studies show that HPV screening compared with cytology increases the sensitivity to detect HSIL but with the cost of less specificity [331]. Combining HPV screening with cytology may improve cervical cancer screening [332]. HPV screening may tentatively be used particularly for risk patients such as patients with HIV, other immunodeficient patients and sex workers.

5.1.6 Vaccine does not cover all prevalent HR-HPV strains

The current vaccine used in Rwanda (Gardasil®) protects against HPV16, HPV18, HPV6 and HPV11. The vaccine may also give cross-protection against HPV33, HPV31, HPV45, and HPV51 [333]. In view of HPV52 being prevalent in Rwanda, while HPV16 and HPV18 being much less common than in the Western world, our data indicate that the present vaccine may give an insufficient coverage against cervical cancer. An introduction of a vaccine with a broader HPV coverage, such as the nonavalent vaccine Gardasil® 9, into the vaccination program in the future may therefore be of particular importance in Rwanda. Pharmaceutical company-sponsored phase III HPV vaccine trials in Africa are uncommon [334]. A variety of different HPV strains was detected among Rwandan women. HPV16 was the most common strain, however, it constituted only 13.45% of all HPV infections. Besides protecting against HPV6, 11, 16 and HPV18, the currently used vaccine in Rwanda Gardasil® may give cross-protection against HPV31 [323]. Based on the current data, Gardasil® would, however, only have given protection against 1 out of 4 HR-HPV infections and 1 out of 10 persistent HR-HPV infections in our cohort. Therefore, Gardasil® may be insufficient to give HPV protection in the Rwandan population. Our finding is consistent with other studies from East Africa, *i.e.*, for example neither Cervarix® (protects against HPV16 and HPV18) nor Gardasil® are anticipated to give full protection against cervical cancer in Ethiopia due to different compositions of HPV strains such as a high prevalence of HPV52 [335]. Studies from The Democratic Republic of Congo, Zambia and Nigeria also show a higher prevalence of HPV52 than HPV16 [326, 336, 337].

5.1.7 Attitudes to cervical cancer screening

Among HIV+ women, 27% had undergone a cervical cancer-screening test before the study compared with only 7% of HIV- women ($p<0.001$). While HIV+ women often were recommended to do a cervical cancer-screening test by their physician, HIV- women reported more frequently that the idea to do the test was their own. The reason for not undergoing cervical cancer screening was that many women reported not knowing about the test/cervical cancer. Financial reasons for not undergoing cervical cancer screening test was more common among HIV+ women compared with HIV- women (25% *vs*. 7%, respectively; $p<0.001$). In view of HIV being a risk factor for cervical cancer, it is notable that only 27.7% of HIV+ women had previously underwent a cervical cancer screening. Both in HIV+ and HIV- women, the unawareness of the test was high. About two third of the cohorts were not aware of the cervical cancer screening test, which is lower in comparison to countries such as Kenya and Uganda [338, 339]. A large proportion of HIV+ women reported economical reasons for not undergoing cervical cancer screening, which is commonly reported in developing countries [340]. Being widowed was more common among HIV+ women than HIV- women, which most likely contributes to a worse economical situation where health care issues may not be prioritized. Studies from Rwanda´s neighbour, the Democratic Republic of Congo, show that less than one third of women are willing to pay for a Pap smear test [341]. Information campaigns about cervical cancer, targeting HIV+ women are at present needed. Moreover, it is important to reduce the costs to undergo cervical cancer screening particularly for risk groups such as patients with HIV.

5.1.8 Prevalence of RTIs in HIV+ and HIV- women

In paper I (cohort A (N=400), the prevalence of different STIs and RTIs was reported. In paper III, cohort B (subgroup of cohort A; N=237) was called back for second testing of STIs and RTIs. STIs and RTIs were similarly distributed among women regardless of present co-infection of HPV. No correlation between baseline characteristics and prevalence of STIs/RTIs at 9 months was observed.

Candidiasis was the most prevalent genital infection and occurred in around 1 out of 4 women younger than 35 years of age. The prevalence decreased with age both in HIV+ and HIV- women. Persistent candidiasis occurred more often in HIV- women (50% of cases) than in HIV+ women (7.7% of cases; $p<0.05$). One contributing factor was that HIV+

women were on a regular basis in contact with the HIV clinic, which resulted in that more HIV+ women than HIV- women were treated for candidiasis and for other RTIs/STIs. Our prevalence figures of candidiasis are similar to the ones reported from Burkina Faso and Kenya [342-344]. *T. vaginalis* infection decreased with age in the HIV+ group, while it increased with age in the HIV- group. In cohort A, bacterial vaginosis occurred rarely among HIV+ women. However, in cohort B (*i.e.*, subgroup of cohort A) the prevalence of bacterial vaginosis in HIV+ women was instead 8.8-22.6% and in HIV- women 15.0-18.0%. Even though all tests were performed with the same technique and at the same laboratory, technical issues with the analyses at baseline could be an explanation to the discrepancies in prevalence.

5.1.9 Prevalence of STIs in HIV+ and HIV- women

The 9-month incidence of syphilis was 10.9% in HIV+ women compared with 0% in HIV- women ($p<0.01$). Our data corroborates previous studies showing that syphilis is more common among HIV+ women than among HIV- women [345-348]. Our study revealed a higher prevalence of syphilis among HIV- women than previous studies [346]. The seroprevalence of syphilis of cohort A was 6% in HIV- women. Even if considering the small number of identified cases (6 of 100) among HIV- women, the seroprevalence of syphilis was noteworthy higher than the prevalence of 0.8% reported in the recently published study from Rwanda by Mutagoma et al. (2016) [346]. The percentage of women reported ever being infected by gonorrhea was higher in the HIV+ group compared with the HIV- group ($p<0.001$) and the percentage increased with age. Only a few women reported to have been infected by chlamydia, though, the proportion of who stated “I don´t know” was much higher in the HIV+ group than in the HIV- group ($p<0.001$). In cohort A, the number of women tested positive for HBsAg and anti-HCV was small both among HIV+ women (1.0% and 2.1%, respectively) and HIV- women (3.4% and 2.6% respectively).

5.1.10 Rwandan women did not seek treatment for genital infections

Among women who were tested positive for any STI/RTI at baseline, only 13/53 women (24.5%) contacted their health facility and got treatment. HIV+ women tended to seek treatment more often than HIV- women (36.4% and 16.1%, respectively; $p=0.11$). The number of patients treated for STIs/RTIs was low in both studied cohorts giving low

power to the statistical analysis. When considering all STIs/RTIs combined, 25% of HIV+ women were cured by treatment compared with 80% of HIV- women (n.s.). In HIV- women 4/5 STIs/RTIs (80%) and in HIV+ women 6/11 STIs/RTIs (55%) were cured by treatment (n.s.). In HIV- women 17/32 STIs/RTIs (53%) and in HIV+ women 15/25 STIs/RTIs (60%) were resolved spontaneously (n.s.).

Besides access to health care, some of the barriers preventing African women to seek treatment for genital infections have been extensively studied, *i.e.*, the asymptomatic characteristics of many of genital infections, the way in which women perceive symptoms as not seriously enough to seek treatment, self-medication among women and the stigma attached to seek treatment for STIs [349]. As a consequence to this, complications may arise, *e.g.,* leaving bacterial vaginosis untreated may lead to preterm labor and postpartum endometritis [350]. Well-known is also the risk to develop neurological complications if syphilis is left untreated [351].

5.1.11 Sexual behaviour among Rwandan women

Seventy-five percent of HIV- women and 35% of HIV+ women reported to have had sex during the 9-month follow-up. The majority (87%) claimed to have had only one sexual partner and 41% never used condom for protection. More HIV- women than HIV+ women had had more than one sexual partner (20% *vs* 2.1% respectively; $p<0.001$) and never used condom (57.3% *vs* 14.6%, respectively; $p<0.001$) during the follow-up. We showed that most of HIV+ women claimed not to have had sex during the 9-month follow-up, while those who engaged in sexual activity claimed using condom, which is in line with previous studies in Africa [352]. However, our study shows that 30 out of 32 cases of all infections observed at 9 months were new and were contracted during the follow-up period and that 82% were found among HIV+ women. The paradox of high rate of condom use among HIV+ and high rate of new infections has been demonstrated in several other African countries [353, 354]. Religious beliefs led to the less consistent use of condoms and that women had difficulties in negotiating condom use with their partners. The social desirability bias among HIV+ women may possibly also contribute to the paradox. Whether women are living with polygynous partners was not covered in our questionnaire. However, the paradox of high degree of STIs and few sexual partners among Rwandan women may possibly depend on STIs coming from polygamous partners.

While all unmarried HIV+ women and the majority of all married HIV+ women (82%) reported using condoms, the corresponding figures for unmarried and married HIV- women were 30% and 45%, respectively. Neither did the awareness of present STI affect attitudes to condom use among women. Almost all STI positive HIV- women (87.5%) reported not to have used condoms during sexual intercourse during the 9-month follow-up. The low percentage of condoms use in people who are HIV negative or not aware of HIV status is consistent with previous studies [355, 356]. Previous studies also show a low use of condoms among married and partnered people in SSA [357]. Condom use has decreased the last years in Rwanda and Uganda, while the use of injectable contraceptives has increased as a contraceptive method [358]. This may as a consequence, lead to an increased exposure to STIs including HIV.

5.1.12 Participant's characteristics associated with PHR/HR-HPV infection and squamous intraepithelial lesions

PHR/HR-HPV infection at baseline was associated with being divorced (OR=6.48; p<0.05) in HIV- women .It was also associated with ever having a genital gonorrhea infection (OR=4.88; p<0.001; AOR=3.01; p=0.07) and not knowing if having been infected by chlamydia (OR=1.78; p<0.01; AOR=2.61; p=0.12) in HIV+ women. HIV+ women have a higher degree of susceptibility to various genital infections and may be exposed to more genital infections than HIV- women [359]. PHR/HR-HPV infection at baseline was negatively associated with being under ART (OR=0.20; p<0.05) and a CD4 count>500 cells/mm^3 (OR=0.26; p=0.09; AOR=0.18; p=0.05).

Squamous intraepithelial lesions at baseline were associated with hepatitis B infection (OR=18.80; p<0.01; AOR=50.25; p<0.01), with *Trichomonas vaginalis* infection (OR= 6.71; p<0.05; AOR=30.98; p<0.05) and with PHR/HR-HPV (OR=16.89; p<0.001; AOR=16.33; p<0.001), in HIV- women. Both HPV and hepatitis B are transmitted sexually and previous reports suggest an association between hepatitis B and cervical HPV infections [360].

In HIV+ women the number of live births (OR=1.44; p<0.01; AOR=1.55; p<0.05) was correlated with SIL, which is supported by previous reports [361]. HPV infection and the generation of HSIL may be facilitated by the immunosuppressive state pregnancy induces [362]. Being infected by PHR/HR-HPV (OR=14.65; p<0.001; AOR=13.02; p<0.001) was positively associated to SIL, while being under ARV (OR=0.14; p<0.01) and having a CD4

count>500 cells/mm^3 (OR=0.07; p<0.05) were negatively associated to SIL. These findings are in line with previous reports showing that normal CD4 count in HIV+ patients leads to a decreased risk of being infected by HPV and being diagnosed with SIL [363, 364]. Still the prevalence of HPV was higher in the group of well-treated HIV+ women compared to HIV- women. ARV increases the chance of clearing HPV infections, however, some HR-HPV may still be difficult to eradicate under ARV [325]. Even if HIV+ patients under ARV may have normal CD4 count, HPV-specific T-cell immunity may be impaired contributing to the difficulty in clearing HPV infections [365].

5.1.13 Risk factors for developing HR-HPV persistence and HSIL or worse cytology

Being unmarried (OR=3.09; p<0.01), two or more life-time sexual partners (OR=2.59; p<0.05), being infected by HPV16/18 (OR=5.82; p<0.01; AOR=6.18; p<0.05), being infected by HR-HPVs other than HPV16/18 (OR=16.00; p<0.001; AOR=14.18; p<0.001) and HIV positivity (OR=9.46; p<0.001; AOR=6.72; p<0.01) at baseline were positively correlated, whereas age at first intercourse of 21 years or over was negatively correlated (OR=0.38; p<0.05) with HR-HPV persistence. Being infected by HR-HPVs other than HPV16/18 (OR=4.35; p<0.01; AOR=6.57; p<0.001) and other STIs/RTIs (OR=2.37; p<0.05; AOR=2.20; p=0.09) at baseline were positively correlated with LSIL. Being unmarried (OR=0.39; p<0.05; AOR=0.29; p<0.05) and using hormonal contraceptives (OR=0.49; p=0.09; AOR=0.35; p<0.05) were negatively correlated with LSIL. Being unmarried (OR=3.85; CI: 1.07-13.89; p<0.05), being infected by HPV16/18 (OR=4.99; p<0.001; AOR=3.74; p<0.05) and being infected by HR-HPVs other than HPV16/18 (OR=5.82; p<0.05; AOR=5.83; p<0.05) at baseline were positively correlated with development of HSIL or worse cytology. HIV positivity tended to be positively correlated to HSIL or worse cytology in the univariate analysis (OR=3.39; CI: 0.94-12.23; p=0.06).

In a study from Brazil it was demonstrated that being unmarried was correlated with HPV re-infection among pregnant HIV+ women in Brazil [366]. Divorced, widowed or single women were demonstrated to be at higher risk of contracting incident HPV infections in a study from Costa Rica [367]. Unmarried women were at a greater risk of contracting HPV in a study from Canada [368]. In line with many reports, HR-HPV persistency was associated with development of cervical lesions. In HIV+ women with persistent HR-HPV infections, 86% presented with abnormal cytology at one or multiple occasions during the two-year follow-

up. Previous studies have identified HIV status as an independent risk factor for HSIL [369, 370]. While a higher proportion of HIV+ women than HIV- women developed persistent HR-HPV infections and HSIL, HIV positivity was not retained in the multivariate analysis as an independent risk factor for the development of HSIL. Our study was, however, underpowered, to reveal such a correlation, since only few women developed HSIL in our cohort. It is suggested in some studies that older women more often than younger women develop persistent HR-HPV infections, [371-373]. Age was, however, not retained as a risk factor to HR-HPV persistence. Other large prospective cohort studies have also shown that age does not constitute a risk factor for HPV persistence [374-376].

Infection by HPV 16/18 at baseline was the only risk factor for HSIL but did not constitute a risk factor for LSIL. Infections at baseline, by other PHR/HR strains besides HVP 16/18 were, however, a risk factor for development of both LSIL and HSIL. While HPV persistence is a strong predictor of HSIL and risk of development of cervical cancer, LSIL may instead be associated with present chlamydia infection [377], trichomoniasis [378] and bacterial vaginosis [379]. Since the number of cases of individual STIs and RTIs was low, we did not stratify individual RTIs and STIs in the univariate analysis. While we could not observe a correlation between the use of oral contraceptives and HSIL//worse cytology, other studies show that the risk of cervical cancer increases with the duration of use of oral contraceptives [135]. Instead women ever using oral contraceptives were less likely to develop LSIL. A study from Colombia also demonstrated a reduced risk of LSIL among oral contraceptive users [380].

5.1.14 SNP in IL28B correlated with susceptibility to HPVs infection

Women contracting HPV had the C/T and T/T genotypes of the IL28B SNP (rs12979860) more commonly than women *not* contracting HPV in log-additive and in dominant models ($p<0.05$) .The polymorphism was, however, correlated neither with HR-HPV persistence and nor with the development of HSIL or worse cytology. No differences in the distribution of genotypes between HIV+ and HIV- women were observed. The IFNλ3 rs12979860 SNP has previously been linked to susceptibility for viral infections. Hence, the T allele was overrepresented among hepatitis C infected Uruguayan patients compared with uninfected controls [381] and was linked to susceptibility for hepatitis B infection in the Chinese population [382]. In

contrast, the T allele appeared slightly underrepresented in HIV-1 infected subjects [383]. In our study we found no correlation between rs12979860 SNP and HIV, but found that the CT and TT genotypes of the IFNλ3 rs12979860 SNP were more common among women infected by HPV than among women *not* infected by HPV. While the polymorphism is associated with clearance of hepatitis C [384, 385], the present data showed no association between the polymorphism and the chance of clearing HPV infection.

5.1.15 Strengths and limitations of Paper I-III

Our study has strengths and limitations: First, the cervical lesions were characterised based only on cytology test results, misclassification resulting in low sensitivity of cytology may have lead us to underestimate cervical abnormalities so that may be women were classified as < HSIL while they are really HSIL or worse but we know that up to now no other gold standard test available next to cytology. As many other follow up studies in long time, we also experienced a problem of participants lost to follow, which limited our power during the analysis, however we assume that the longitudinal design is the most suitable for the natural history of the disease study. As our study objectives were directed to sensitive issues, such as women sexual life, the results in relation to condom use and number of lifetime sexual partners should be interpreted with caution. In addition, as some of the research questions referred to past event, we could not exclude a recall bias during our results interpretation. However, in spite of limitations, to our knowledge, this is the first study report on natural history on cervical HPV cancer in Rwanda for a follow up time extended up 2 years of observations. In addition, our study population were suitable for natural history of cervical HPV cancer .In fact, most of the factors known to interact with the course of cervical cancer evolution such as smoking, HPV vaccination and cervical cancer screening were observed at low proportion. In addition, the study was designed to observe participants with multiple sampling point times. This gave us the opportunity to define persistence/clearance infection for at least two consecutives positive/negative test results.

5.2 Preclinical studies (Paper IV-V)

5.2.1 Radiation did not induce morphological changes in rat cervix

In paper IV, we wanted to assess how the immune system and antioxidative responses of the uterine cervix are affected by cervical irradiation. In paper V, we wanted to assess whether HBOT may reverse changes induced by cervical irradiation. Histological examination of the cervix showed that the irradiation seemed not to have induced any apparent changes of the cervix morphology. We did not observe any morphological changes (e.g. fibrosis, oedema) in the cervix 28 days after cervical irradiation, even though the equivalent dose of radiation given was large. Likewise, at earlier time points after cervical irradiation (1-14 days post-irradiation) we did not observe changes in the morphology of the cervix [386]. In previous studies from the research group studying the pathophysiological processes following urinary bladder irradiation, the morphology of the urinary bladder 1-28 days post-irradiation seemed intact, although changes in oxidative stress and in the immune response occurred [291, 315]. Tissue changes induced by irradiation may take long time to develop. For example, irradiation of the rectum induces changes in the crypts 6 weeks after irradiation [295]. Typical radiological signs of lung fibrosis do not develop until 10 weeks after thoracic irradiation of the mouse [387]. Still, our findings indicate that tissue changes occur after cervical irradiation involving oxidative stress, antioxidative responses and immune-regulatory pathways. These responses may tentatively later result in advanced changes in the cervical tissue, such as fibrosis, similar to the clinical situation in patients exposed to radiotherapy due to for example cervical cancer.

5.2.2 Radiation induces oxidative stress and changes in TLRs expression in rat cervix

Western blot showed the expression of TLRs 2-9 in control and irradiated cervical tissue. An increase in cervical TLR5 expression in response to cervical irradiation occurred in the cervical epithelium ($p<0.05$). TLRs 2-9 were expressed in the epithelium. In the submucosa, TLR2, TLR4, TLR5, TLR6 and TLR9 were expressed. In the smooth muscle TLR2-9 except TLR8 were expressed. TLR4, TLR5, TLR6 and TLR7 were also expressed in blood vessels. MyD88 and TRIF were expressed in the cervical epithelium. Western blot analysis showed that toll-like receptor downstream molecules TRIF and NF-κB were increased in the irradiated cervix, while

MyD88 instead was decreased. SOD-1, SOD-2, catalase and 8-OHdG were expressed in the cervical epithelium and in blood vessels of the cervical submucosa. Cervical irradiation induced up-regulation of 8-OHdG, SOD-1 and catalase in the epithelium and in submucosal blood vessels of the cervix.

Twenty-eight days after cervical irradiation, no obvious microscopic changes in the cervix could be observed. TLR5, TRIF and MyD88 expressions assessed by western blot were neither affected by cervical irradiation and nor by HBOT. However, TRIF expression in the cervix was lower in irradiated rats exposed to HBOT compared with those exposed to solely cervical irradiation ($p<0.05$). The expression of 8-OHdG was increased in the cervical epithelium after cervical irradiation, while HBOT reversed the expression to levels observed in control animals ($p<0.05$). Similarly, the expression of 8-OHdG in submucosal blood vessels tended to be increased by cervical irradiation and be reversed by HBOT, however, statistical significance was not attained (n.s.; n=5). Catalase was decreased in the cervix after irradiation and the decrease was normalized in the groups exposed to HBOT ($p<0.01$-0.05; n=5-11). The expressions of SOD-1 and SOD-2 were neither affected by irradiation nor by HBOT (n.s.; n=5-11).

The expression of TLRs (TLR1-9) in the female reproductive tract has been demonstrated in previous studies [184, 388-390]. It is also established that radiation induces TLR-driven mechanisms in cancer [391]. However, knowledge about how radiation affects the expression of TLRs in the normal cervical tissue is at present lacking. The most apparent finding of our study was that cervical irradiation induced increases of TLR5 in the epithelium concomitant with changes in cervical levels of TLR adaptor molecules MyD88, TRIF and NF-κB. The cervical epithelium expressed TLRs 2-9, which indicates that the cervical epithelium may respond to a high number of PAMPs and DAMPs. In the epithelium, TLR4, TLR6 and TLR9 were more expressed than TLR2, TLR3, TLR5, TLR7 and TLR8. Previous studies on cervical human epithelial cell lines showed the presence of all TLRs (1-9), where stimulation of TLR2, TLR3, TLR5 and TLR6 induced the release of cytokines [392]. The myometrium expresses TLR2, TLR3 and TLR5 and stimulation of these TLRs may induce the release of pro-inflammatory and pro-labour mediators [393-395]. TLRs (such as TLR2, TLR4 and TLR5) may use the MyD88/IRAK/NF-κB signal and the TRIF transduction pathways. Our data show that TRIF and NF-κB increased while MyD88 decreased in the irradiated cervix. The increase in TRIF

appeared in the epithelium concomitant with the up-regulation of TLR5. Studies show that TLR5 may interact with the TRIF pathway in the gut epithelium [396]. TLR5 may mitigate radiation-induced damage in the irradiated head and neck area of the mouse [397]. TLR5 agonists may also reduce apoptosis after irradiation of the gut thereby improving tissue remodelling after rectal irradiation [398, 399]. The TLR5 agonist flagellin protects mice exposed to a lethal dose of whole-body γ-irradiation via MyD88 dependent pathways [400]. Radioprotective effects have also been shown to be generated via other TLRs such as TLR2, TLR3, TLR4 and TLR9 [401]. Western blot showed the presence of two bands of TLR8 (90 kDa and 95 kDa). Radiation tended to increase and decrease the expression of the denser and lighter band, respectively, which may indicate that TLR8 may undergo processing in response to irradiation. Previous studies show that TLR8 undergoes proteolytic processing in the endolysosomes to generate functional receptors in human monocytes and macrophages [402].

Our study showed that oxidative stress (reflected by 8-OHdG staining) increased successively till 14 days after cervical irradiation, while SOD-1 and catalase increased in the cervical tissue already 24 hours after cervical irradiation. Changes in oxidative stress and antioxidants occurred particularly in the epithelium and in the submucosal blood vessels of the cervix. The present findings are in line with previous reports showing that oxidative stress and anti-oxidative responses appear particularly in the urothelium and in submucosal blood vessels 28 days following irradiation of the rat urinary bladder [403]. Our data may indicate that oxidative stress induced by cervical irradiation activated TLR5 and TLR-connected downstream molecules TRIF and NF-κB thereby mitigating oxidative stress via the release of antioxidants SOD-1 and catalase in the cervical tissue.

Fourteen days following cervical irradiation the dominant effect on the cervical tissue was an anti-inflammatory response reflected by a reduction in important pro-inflammatory cytokines and chemokines. G-CSF, M-CSF, IL-10, IL- 17A, IL-18 and RANTES expressions in the cervix decreased two weeks after cervical irradiation ($p<0.001$-0.05). GM-CSF and MCP-1 tended to decrease but significance was not attained ($p=0.05$ and $p=0.06$, respectively). IL-17 was decreased concomitant with a decrease in G-CSF, M-CSF and IL-10 levels in the cervix. The present findings are in line with studies showing that pro-inflammatory mediators are reduced 14 days following urinary bladder irradiation [291, 403]. IL-17 plays an important

role in the female genital tract and may be released by Th17 cells and mucosal-associated invariant T (MAIT) cells [404, 405]. Our findings with a reduced IL-17 in response to cervical irradiation may indicate that radiation may lead to a weaker response to pathogens. An imbalance between Th1 and Th2 cytokines may also favour the development of adverse functional side effects to radiation and fibrosis. A number of studies have shown that Th17 cells and IL-17 play important roles in the development of radiation-induced functional disorders and fibrosis, *e.g.*, radiation-induced proctitis [290], liver fibrosis [406, 407] and radiation-induced pulmonary fibrosis [408, 409]. Interestingly, IL-10 and IL-17 levels in bronchoalveolar lavage, after pulmonary irradiation, were inversely correlated to the severity of pulmonary fibrosis [408]. Speculatively, our findings of reduced levels of IL-10 and IL-17 may indicate that a pro-fibrotic microenvironment may have been developed in the cervix. The development of fibrosis seems, however, to take time since we could not either observe fibrosis in the rat cervical tissue 28 days after cervical irradiation (unpublished data).

5.2.3 HBOT reverse the radiation induced-oxidative stress in rat cervix

In an animal model we here show that HBOT may reverse changes in oxidative stress induced by ionisation radiation. Our previous study examining the pathophysiological processes 1-14 days following cervical irradiation showed that irradiation may induce changes in the immune system of the cervix [386]. Several TLRs may be important for radioprotection [410]. Stimulation of TLR5 may reverse oxidative stress after irradiation of rat testis [411], may induce tissue remodelling and immune capacity in radiation-induced proctitis [398] and may stimulate hematopoietic progenitor cell proliferation in lethally irradiated mice [412]. In our previous study we observed an up-regulation of TLR5 and activation of the connected intracellular signalling pathways TRIF and MyD88 [386]. The present study shows that TLR5 activation seems to have subsided 28 days after cervical irradiation. Albeit TLR5 activation was not observed, oxidative stress as assessed by 8-OHdG was present at 28 days after irradiation as well as a reduced expression of catalase in the cervix compared with controls. In our previous study we showed that SOD-1 and catalase were activated in the early events following cervical irradiation [386]. The expression of SOD-1 and catalase were normalized 14 days following cervical irradiation. For catalase, the expression at 14 days even tended to be below normal expression, which is consistent with the current findings that catalase was decreased at 28 days after cervical irradiation. In rats exposed to cervical irradiation followed

by HBOT, the expression of catalase was normalized most plausibly as a consequence of reduced levels of oxidative stress.

5.2.4 Strengths and limitations of Paper IV-V

In paper IV and V, we are presenting results from western blot and immunohistochemistry technics. Both technics are characterised by a multiple steps of preparation and washing before the last step of protein expression visualisation and quantification. Even if we were using a well-validated protocol, we could not however minimize potential loss of protein during the whole process, which lead to underestimation of the reported results. This is why we reported the relative quantities. In addition, reporting results from immunohistochemistry should be associated with an excessive level of subjectivity. To minimize this limitation, sections were prepared and blinded before a second person read and analyse them.

6 CONCLUDING REMARKS

6.1 Clinical studies (Paper I-III)

To summarize, the results from paper I-III indicate the following:

1. The overall prevalence of HPV infection and the prevalence in HR-HPV infection from this study are quite high even if they fit into the range of those previously reported in Rwanda. They also confirm that Rwanda is among East African countries with high prevalence in HPV infection;
2. A high level in HPV incidence especially among previously negative tested women is bad indication that, if nothing is done, the predicted increase in HPV infections among SSA countries should become true;
3. HPV 16, 52, 58, 66 and HPV70 were the most five prevalent types among Rwandan women, with HPV 16 being the most persistent and the most associated with cervical precancerous lesions. This supports previously provided evidences that the vaccine currently (Gardasil®) in use in Rwanda, should miss an important proportion of women and emphasise the need for a more protective vaccine;
4. Consistent with other natural history of cervical HPV infection studies, most of the infections, even those related to HR-HPV types are transients. Only a small fraction of women were found with persistent infection. This small fraction corresponds to the fraction of women with HSIL who were presented to the study with HR-HPV infection. This constitutes a good tool for decision makers in Rwanda when designing a screening program;
5. Unexpectedly, a fraction of 5/237 of women with HSIL was negative for HR-HPV and a non-negligible fraction of other cervical abnormalities were shown not related to HPV infection. If we agree with sensitivity of cytology test, this result requires confirmation from other natural studies at national level;
6. A shown overall high prevalence in STIs/RTIs with endemic prevalence in bacterial vaginitis vaginal candidiasis and syphilis infection among HIV infected women, recall for an urgent need preventive measures in Rwandan population by targeting high-risk groups such as HIV infected women. This will reduce a

high proportion of women with cervical low-grade lesions and probably the incidence of HPV infection;

7. HIV infection is the most risk factor for contracting HPV, HR-HPV infection. Furthermore, we showed that specific HR-HPV DNA test should be used as a cervical cancer screening test in HIV infected women;
8. The results from this study show that women sexual behaviours such as early age at first intercourse, high number of lifetime sexual partners, being unmarried, history of gonorrhoea infection constituted risk factors for HR-HPV acquisition and persistence, especially among HIV infected women. The results also show that the infection by HR-HPV types other than HPV 16/18 and the infection by STIs/RTIs at baseline predicted more LSIL while the infection of any HR-HPV presented at baseline and being unmarried constituted the independent risk factors for developing HSIL;
9. Finally, the C/T and T/T genotypes of the IL28B SNP rs12979860 increase women's susceptibility to HPV infection.

6.2 Preclinical studies (Paper IV-V)

The results from paper IV and V lead us to the following conclusions:

10. The uterine cervix expresses the TLRs 2-9;
11. Radiation treatment induces upregulation of TLR5 coupled with depletion in important pro-inflammatory cytokines in rat cervix;
12. Radiation treatment induces oxidative stress, in the rat cervix, which can be visualised by oxidant and/antioxidant markers;
13. Radiation treatment induces long-lasting changes in oxidative stress in the uterine cervix and HBOT may tentatively be used to help women who have developed radiotherapy-induced adverse effects of the genital tract;
14. More studies on effect of radiation in rat cervix are needed to validate its use as model in studies of immunological aspects of human cervix

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