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Mucosal microenvironment and host immunity in HPV-driven carcinogenesis

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1. INTRODUCTION

1.1 HUMAN PAPILLOMAVIRUS AND HIV CO-INFECTION: GENERAL FEATURES

Human papillomaviruses (HPVs) are small, non-enveloped, epitheliotropic, double-stranded DNA viruses that infect mucosal and cutaneous epithelia in a wide variety of higher vertebrates (IARC Monographs, 2007). HPVs belong to the *Papillomaviridae* family (IARC Monographs, 2007) and represent one of the most common causes of sexually transmitted disease in both men and women worldwide and probably the most common sexually transmitted viral disease (Milner, 2015; Geskus et al. 2016). Since Harald zur Hausen proposed the link between HPV and cervical cancer in the early 1970s (zur Hausen et al., 1975), knowledge on HPV and HPV-related diseases has rapidly grown.

HPV infection is commonly found out in the male and female anogenital tract, and its association with different tumours has been observed for vulva, vagina, penis, and anal canal. Among HPV-related anogenital cancers, HPV-16 is the most commonly type detected, followed by HPV-18 (IARC monographs-100B, 2011). It is also well established that the risk for anogenital cancers is higher among HIV-positive people compared to the general population, despite combined antiretroviral therapy (cART) (Palefsky, 2009).

Furthermore, a low CD4-T cell count, upon the onset of AIDS, is associated with a statistically significant increase in the risk of developing invasive anal canal tumors among HIV-1 positive men (Piketty et al., 2008). To this extent, the immunosuppression degree observed in HIV-1 population is associated with an increased persistence of HPV and a progression of HPV-lesion severity (Moscicki et al., 2004, Shiels et al., 2017; Hernández-Ramírez et al., 2017). In this context, the incidence of anal cancer has been rapidly increasing in Men who have sex with men (MSM), that also display an increased risk of HPV infection, due to their high-risk sexual behaviors (Colón-López et al., 2018; Chiao et al., 2006; D'Souza et al., 2008). Indeed, the interaction between HIV and HPV, which share

the same risk factors, has been reported to increase the risk of HPV and reduce the clearance rate among people living with HIV (Yuan et al., 2019).

It has been also hypothesized that during the period of HAART, the increased life expectancy provides enough time for progression of premalignant lesions as well as actual invasive tumors development (Chaturvedi et al., 2009; Ferlay et al., 2010; Teeraananchai et al., 2017). In addition, immunosuppression due to HIV-1 can influence the last stages of HPV-related cancer progression, especially at the mucosal level (Hernández-Ramírez et al., 2017); in fact, for the resolution of HPV infection, both the innate immune and the antigen-specific responses are considered essential, with a prominent role in the anogenital mucosa.

1.2 HIV-1 IMMUNOSUPPRESSION AND HPV-RELATED LESIONS

The strong relationship between HIV and HPV has been well established and appears to be related to the alteration in cell-mediated immunity that characterizes HIV-1 infection (Houlihan et al., 2012). This component of the immune response is crucial for HPV clearance, and its failing would increase the susceptibility and the potential reactivation of latent HPV infections. At the same time, HPV infection appears to be associated with a greater likelihood of HIV transmission (Houlihan et al., 2012). Although the mechanism is not yet really established, it seems that the inflammatory response triggered by HPV may accurately stimulate all the cells that are most vulnerable to HIV infection (Moscicki et al., 2004). Therefore, this could be an important topic to investigate because it could give to HPV prevention the appropriate importance. Although anal cancer precancerous lesions can be identified through anal cytology and High Resolution Anoscopy (HRA) as an easy and effective method, the impact of these screening activities on the reduction of anal cancer has yet to be demonstrated. Several studies are focusing on the identification of possible alternatives to prevent HPV-related cancer, as the safety, immunogenicity, and efficacy of current HPV vaccines in HIV-1 positive subjects.

However, the exact cause of the highest risk of anal cancer development among the MSM population is not completely defined but probably reflects the higher rate of receptive anal intercourse that expose to an increased risk of anal HPV infection and the contraction of other sexually transmitted diseases and chronic anal inflammation (Chelimo et al., 2013). Moreover, a crucial HPV risk factor in this population is represented by immunosuppression (Bertisch et al., 2013; Coghill, et al., 2016). However, it seems that the introduction of an effective antiretroviral therapy did not affect the incidence of anal cancer and the risk seems rather related to the state of immunosuppression and the HIV load before the onset of therapy intake (Patel et al., 2008; Piketty et al., 2008; D'Souza et al., 2008; Seaberg et al., 2010; Simard et al., 2010).

In this context, among HIV-1 positive subjects, the most frequent anal cancer histological type is represented by squamous cell carcinoma (SCC), while a smaller percentage consist of adenocarcinoma and small cell neuroendocrine carcinoma (D'Souza, et al., 2008).

To date, there are still no guidelines for anal canal screening, nor any randomized clinical trials have been carried out to validate its effectiveness. Any possible lesions are identified with anal cytology, HRA and HRA-guided biopsies and any potential treatment is aimed at preventing invasive carcinoma, reducing related mortality and morbidity.

However, HPV-anal infection in men often occurs with no initial signs or symptoms of infection, even in the absence of anal receptive intercourse, and its prevalence (25%) suggests that this asymptomatic HPV may be a frequent anal infection (Olesen et al., 2019). Likewise, the identification of anal SIL (squamous intraepithelial lesion) is more frequent in HIV-1 positive than HIV-1 negative homosexuals, and the risk of progression from a low to a higher grade SIL is always greater in HIV-1 positive MSM (Abramowitz et al. 2007). Moreover, HIV-positive men and women are more likely to be infected with multiple HR HPV types than HIV-negative subjects, and persistent HPV prevalence results increased due to immunosuppression. Notably, in developed countries, the rate of cervical HPV infection reaches its maximum around 20-25 years of age, and then decreases, as a consequence of the mechanisms of acquisition and

clearance (Bosch et al, 2013). This age-related decline was not found in MSM and this may be due to impaired clearance of pre-existing infections, the reactivation of latent infections and the continuous acquisition of new infections, sometimes due to a frequent change of partner.

Given this PhD project focuses on the characterization of HPV infection and the related immune response during HIV-1 infection, a brief description of HPV characteristics and oncogenic properties will be provided below.

1.3 HPV VIRAL PARTICLE, GENOMIC ORGANIZATION AND LIFE CYCLE

HPVs are non-enveloped, small circular double-stranded DNA viruses with an icosahedral capsid, consisting of 72 capsomers, with a 55 nm diameter (Figure 1) (IARC monograph, 2007). The viral genome is organized into three regions of unequal size, consisting of 8 overlapping open reading frames (ORFs): early region (E), late region (L) (encoded by 50% and 40% of the genome, respectively) and long control region (LCR), also named upstream regulatory region (URR) (Figure 2). About 10% of the genome is contained within LCR and regulates transcription and replication of the viral DNA (Stünkel, et al., 1999). Amongst all HPVs, these three regions, are separated by two polyadenylation sites (pA): early pA (AE) and late pA (AL) (IARC monograph, 2007).

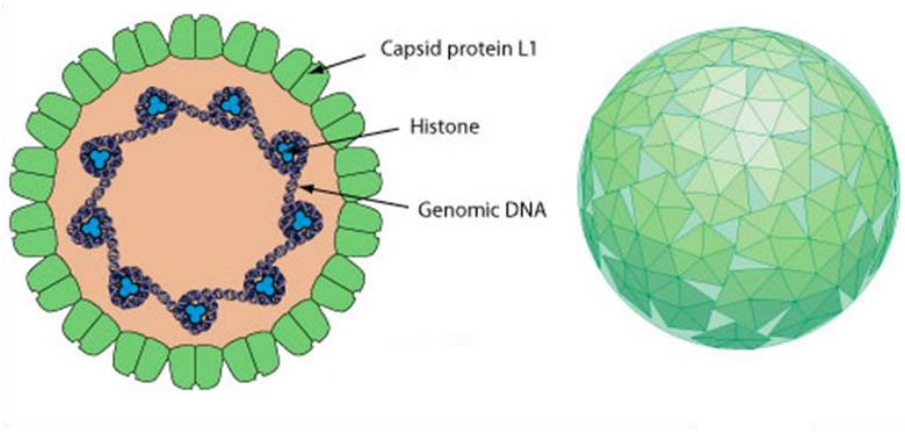

Figure 1. HPV virion structure (modified from https://viralzone.expasy.org/5)

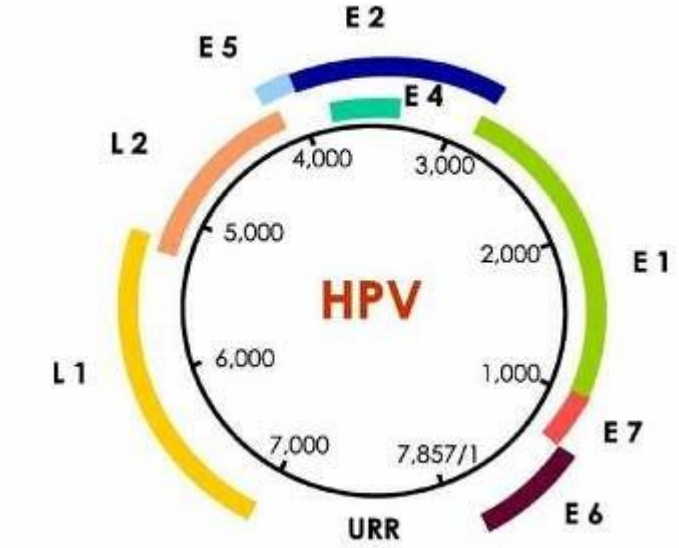

Figure 2. Schematic presentation of the HPV genome showing the arrangement of the early E or nonstructural genes, the capsid genes (L1 and L2) and the upstream regulatory region (URR). (Muñoz et al. 2006).

The Early region encodes for six ORFs (E1, E2, E4, E5, E6 and E7, Figure 2), coding for genes involved in regulatory functions, DNA replication, activation of the lytic cycle, and may have transformation potential (Streenbergen et al., 2005).

The Late region is located downstream of the early region and it contains the ORFs encoding the major and minor capsid proteins, L1 and L2, respectively (Figure 2).

The LCR is a non-coding segment of about 850 bp, that contains the origin of replication (ORI), as well as binding sites for different factors involved in the regulation of early and late promoters' transcription (Figure 2). A brief description of all HPV protein's function is provided below.

E1 is a highly conserved HPV protein with both ATPase and helicase activities (Hughes and Romanes. 1993, Bergvall, et al., 2013). Through a complex with the E2 protein, E1 forms hexamers with high binding affinity for DNA and initiate DNA replication (Longworth and Laimins, 2004).

Accordingly, the E2 dimer (IARC monograph, 2007), which acts as an important regulator of DNA replication and viral transcription, recruits the viral helicase E1 to the origin of DNA replication (Desaintes et al., 1996). Furthermore, E2 also regulates E6 and E7 levels (Gammoh et al., 2006), but E2 loss, following viral genome integration, represents one of the first step for neoplastic transformation (Pett et al., 2004).

The E4 protein is a fusion product with a 5-amino acid sequence from the N-terminus of E1, resulting in an E1^E4 protein, where the E1 sequence is used for initiation of translation (Hebner et al., 2006). Of note, E1^E4 transcripts are expressed throughout the HPV life cycle; E4 role is linked to productive infection: it interacts with the keratin intermediate filaments, facilitating the release of viral particles.

The E5 ORF encodes for a small hydrophobic membrane-localised protein with only weak transforming abilities in cell culture, (Hebner et al., 2006). The E5 ORF is encoded in most HPV early and late transcripts and it stimulates cell proliferation (Venuti et al., 2011), activates

signal transduction for mitosis (Fehrmann et al., 2003), inactivates the p21 protein (Venuti et al., 2011) and prevents apoptosis following DNA damage (Venuti et al., 2011).

The E6 protein is considered as a major HR-HPV oncoprotein that can transform human mammary cells and cooperate with E7 in transforming primary foreskin keratinocytes (Hebner et al., 2006). In HR-HPV, E6 is directly and indirectly associated with the tumour suppressor protein p53 (Ruttkay-Nedecky et al., 2013, Tommasino et al., 2003) degradation, leading to abnormal cell growth and blockage of apoptosis (Moody e Laimins, 2010; Figure 3). Due to the key role of p53 in maintaining genomic integrity, the HPV infection favour chromosomal abnormalities, considerably increasing the probability of these infected cells to evolve towards a malignant state, in particular during HPV-16 infection (Duensing et al., 2004; Münger et al., 2004). By contrast, no degradation of p53 was observed in keratinocytes infected with a low-risk HPV genotype (Mesplède et al., 2012). E6 also interacts with Interferon Regulatory Factor-3 (IRF3), a regulator positive transcription of the interferon (IFN) promoter, inhibiting the transactivation function of IRF3 and this may explain the ability of HR-HPVs to evade the immune response. Furthermore, E6 binding to Tyrosine kinase 2 (Tyk2) abolishes the intracellular JAK-STAT pathway (Li et al., 1999).

The E7 nuclear protein induce terminally differentiated cells to enter the cell cycle, binding the product of the retinoblastoma susceptibility locus (pRb) and the related pocket protein family members p107 and p130, leading to ubiquitin-dependent degradation (Dyson et al., 1989). The E7 protein contains three conserved regions (CR), known as CR1, CR2, and CR3, required for abrogation of epithelial cell quiescence and contribute to cellular transformation (Phelps et al., 1992).

Given the HPV life cycle phases depend on the epithelial differentiation, it results difficult to study the mechanisms that regulate the production and assembly of HPV virions. However, the capsid proteins, L1 and L2, are expressed in the late HPV life cycle in highly differentiated suprabasal cells and are the main components of HPV capsid (Ozbun et al., 1997). L1

monomers are organized as pentameric structures**,** known as capsomeres, to which L2 copies are associated (Chen et al., 2000; Modis et al., 2002, Becker et al., 2004; Finnen et al., 2003). As other viruses, HPV capsids may undergo a further maturation process before being released from the cell (Buck et al., 2005). However, L2 can bind DNA and localise to ND10 domains in the nucleus, which are the major sites of DNA replication (Zhou et al., 1994; Day et al., 2004; Ishov et al., 1996) and subsequently recruit L1 to create new virions.

As previously described, HPVs are a large and diversified group of viruses, displaying a tropism for several specific tissues. They are exclusively intraepithelial pathogens, and virus growth depends on the expression of the complete program of keratinocyte differentiation (Pinidis et al., 2016). All HPVs infect epithelial cells, and they require the terminal differentiation of the host cell to produce infectious virions (zurHausen. 2002; Hebner and Laimins. 2005).

The HPV replication cycle is divided into early and late phase and is closely associated with the epithelial cell differentiation program (Egawa et al., 2015). For productive infection to occur, HPVs must gain access to the basal epithelium through an abrasion or other type of damage in the skin (Christensen et al., 2016).

Through the interaction of the L1 protein with the heparan-sulfate proteoglycans, the viral particles bind efficiently to the basement membrane (BM**)**; L1 and L2 proteins undergo conformational changes that allow the binding to keratinocytes receptor, near the epithelial lesion (Schiller et al., 2010). Of note, HPVs are among the few viruses that initiate the infection process at an extracellular site (Schiller et al., 2010; Figure 3).

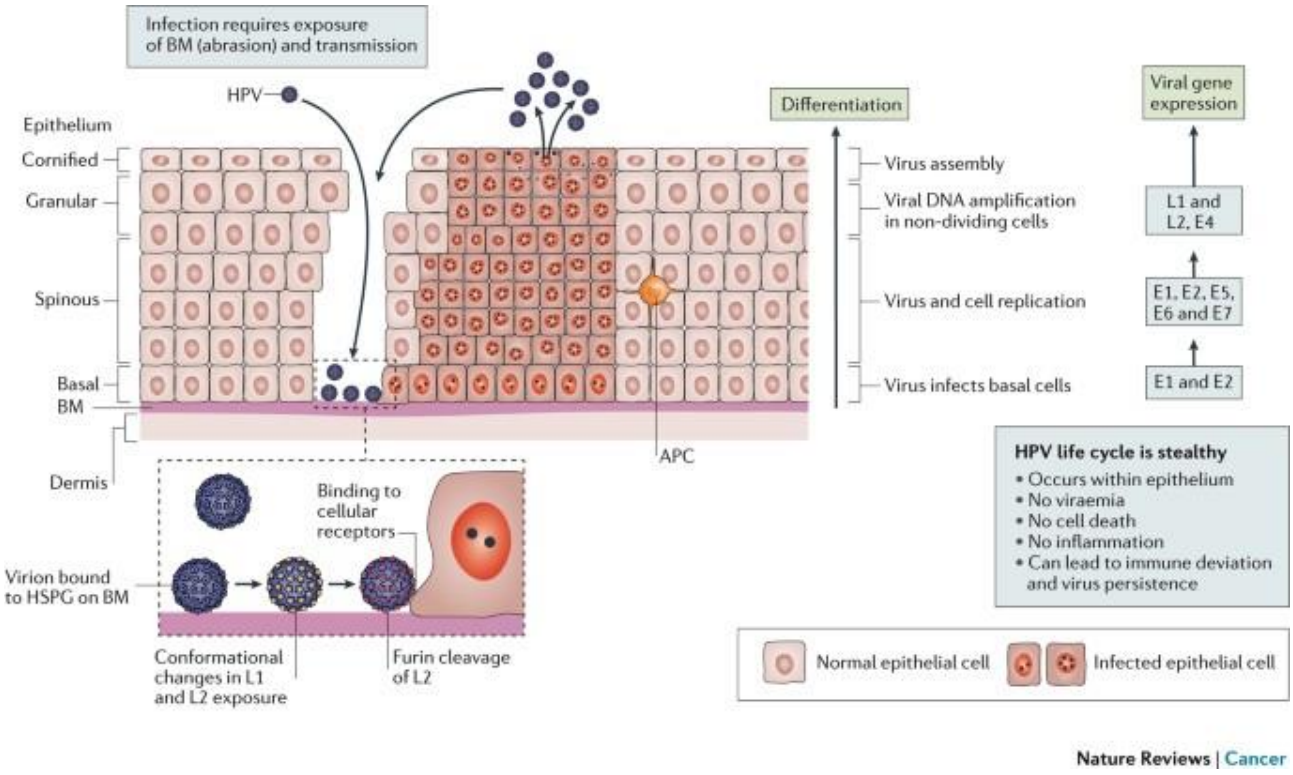

Figure 3. Schematic representation of different phases of HPV infection and life cycle (Roden, et al.,2018).

However, the mechanism through which the virus enters the host cell is not entirely understood, but it probably involves also cellular integrins (zurHausen. 2002; Hebner and Laimins. 2005). Following viral uncoating and transport to the nucleus, a cell cycle-indipendent burst of viral replication amplifies the viral copy number (50 to 100 copies per cell) (zurHausen. 2002; Hebner and Laimins. 2005, Pinidis et al., 2006). Then, the HPV infected cell enters the transit amplifying proliferative compartment of the epithelium, where there is a phase of plasmid or episomal maintenance with an invariable viral copy number, minimal viral gene expression but the virus replicates in synchrony with the S-phase of the host cell (zurHausen. 2002; Hebner and Laimins. 2005, Stanley et al., 2012).

Of note, during this phase of the HR-HPVs life cycle, the expression of the potent oncogenes E6 and E7 is under strict control (Gadducci et al., 2013).

Although the actual pattern of viral gene expression in the basal cells is not well defined, it seems that E1 and E2 are expressed to maintain the viral DNA in episomal form, while the viral genes E5, E6 and E7 enhance the proliferation of the infected cells and their lateral expansion (Wilson et al., 2002). In the upper layers of the mucosa, E1, E2, E6 and probably E7 genes expression result in genome replication assembly, maturation, and release of the viral particle. (Hubert et al., 2002; Stubenrauch et al., 1998; Thomas et al., 1999; Flores et al., 2000).

The dis-regulated production of HPV oncoproteins E5, E6 and E7, interacting with cellular tumour suppressor proteins, transcription factors and several cell pathways that are required for regulation of the cell-cycle progression in response to DNA damage (Palefsky et al., 1995; Yokota et al., 1993), may create a cellular environment in which normal cell- cycle controls are lost, allowing mutations to occur. Therefore, this accumulation of mutations promotes HPV related carcinogenesis (zur Hausen, 2000). On average, it takes approximately 20 years between the HPV infection and malignant transformation leading to cancer development (Frazer et al., 2006). In this context, HPV integration into the host genome is a critical event in malignant transformation, which assures the persistent expression of the E6 and E7 HPV oncoproteins in the basal and parabasal cells of the anogenital epithelium (Duensing et al., 2004).

1.4 HPV TAXONOMY AND CLASSIFICATION: HIGH RISK AND LOW RISK HPVs

HPV isolates are traditionally described as ‘‘types’’ or genotype. The first HPV types were isolated as early as 40 years ago (Orth et al., 1977; Coggin et al, 1979) and the difficulty to find appropriate cell culture systems (i.e., terminally differentiating epithelia) to propagate these viruses has hampered progress in studying viral functions and limited the establishment of a taxonomy based on biological properties. To date, more than 200 HPV genotypes (n=228)

have been completely characterized in humans, based on the nucleotide sequence analysis of PCR products derived from the L1 ORF (de Villiers et al., 2004) (Figure 4). To define an HPV type as a new genotype, the L1 gene sequence needs to be at least 10% different from any other known genotypes; each HPV is identified by a number based on the order of their identification (de Villiers et al., 2004; Bernard et al., 2006). Differences between 2% and 10% homology define a subtype and less than 2% a variant.

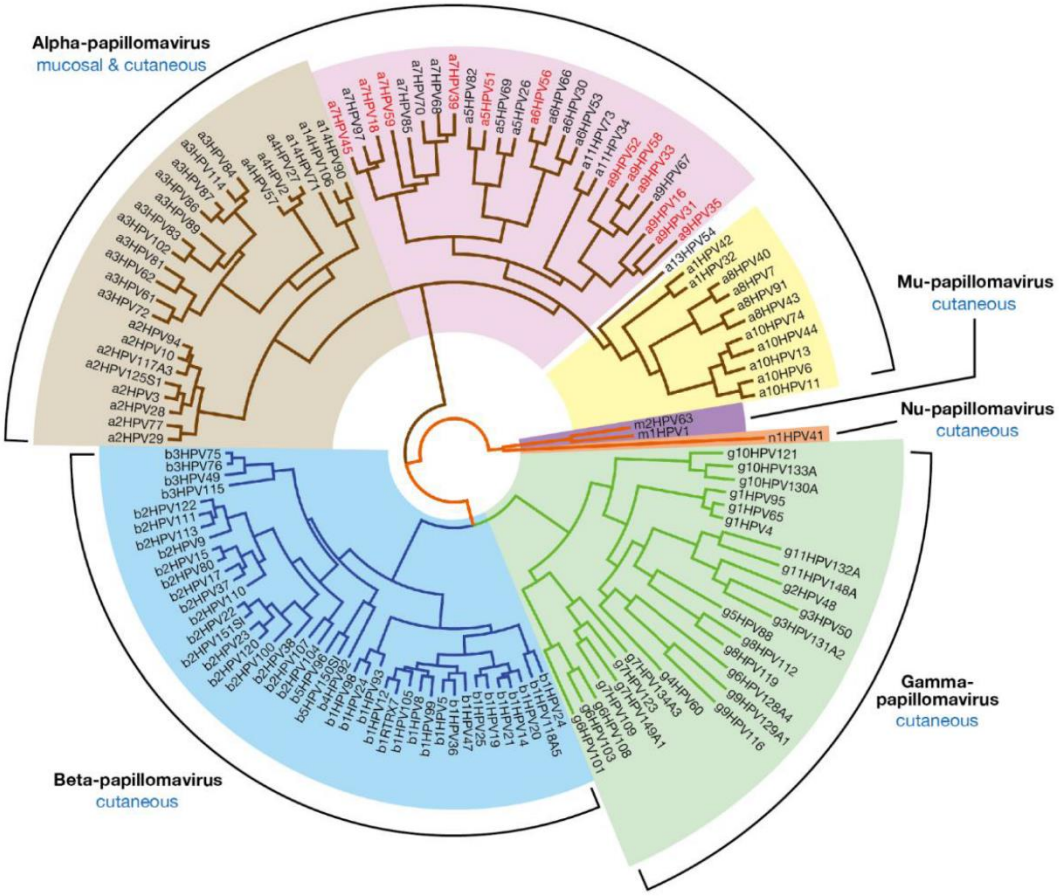

Figure 4. Evolutionary Relationship between Human Papillomaviruses. The HPV types found in humans are included into five genera, with the Alpha-, Beta- (blue) and Gammapapillomavirus (green) representing the largest groups; HPV types from the Alphapapillomavirus genus are often classified as low-risk cutaneous (light brown); low-risk mucosal (yellow); or high-risk (pink) according to their association with the development of cancer. The high-risk types highlighted with red text are confirmed as "human carcinogens" based on epidemiological data. The remaining high-risk types are "probable" or "possible" carcinogens. (Egawa et al., 2015).

Based on their tropism, HPVs can be categorized as cutaneous or mucosal types, infecting basal epithelial cells of the skin or inner lining of tissues (Egawa et al., 2015; Gheit et al., 2019). Cutaneous HPV types are epidermotropic and infect the keratinized surface of the skin, targeting hands and feet skin (Coscia et al., 2015); on the other hand, mucosal types infect the lining of the mouth, throat, respiratory tract, or anogenital epithelium (Coscia et al., 2015). Although most HPV infections are benign, all HPV types might be associated with a variety of clinical conditions that range from innocuous lesions to cancer.

Depending on their isolation from benign or malignant lesions, HPV can be divided into different groups called low-risk (LR), probable high-risk (HR), HR and undetermined HPV types (Lorincz et al. 1992) (Table 1). Notably, HPV types that are preferentially detected in cervical and other anogenital cancers have been designated as high-risk types (zurHausen. 1986; Munoz et al., 2003). By contrast, those found primarily in genital warts and non-malignant lesions were labelled as LR type. HR oncogenic viruses include HPV16, 18, 31, 33, and 35 (de Villiers et al., 2004; Munoz et al. 2003). HPV types 39, 45, 51, 52, 56, 57, 58, 59, and 68 may also be present in dysplastic and malignant lesions of other anogenital sites, although less frequently detected. The LR-HPV types include HPV6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89, which are usually associated with benign lesions such as cervical condylomas (de Villiers et al., 2004; Munoz et al. 2003).

Table 1: Classification of LR-HPV and HR-HPV types

GROUP	HPV GENOTYPES
Low-Risk (LR)	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 89
Probable High-Risk	26, 34, 53, 66, 70, 73, 82
High-Risk (HR)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
Undetermined Risk	30, 62, 67, 74, 83, 84, 86, 87, 91, 114

Functional differences between HR and LR-HPV types strongly correlate with malignant conversion of infected cells. In particular, HR HPV types induce increased chromosomal abnormalities and aneuploidy in the cell and their oncoproteins (E6, E7) that interact specifically with cellular proteins (p53, Rb) are more engaged in the regulation of cell growth and proliferation (Yim et al., 2005). About 40 mucosal HPV genotypes have been identified as they spread through sexual contact and infect primarily the cervix, vagina, vulva, penis, and anus. In this context, some HPV types have been associated with different pre-malignant and malignant SIL degrees and are considered the main etiological factor determining uterine cervix, anal and other types of epithelial cancer. Regarding the grade of oncogenic risk, four HPV genotypes are most often found within the malignant cells of cervical cancers, with HPV 16 accounting for about half of the cases in the United States and Europe and HPV types 18, 31, and 45 accounting for an additional 25 to 30% of cases (Harro et al., 2001). The most recent International Agency for Research on Cancer (IARC) classification has grouped the HR-HPVs into Group 1, that includes HPV strains that are certainly carcinogenic to humans, and Groups 2A and 2B, respectively possible and probable carcinogens for humans; by contrast, the LR-HPV group include genotypes that cannot be classified as carcinogenic (Group 3, Table 2).

Table 2. Classification of HPV types according to the oncogenic risk

		Definition	HPV Types
HR-HPV	Group 1	Carcinogenic to humans	16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
	Group 2A	Possible carcinogenic to humans	68
	Group 2B	Probable carcinogenic to humans	26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97
LR-HPV	Group 3	Not classifiable as carcinogenic to humans	6, 11, 28, 32, 40, 42, 43, 44, 54, 55, 57, 61, 62, 71, 72, 74, 81, 83, 84, 86, 87, 89

(IARC monographs-100B, 2011)

1.5 HPV AND ANAL CARCINOGENESIS

Biological and functional studies have finally demonstrated the direct role of HPV infection in the development of several human cancers (Tulay et al. 2016). Nonetheless, many HPV infections do not lead to cytological anomalies or cancer, but they are cleared by the immune system in a relatively short time (6–12 months) (Rositch et al., 2013; Cho et al., 2015).

HPV is responsible for nearly 100% of cervical cancers and around 88% of anal cancers, most of which caused by HPV 16 or 18 (Arbyn et al. 2012). Given the association with HR-HPVs, and similarities between histology features, and their precursor lesions, anal cancer and cervical cancer are very similar diseases. Overall, the incidence of anal cancer among HIV-1 uninfected MSM is greater than the incidence of cervical cancer in the general population of women (Daling et al., 1987; Ries et al., 2007).

The morphologic changes, caused by HPV infection in either the cervix or the anus, are similar on both cytology and histopathology and the spectrum of HPV-related cutaneous perianal

disease is essentially equivalent to vulvar neoplasia (Darragh et al., 2011). Anyway, the objective of anal cytology is to sample the surface epithelium of the entire anal canal, from the distal rectal vault to the anal verge, to obtain an adequate cytologic specimen (Darragh et al., 2011). The interpretive categories used for anal cytology are the same as for Pap tests: negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells cannot exclude HSIL (ASC-H), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC). Despite anal cytology has operational characteristics similar to the Pap test, sampling errors may play a larger contribution than in cervical cytology since these samples are collected without direct visualization of the anal canal (Palefsky et al., 1997). The sensitivity of anal cytology for the detection of high-grade anal intraepithelial neoplasia (HGAIN) is highest in HIV-infected MSM, probably due to the larger lesion size and burden of disease usually experienced from this population. Generally, normal anal cytology samples include rectal columnar cells, squamous metaplastic cells, nucleated squamous cells, and anucleated squames, which might be considered as a sample quality indicator (Figure 5) (Darragh et al., 2004; Darragh et al., 2011).

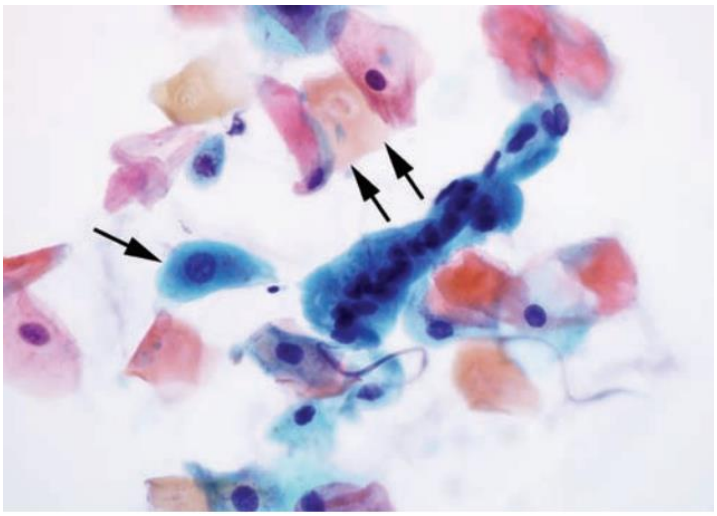

Figure 5. Cellular components on normal anal cytology. The nucleated squamous cells, squamous metaplastic cell (single arrow), anucleate squames (double arrow), and cluster of rectal columnar cells (Anal ThinPrep®, high magnification) are represented (Darragh et al., 2011).

Besides, it has been suggested that cytology-based anal cancer screening programs may lead to a reduction in anal cancer incidence (Leeds et al., 2016). However, these programs have been implemented in a limited number of scenarios, mostly in clinical settings that provide care to MSM with HIV (Goldstone, 2010; Palefsky rt al., 1997; Palefsky et al., 2005). As previously reported, the transformation of HPV-infected cells into cancer is a multi-step process (Egawa et al. 2015), during which E6 and E7 oncoproteins act to enhance cellular proliferation, resulting in increased numbers of infected cells and infectious virions (Hamid et al., 2009). Currently, it is believed that LR-HPV types do not cause malignancy due to weaker binding of their E6 and E7 to their target proteins, differences in promoter positioning and regulation, and pattern of mRNA splicing compared with E6 and E7 from the high-risk HPV types (Doorbar J, et al. 2012; Klingelhutz et al., 2012). To date, current data support the 2-tiered system of low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesion (HSIL) which may be further qualified with the appropriate intraepithelial neoplasia (IN) terminology for specific location (Darragh et al., 2012). Therefore, LSIL includes condyloma and anal

intraepithelial neoplasia (AIN) 1 and are not considered to be precancerous. By contrast, HSIL includes p16-positive AIN 2 and AIN 3. HSIL are considered as true cancer precursors (Darragh et al., 2012).

1.6 ANAL CANCER: RISK FACTORS AND PREVENTION

Prior to the HIV-1 epidemic, the main risk factors identified for anal cancer were smoking, history of male homosexual contact, and history of genital warts and other sexually transmitted infections (presumably reflecting exposure to HPV) (Daling et al., 1987). Moreover, other sexual practices such as exposure to HPV-infected skin surfaces without anal penetration could lead to HPV inoculation of the anal canal (Gheit et al., 2019).

Finally, an increasingly important risk factor for anal cancer is immunosuppression. At least some of the population-based increase in anal cancer, described above, may reflect growing numbers of immunosuppressed individuals. Solid organ transplant recipients, including kidney, heart, and lung transplants, are also at increased risk of anal cancer (Collett et al., 2010.)

Risk factors for anal HPV infection in HIV-infected MSM have been difficult to determine since the proportion of people infected with HPV is so high. One report showed that oncogenic anal HPV infection was associated with receptive anal intercourse (Wilkin et al., 2004). It is clear, however, that as with anal cancer, having receptive intercourse is not necessary to acquire anal HPV infection, as shown in studies of men who have sex only with women (Nyitray et al., 2008). In addition, condom usage may not adequately protect individuals from exposure to HPV since HPV can be transmitted by contact with infected labial, scrotal, or anal tissues that are not protected by a condom (Burd et al., 2003).

Furthermore, in HIV-1 positive MSM, since HIV-1 diagnosis, a higher nadir CD4+ T cell count, a younger age, and higher number of recent sexual partners have been identified as risk factors of anal HPV infection (Richel et al., 2014; Burgos et al., 2015).

Researchers and health-care practitioners believe that, as cervical HSIL and cervical cancer, untreated anal HSIL is the main cause of anal cancer (Darragh et al., 2011). However, HPV-related anal cancer can be prevented via primary (HPV vaccination) and secondary (anal dysplasia and HSIL screenings) prevention. For primary prevention, two commercially available HPV vaccines (a 9-valent vaccine and a 4-valent vaccine) offer the potential for immunity against HPV 16/18/31/33/45/52/58 (oncogenic strains) as well as HPV 6 and 11 (no-oncogenic strains) (Palefsky et al., 2011; Shiels et al., 2009). To be most effective in preventing disease, all individuals should receive the vaccine prior to sexual debut (Kim, 2010) and it is also safe and recommended for HIV-1 infected individuals between the ages of 9 and 26 years (Meites, 2016). For secondary anal cancer prevention, the anal Papanicolaou (Pap) smear is recommended to detect anal dysplasia among individuals asymptomatic for anal cancer and particularly for HIV-1 infected individuals (Wells et al., 2014). While HRA is a more sensitive and specific screening for detecting HSIL, numerous barriers (eg, lack of provider training to perform the test and lack of HRA equipment) prohibit using HRA as the initial screening test (Sowah et al., 2015, Davis et al., 2013).

Previous research identified barriers and facilitators to screening for anal cancer among HIV-1 infected gay and bisexual men (GBM) and MSM populations (Newman et al., 2008; Reed et al., 2010). Those obstacles included lack of awareness to screen, fear, the stigma related to receiving the anal Pap smear and screening costs (Newman et al., 2008).

1.7 THE ROLE OF E6 AND E7 IN ANAL CARCINOGENESIS

The normal productive viral life cycle of the HR-HPV types is a highly regulated and coordinated process (Tomaić et al., 2016). However, mainly during persistent infection, the HPV DNA is randomly integrated into the host genome, leading to cellular immortalization and eventually to malignant progression (Tomaić et al., 2016). As a consequence of this process,

the HPV replicative capacity collapses, with the loss of most of the viral genes and the uncontrollably expression of E6 and E7, leading to cellular immortalization, cellular transformation, and lastly resulting in cancer development (Androphy et al., 1987; Smotkin et al., 1986). The importance of E6 and E7 in maintaining the transformed phenotype can be ascertained by their constant expression in tumours and derived cell lines even many years after the primary immortalizing events. Moreover, it has been described that E6 interferes with cell survival pathways and E7 promotes cellular proliferation (Barbosa et al., 1989; Hawley-Nelson P., et al., 1989; Mantovani et al., 2001). Thus, these two oncoproteins are considered as excellent targets for therapeutic intervention, and understanding the molecular mechanisms underlying their respective functions is critical for developing such antiviral therapies.

1.8 HPV AND IMMUNE SYSTEM

Although HR-HPV types are clearly associated with anogenital carcinogenesis, many HPV infections remains asymptomatic and are usually cleared by the immune system in approximately 6–18 months. To this extent, the persistence of the infection is strongly influenced by the immune system status and host genetic features (Rodriguez et al., 2008).

HPV has several peculiarities, such as a non-lytic replication cycle, low protein expression in immunocompetent tissue and apical release of viral particles, which minimize or prevent its exposure to the host defences. In addition, HPV oncoproteins, E6 and E7, can directly interfere with immune-response related pathways favouring viral persistence (Einstein et al., 2009).

E6 can reduce the levels of cell surface E-cadherin on keratinocytes, thereby limiting the presentation of viral antigens to the Langerhans cells and promoting HPV survival (Caberg et al., 2008; Hubert et al., 2005).

On the other side, a well-known mechanism by which E7 oncoprotein can suppress cytotoxic response is through the downregulation of the transporter associated with antigen protein 1

(TAP1) (Li et al., 2010; Zhou et al., 2013), subsequently reducing Major Histocompatibility Complex (MHC) I-dependent antigen presentation and dramatically impairing the CTL response (Li et al., 2010; Zhou et al., 2013).

It is well known that cytokines and chemokines specifically targeting cells of the immune system are fundamental molecules responsible for orchestrating the immune response against pathogens (Turner et al., 2014). HPVs, similarly to other viruses, have developed mechanisms to circumvent the immune surveillance by altering the cytokine expression pattern (Grabowska et al., 2012), especially in the IFN family. Among others, the down-regulation of TNF-alpha expression and a concomitant attenuated response to this proinflammatory cytokine has been observed in progressing cervical cancer lesions (Tummers et al., 2015), while the anti-inflammatory molecule IL-10 appears to be up-regulated, limiting the migration of non-resident immune cells to the site of infection (Berti et al., 2017).

Likewise, Natural killer (NK) cells, as a key compartment of innate immune system, also displayed a specific role against viruses transformed cells, which exhibit an abnormal expression of MHC molecules and are immediately eliminated via particle dependent cytotoxicity, a target cell apoptosis pathway and antibody dependent cytotoxicity (ADCC) (Orange et al., 2013), or via cytokines and chemokines (such as IFN-γ) secretion to activate other immune cells (Sutlu et al., 2009). Considering the importance of these two components of immune system in the control of HPV spread, a brief discussion on the general characteristics of IFNs and NKs and their role during HPV infection is provided below.

1.9 THE INTERFERON SYSTEM

Interferons (IFNs) are a heterogeneous class of soluble mediators, originally defined for their ability to interfere with the *in vitro* and *in vivo* replication of several viruses (Boasso, 2013), and are specialized in coordinating the host immune responses in a cell-type-specific manner. Overall, IFNs are pleiotropic biological molecules, that can regulate a series of biological activities including cell cycle regulation, cell differentiation, innate and adaptive immunity, angiogenesis, and other biological functions, through the control of thousands of cellular genes (Rusinova et al., 2013).

IFNs are divided into three groups based on their structure, function, and their receptors (Figure 6):

- type I (IFN-α subtypes (n=13), IFN-β, IFN-ε, IFN-ω and IFN-κ),
- type II (IFN-γ)
- type III (IFN-λ1, 2, 3, 4).

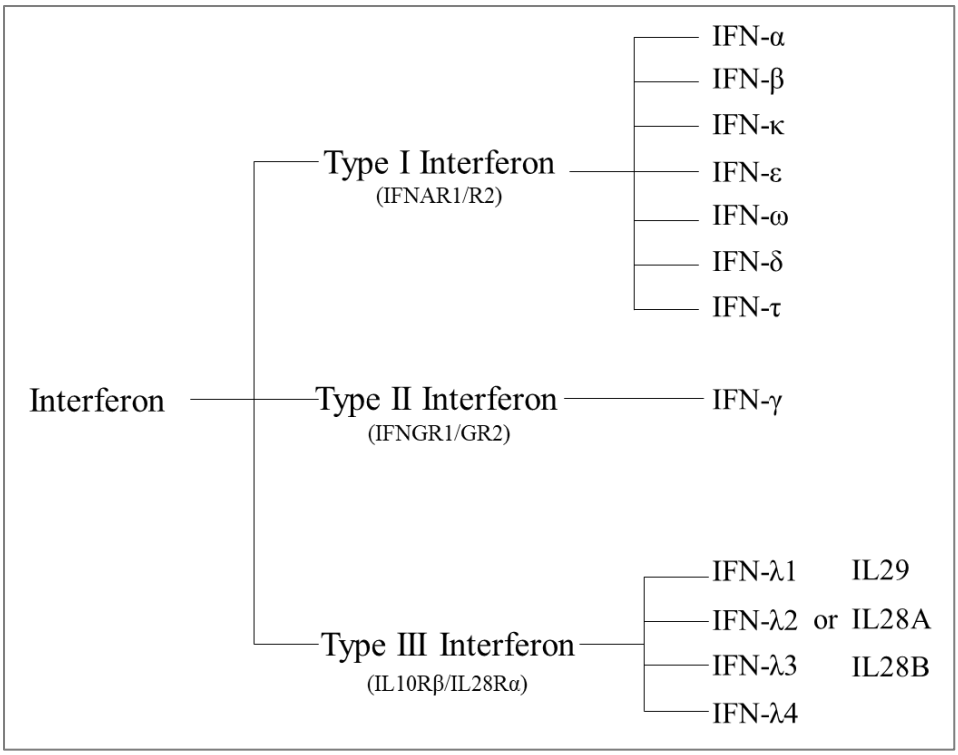

Figure 6: Classification of IFN. Based on the type of receptor through which they signal, IFNs have been classified into three major types and each of them contains subtypes, except Type II IFN (modified from Wang H et al., 2017).

IFNs belong to a broader family known as type II cytokines, which includes some cytokines related to IL-10 (Pestka et al., 2004). These class II cytokines signal through receptors that share extracellular domains, namely CRF2. Although the tertiary structure of IFNs is similar to that of IL-10, their primary structure differs from all CRF2 receptor ligands.

Type I and III IFN can be mainly produced by all virus-infected cells, but the mechanisms involved in the sensing of viral products, as well as the amount of IFN produced and the power of the cellular response, may differ according to cell types.

Since my PhD thesis focused on the expression of the type I and III IFN during anal HPV infection, the following section will deal with the main characteristics of IFNs, except for type II IFN.

Numerous transcription factors are involved in activation and modulation of the expression of IFN genes (Figure 7). Studies focused on the induction of type III IFN suggest that these "cytokines" are produced by the same stimuli and transcription factors that regulate the expression of type I IFN (Levy et al., 2011). The intracellular signals that they are activated following the binding of the IFN molecules produced with their own receptor are numerous and independent. In particular, the stimulation of innate immunity, as type I and III IFN pathways, arises from the interaction of Pathogen Associated Molecular Pattern (PAMPs) with cellular receptors encoded by the germ line, the Pattern Recognition Receptors (PRR) (Table 3). These comprise the Toll Like Receptors (TLRs), Nucleotide binding Oligomerization Domain (NOD) proteins, C-type lectin receptors (CLRs), RIG-1 like receptors (RLRs), and a long list of cytosolic receptors that bind the DNA or RNA (Blasius et al., 2010; Takeuchi et al., 2010; Elinav et al., 2011; Sancho et al., 2012; Paludan et al., 2013; Wu et al., 2014; Xiao et al., 2013) (Table 3). Based on the pathogen and the cells that must activate the cellular response, specific sensors are recruited and activate a signaling cascade that leads to the induction of antiviral and inflammatory genes and pro-inflammatory cytokines.

The IFN exerts its effects in an autocrine and paracrine way through binding to specific receptors present on the cell membrane, resulting in signal transduction through the JAK / STAT pathway, leading to production of effector proteins that have the role of making the cell resistant to viral infections (Stark et al., 2012) (Figure 7).

Table 3: Pattern recognition Receptors Localization and Function

Family	Localization	Function
TLR (TLR1-TLR11)	Cell surface (TLR 1,2,4,5,6,11,19) Intracellular compartment (TLR 3,7,8,9)	Viral glycoprotein and bacterial PAMPSs detection, immune system cell activation
NOD (NOD1, NOD2)	Cytosol	Intracellular microbial sensors
RLR (RIG-I, MDA5, LGP2)	Cytosol	Recognition of viral PAMPs (ssRNA), intracellular sensor, antiviral response
CLR (Dectin-1, Dectin-2, MINCLE)	Membrane	Pathogen phagocytosis, recognition of endogenous ligands
cGAS	Cytosol	cytosolic DNA sensor, inflammatory response activation
STING	Endoplasmic reticulum	DNA sensor, adaptor protein for type I IFN signaling
IFI16	Nucleus and cytosol	recognition of synthetic dsDNA and viral DNA inflammasome activation, DNA damage responses in apoptotic cells
AIM2	Cytosol	DNA sensor,
DDX41	Nucleus	DNA recognition and regulation of DNA virus infection in immunocompetent cells

Type I and III IFNs share the same post-receptor signaling components; consequently, the binding of both IFN types to their receptor on the plasma membrane activates "Janus kinase 1" (Jak1) and Tyk2, by a phosphorylation of tyrosine residues; Jak1, in turn, phosphorylates the

residues of tyrosine on STAT1 and STAT2 proteins (Levy et al., 2011; Samuel et al., 2001). Phosphorylated STAT1 and STAT2 proteins bind to IRF9 factor to form an IFN-Stimulated Gene Factor transcription complex 3 "(ISGF3), which moves into the nucleus and recognizes and binds ISRE, allowing the expression of the "IFN stimulated gene" (ISGs), whose products act on viral replication (Weber, 2020). Only about 37% of ISGs are specifically induced by IFN type I, while the other ones are also regulated by type II and III IFNs (Hertzog et al., 2011) (Figure 7).

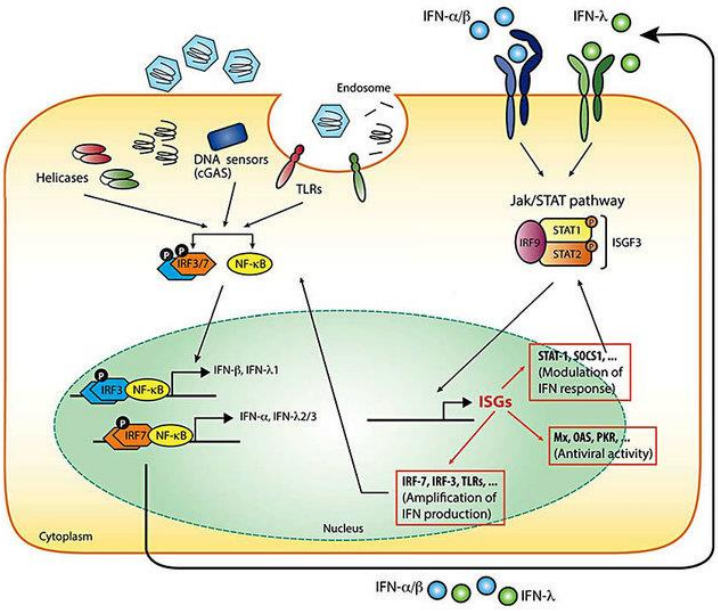

Figure 7: Type I and type III IFN signal transduction pathways.

The proteins encoded by ISGs can repress pathogens through several mechanisms, such as the inhibition of viral transcription, translation and replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism. Moreover, the expression of specific ISGs can be associated with the pathogenesis of some viral infections, as well as the occurrence of inflammatory disorders, autoimmune diseases, and cancer.

The most characterized ISG are 2'-5 'oligoadenylate synthetase (2'-5' OAS), the dsRNA-dependent protein kinase (PKR), Mx proteins, especially humans Mixovirus resistance protein A (MxA), the ISG56 or IFIT1 or p56 protein, ISG15 (ISG15 ubiquitin-like modifier). Janus

kinase-signal transducer and activator of transcription (JAK-STAT) is the predominant, canonical pathway that regulates ISG transcription. Broadly speaking, an ISG is any gene whose expression is induced by IFN signaling. Advances in RNA-sequencing (RNA-seq) technology have enabled the identification of ISGs across varied cell lines by measuring changes in the transcriptome in response to IFN stimulation. A subset of ISGs are direct targets of IRF3/7 and can be induced with or without downstream IFN signaling (Figure 8) (Au-Yeung et al., 2018). Other ISGs are both basally expressed and IFN-inducible, while still others are cell-type specific (Schneider et al., 2014; Schoggins, 2019).

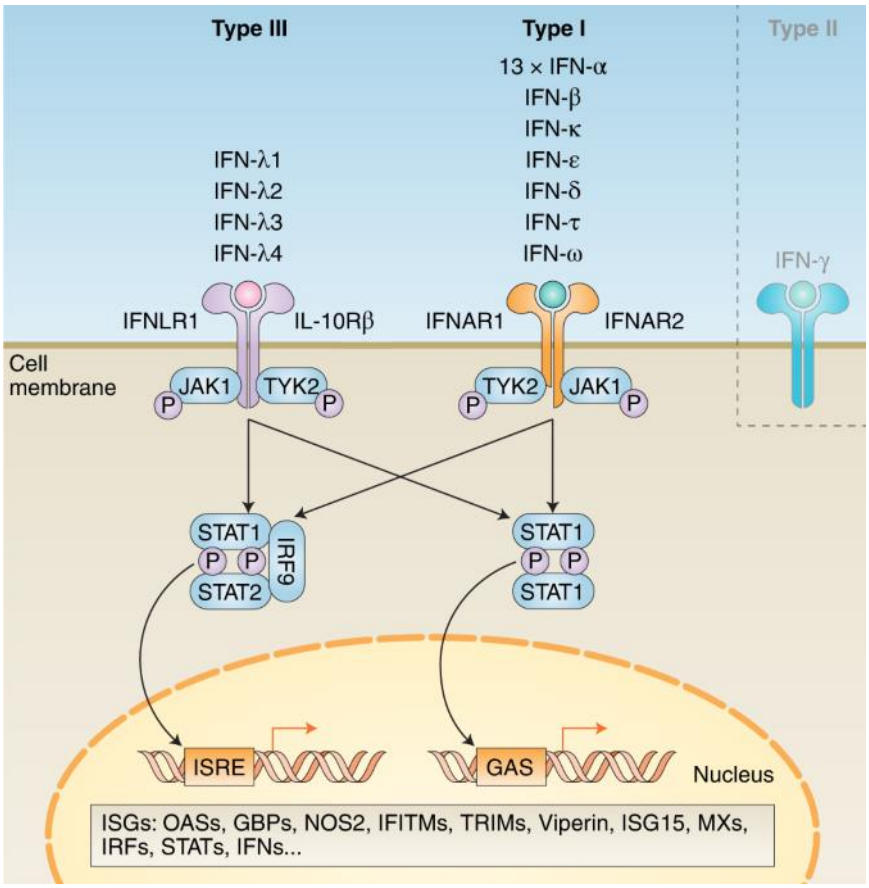

Figure 8: IFN Signal transduction induces the expression of ISGs

1.9.1 TYPE I IFN

Each type I IFN is encoded by a single gene, except for IFN-α, which in humans includes 13 subtypes (Kalliolias et al., 2010) (Table 4). Human IFN-α proteins are encoded by genes without introns, located on human chromosome 9, and share a conserved α-helix structure for 80% of the total 166 amino acids. IFN-α and IFN-β function as monomers, unlike IFN-γ (type II IFN) which functions as homodimer.

Table 4. Main components of the IFN family

IFN type	Name (no. of genes)	Location in human chromosomes	Receptors
Type I	IFNα (14) IFNβ (1) IFNκ (1) IFNω (1) IFNε (1)	Chromosome 9	IFNαR1 and IFNαR2 (also known as IFNAR1 and IFNAR2)
Type II	IFNγ (1)	Chromosome 12	IFNγR1 and IFNγR2 (also known as IFNGR1 and IFNGR2)
Type III	IFNλ (3)	Chromosome 19	IFNλR1 (also known as IL-28RA) and IL-10R2

(Capobianchi et al., 2015)

Despite being antiviral mediators, the role of different IFNs can vary. In particular, type I IFNs have four main functions:

1. induction of an antimicrobial state in the infected cell and adjacent cells, to reduce the spread of the pathogen, especially viruses;

2. modulation of the innate immune response to promote antigen presentation, natural killers' activity and regulate pro-inflammatory pathways and cytokines production;

3. activation of the adaptive immune system, promoting the development of an antigen specific cellular response of T and B cells and an immunological memory (Ivashkiv et al., 2014) (Figure 9);

4. exhibition of an antiproliferative activity linked to the induction of autophagy, mediated by type I IFN. This new function could play an important role in the viruses' elimination, antigen

presentation, inhibition of proliferation and development of a positive feedback for type I IFN production (Schmeisser et al., 2014).

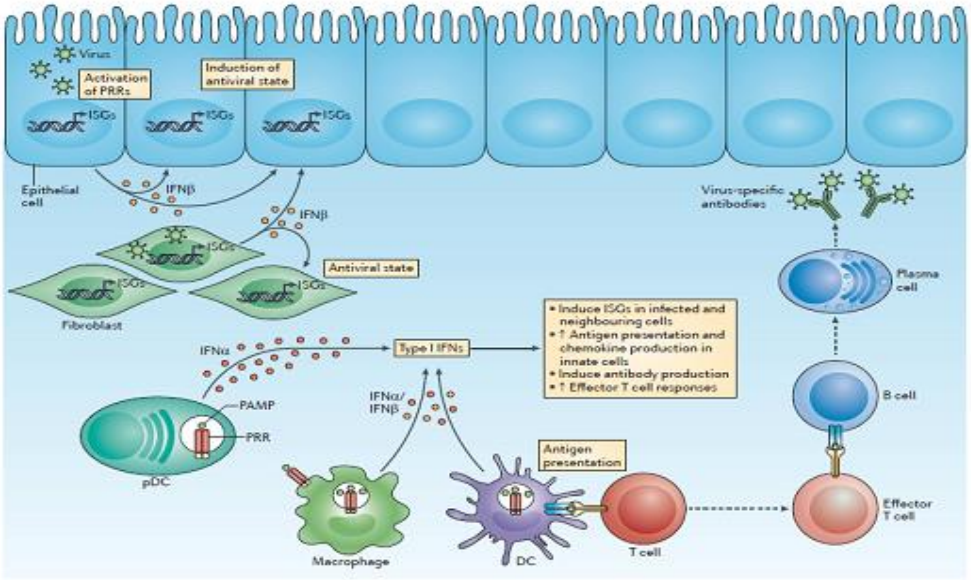

Figure 9. Type I IFN control of innate and adaptive immunity.

Type I IFNs provide resistance to acute viral infections but may have a dual role, protective or deleterious, during bacterial infections or autoimmune diseases. Most cells can produce IFNβ, while haematopoietic cells, particularly Plasmacytoid Dendritic Cells (pDCs), are the main producers of IFN-α.

All type I IFNs exert their biological activity through binding to their heterodimeric receptors composed by the R1 chain (IFNAR1) and the R2 chain (IFNAR2) (Donnelly et al., 2010). In the type I IFN-induced pathway, the activation of IFNAR receptors activates JAK1 and TYK2, which in turn phosphorylate two cytoplasmic transcription factors, STAT1 and STAT2. These phosphorylated transcription factors dimerize and translocate to the nucleus where they assemble with IRF9 to form a trimeric complex known as ISGF3 (Interferon Stimulated Gene Factor 3 transcription complex).

Considering the importance of the IFN system in the antiviral response, it is not surprising that genetic and epigenetic variations in IFNs and related genes are associated with variations in the specific virus-associated disease.

Based on the crosstalk between type I IFNs, there seems to be a specific and characteristic expression of the various ISGs which in turn depends on the cell type considered. Therefore, viral infection induces the production of type I IFN, which can have multiple effects such as clonal expansion of specific cell lines, cell differentiation and survival. Often type I and II IFNs work together to activate innate and adaptive immune responses, favouring the elimination of viral infection (Sainz et al., 2005).

The regulation and modulation of type I IFNs is complex and involves a cascade of sensor molecules, adapters, kinases, and transcription factors, which guide innate immunity and activate the adaptive response. The binding affinity, competition for cytosolic molecules and the timing of activation influence the production of type I IFNs.

However, type I IFNs can also exert pro-apoptotic and anti-proliferative roles within the cell and the excessive production of these molecules can affect cell viability, promoting various phenomena such as autophagy, cell migration and vasculogenesis. The IFNs antiproliferative activity helps the host to eliminate infected cells. Since IFN-β has a greater binding affinity with its receptor than IFN-α, it displayed a better antiproliferative and immunoregulatory activity (Jaitin et al., 2006). Due to the potential use in the therapy of melanomas, the action of type I IFN has been extensively studied in terminally differentiated keratinocytes and melanocytes, for its association with cell proliferation arrest (Ismail et al., 2014).

IFNs are also strong immunomodulators, inducing the expression of MHC, and in the modulation of the activation and differentiation of some effector cells such as monocytes, macrophages, NK cells, Dendritic Cells (DCs) and T and B lymphocytes (Zoon et al.,1986; Bekisz et al., 2013).

Furthermore, during persistent infections, chronic exposure to type I IFN activity can compromise the protective immune response against viruses and cause an increased susceptibility of the host to collateral damage.

1.9.2 TYPE III IFN

Type III IFNs comprise a family of 4 members: IFNλ1, IFNλ2, IFNλ3 (known as IL29, IL28, IL28B) and the more recent IFNλ4. These genes are located on human chromosome 19 (Egli et al., 2014). The IFN λ genes are located on chromosome 19 and their encoded proteins are highly similar to each other (Figure 10).

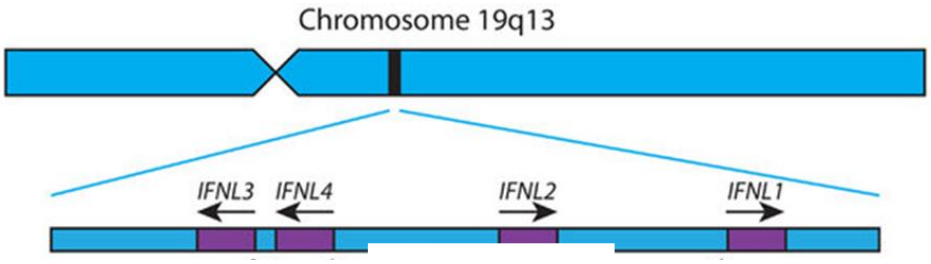

Figure 10. Chromosome 19, locus q13.13 encoding the IFN-λ genes (modified from Laidlaw et al., 2014)

Type III IFNs signal through a receptor complex consisting of IFNLR1 (also called IL-28R), which is the ligand interaction chain, as well as the one that gives receptor specificity, and IL-10R2, the accessory chain (Bartlett et al., 2005). This receptor complex is restricted to cells of epithelial origin and includes epidermal, bronchial, gastrointestinal cells and hepatocytes (Sommereyns et al., 2008; Mordstein et al., 2010). Consequently, IFN-λs have a more limited functional range compared to IFN-α and β, which their have receptors widely expressed on several cell types, despite being functionally related to each other. In fact, they are able to induce the expression of many ISGs, albeit in a different way from type I IFNs (Olagnier et al., 2014).

This limited diffusion of the IFNLR1 subunit could explain how the antiviral activities induced by the IFN-λs are not sufficient to develop systemic protection against viral infections, but a response induced by type I IFNs is also required. The binding of type III IFN to the IFNLR1 receptor induces a conformational change which facilitates the recruitment of the second chain of the complex, IL-10R2 (Gad et al., 2009; Miknis et al., 2010), allowing the formation of the ternary complex (IFN λ, IFNLR1 and IL-10R2). This complex formation leads to the activation of Jak1 and Tyk2 kinases, associated with IFNLR1 and IL-10R2, respectively, at the cytoplasmic level; the three types of IFN-λ, 1, 2, and 3 are recognized by the same receptors, determining the activation of a JAK / STAT type signalling cascade (Durbin et al., 2013).

1.10 HPV MODULATION OF THE IFN SYSTEM

HPV has evolved distinct immune evasion mechanisms to avoid the initial recognition by PRRs as well as to interfere with adaptive immunity. Of note, HPV proteins can modulate the activation, signaling and response to IFNs in several ways. It results crucial to consider that normal keratinocytes constitutively express low levels of IFNs in the absence of viral infection (Wang et al., 1999.), while reduced levels of IFN-inducible genes are observed in HPV-infected cells (Chang et al., 2000; Nees et al., 2001). Several studies have demonstrated that HPV oncoproteins can target components of the IFN system to inhibit their action. Moreover, HR-HPVs can alter immunosurveillance and cellular homeostasis through the deregulation of gene expression in host cells, partially through epigenetic mechanisms. Through E6 and E7 oncoproteins, HR-HPVs stimulate infected cell proliferation as a mechanism to promote viral replication and persistence (Conesa-Zamora, 2013; Plesa et al., 2016; Sen et al., 2018).

Overall, both E6 and E7 can target the innate immune response in different ways (Figure 11). Because E6 and E7 can block the expression of TLR9 and the secretion of cytokines in HPV-positive keratinocytes as well as the activation of the TLR9 pathway by CpG motifs, this TLR

is considered the first target of HPV (Hasan et al., 2007; Hasan et al., 2013). Moreover, HPV oncoproteins suppress the cytotoxic response through the inhibition of TAP1, a fundamental molecule involved in the antigen processing (Gameiro et al., 2017);

By contrast, E5 blocks the degradation of EGFR, also ensuring an increase in EGFR recycling, and its overexpression (Wechsler et al., 2018). In the same scenario, E7 physically binds to IRF1 blocking the transcriptional activation of IFN-β promoter (Park et al., 2000). In parallel, E7 inhibits the transduction signal mediated by IFN-α, by binding p48 / IRF9, blocking the formation of the ISGF3 transcription complex (Barnard et al., 2000).

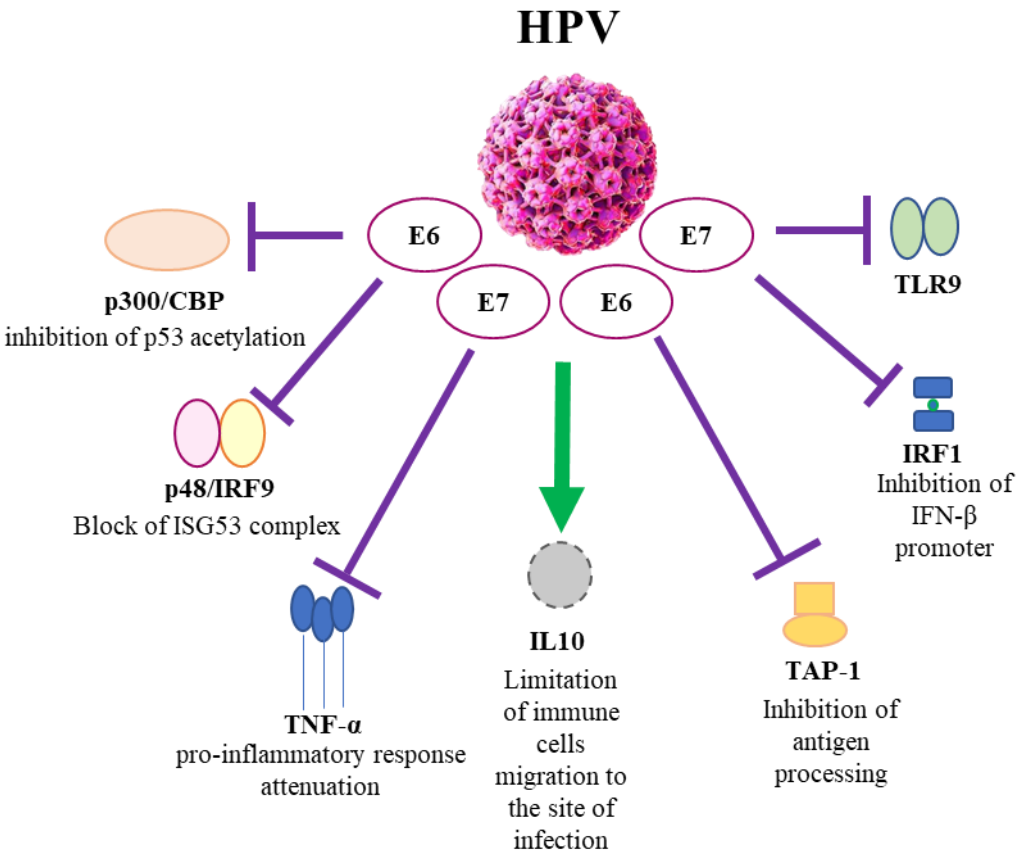

Figure 11. HPV immune evasion strategies

Of note, integrated copies of the HPV genome are usually found in high grade lesions cells. The cooperative action of E6 and E7 in mediating resistance to IFN focuses on the acetylation of p53 by p300 /CBP. E7 protein acts to increase levels of p53, while the E6 protein accelerates

its degradation through the ubiquitin ligase E6AP binding. Protein E6 binds p300/CBP coactivator and inhibits the acetylation of p53, thus contributing to the persistence of HPV in infected cells (Hebner et al., 2007).

To date, although the *in vitro* antiviral and antiproliferative activity of type III IFN has been established, its role in the course of HPV infection is still not well understood; indeed, unlike the type I IFN, there are few data in the literature on the activation of the type III IFN in individuals with HPV infection.

Overall, compared to type I IFN, the type III IFN response is highly cell-specific, being its receptor primarily expressed by epithelial cells. In addition, some studies focused on the specific activity of type III IFN against several viruses showed also an interesting antitumor activity (Lasfar et al., 2011; Liu et al., 2013).

1.11 NATURAL KILLER CELLS AND EVIDENCE FOR A ROLE IN VIRUS-ASSOCIATED TUMORS

NK cells, accounting for 10-15% of peripheral blood cells and originally identified as immune cells with natural cytotoxicity against transformed cells, are granular lymphocytes which recognize and eliminate viral-infected and tumour cells (Herberman et al., 1978; Karre, 2008). Besides their ability to kill cells, it is now well ascertained that NK cells also play a critical role in sculpting innate and adaptive immune responses via cellular cross-talk in several disease contexts (Gasteiger et al., 2014). As opposed to T lymphocytes in the natural tumor immunity, human NK cells displayed a significant role in the elimination of early tumors and metastasis (minimal disease) and are generally found in lower numbers in established tumors. Moreover, as a consequence of their lower specificity, NK cells have a broader reactivity to tumors, effector functions, reduced proliferative capacity and recall response (Bald et al., 2020). Notably, NK cells are a heterogeneous and plastic population, thus they can acquire different phenotypes, depending on the tissue setting or signaling which they are exposed; however,

human NK cells are defined as $CD3^{neg}CD56^{pos}$ lymphocytes and can be divided into functionally distinct subpopulations, depending on CD56 and CD16 expression levels (Cooper et al., 2001):

- $CD56^{bright}CD16^{neg}$ NK cells are immunomodulatory cells displaying a high proliferation potential and secreting a large number of cytokines, especially IFN-γ in response to IL-12, with limited cytotoxic functions. This subset of NK cells is predominantly found in secondary lymphoid organs, such as lymph nodes (Cooper et al., 2001).
- $CD56^{dim}CD16^{pos}$ NK cells have a strong cytolytic activity and a notable capacity to secrete cytokines upon triggering of activating receptors (Cooper et al., 2001). Known as cytotoxic NK cells, they preferentially reside in the blood.
- A subset of $CD56^{neg}CD16^{pos}$ NK cells appears to expand in chronic viral infections including HIV and might represent an exhausted/anergic subset of NK cells.

Unlike other lymphocytes, NK cells lack antigen-specific receptors but lyse target cells following the integration of inhibitory and activating signals, generated by cell surface effector molecules (Lanier et al., 2005). However, the major NK cell receptors, which allow NK cells to discriminate between "self" and a variety of pathological cell states can be divided into three main categories: (i) natural cytotoxicity receptors (NCRs) such as NKp46, NKp30, and NKp44, which can bind to several viral or tumor-associated molecules (Pazina et al., 2017; Barrow et al., 2019); (ii) NKG2A/C/E-CD94 heterodimers and NKG2D homodimers, which are c-type lectins binding to the non-classical Human Leukocyte Antigen E (HLA-E) molecule and stress-induced ligands, respectively, and (iii) the killer-cell immunoglobulin-like receptors (KIRs), which primarily recognize HLA class Ia (HLA-Ia) and Ib (HLA-Ib) molecules and related surface molecules (Pegram et al., 2011).

Tissue-resident NK cells diverge from circulating NK cells and might be found in secondary lymphoid organs as well as in many peripheral tissues including the uterus, lung, and liver (Bjorkstrom et al., 2016; Panda et al., 2019; Sojka et al., 2019). However, it remains unclear if those NK cells represent tissue-resident NK cells, NK cells circulating between tissues and blood, or innate lymphoid cells (ILCs). However, tissue-resident NK cells play a crucial role in particular tissues or organs involved in cancer development and HIV disease.

NK cell development, activation and effector function are regulated by a complex balance between activating and inhibitory signals without prior sensitization and MHC restriction. In addition to the antiviral immune response, NK cells are implicated in tumor surveillance. In this context, NK cells can recognize several MHC-related ligands that are up-regulated on various tumors, including UL16-binding proteins (ULBP1-6) and MHC class I-chain-related proteins A and B (MICA and MICB) (Diefenbach et al., 2000; Salih et al., 2003; Watson et al., 2006). NK cells are also involved in regulatory functions, by improving $CD8^+$ T cell responses against viral infection (Robbins et al., 2007), inhibiting the size/functionality of the T cell response and regulating crosstalk network with DCs and neutrophils to promote or hamper the immune response (Crome et al., 2013; Campbell et al., 2013).

Several immunotherapy protocols have been focused on NK cells manipulation to eliminate cancer cells that are resistant to chemotherapy or to T cell-based immunotherapy (Cheng et al., 2013). To this extent, during tumor progression, some cells are able to evade from NK cell-mediated immunosurveillance, as a high level of MHC-I expression, contributing to cancer development and disease. Recently, the anti-tumour activity of human NK cells has been shown to depend on different activating stimuli (Sanchez-Martinez et al., 2014), especially for solid tumours, which are considered to be more resistant to NK cells than haematological cancer cells (Stojanovic et al., 2011). This low efficacy might be explained by several intrinsic characteristics of solid tumours: i) tumour microenvironment generates immunosuppressant conditions impairing the anti-tumoral activity of immune cells, ii) NK cell extravasation and

infiltration into the solid mass to engage target cells and release cytolytic granules are required for tumor elimination and iii) tumor microregions generated by hypoxic conditions and nutrient restriction influence tumor heterogeneity, differentiation and growth and consequently, might affect its sensitivity to NK cells (Stojanovic et al., 2011; Gras Navarro et al., 2015).

Accordingly to all virus responsible for cancer development, HPV has developed multiple immune evasion strategies to escape CTL- and NK-cell–mediated control, considering long term HPV persistence causing low-grade intraepithelial squamous lesions that may progress to dysplasia, *in situ* carcinoma, and finally invasive carcinoma (Renoux et al., 2011). In fact, function-related receptors of NK cells are down-regulated during HPV infection (Colmenares et al., 2012): it has been demonstrated that NKp46 and NKp30 were significantly decreased in women with cervical cancer and precancerous lesions, consistently with NK cells dysfunction. In addition, studies investigating the role of NK cells in the development of SIL suggest protective effects of NK cells, reporting a decreased NK cell lysis of HPV infected keratinocytes in subjects with SIL or carcinomas as well as a role of NK cells in the regression of SIL (Scott et al., 2001). Actually, the virus-induced tumors are more likely to appear in globally immune-suppressed individuals, who have deficiencies in NK cell activity as well as in other components of the immune system: in particular, co-infection with the immunosuppressive HIV promotes the tumorigenic potential of HPV as well as other viruses, like Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell leukemia virus (HTLV), Kaposi sarcoma virus (KSHV), and Merkel cell polyomavirus (MCV). The ability of these NK cells to control secondary viral infections depends on which viral subtype initiates the secondary infection (Gonzalez et al., 2010).

1.12 NK CELL ROLE DURING HIV-1 INFECTION

During viral infection immune response, the activation of NK cells typically occurs without prior sensitization, but before the induction of T cell mediated immune responses. It is well

described that the immune response processes begin with of type-I interferon secretion (IFN-α) that results in activation of innate effector cells, such as macrophages and DCs, to secrete cytokines, including IL-15 that drives the proliferation of NK cells. This timing of NK cell responses suggests that they may have a role in initial control of HIV infection (Alter et al., 2009), supported by studies highlighting NK cells activity in resistance to HIV, such as the enhanced NK cell functional responses (IFN-γ, TNF-α, CCL3/4/5) (Robertson, 2002). On the other hand, a dramatic change in the peripheral levels of NK cell subsets during HIV infection results in a marked reduction of CD3- CD56+ NK cells and development of a new hypofunctional CD3- CD56- CD16+ NK cell population (Hong et al., 2010). Despite this imbalance in the frequencies of NK cells, their levels appear to remain stable throughout the course of HIV-1 disease.

Of note, NK cells display the following effector functions against HIV-1 infected cells: i) cytolysis of infected cells via the release of perforin and granzymes; ii) release of chemokines that compete with HIV-1 for binding the CCR5 co-receptor; iii) release of cytokines that shape immune responses and Ab mediated NK cell functions, including ADCC (Figure 12). However, few studies have investigated on which specific NK cell population(s) react to HIV-1 infected cells and their functional profiles.

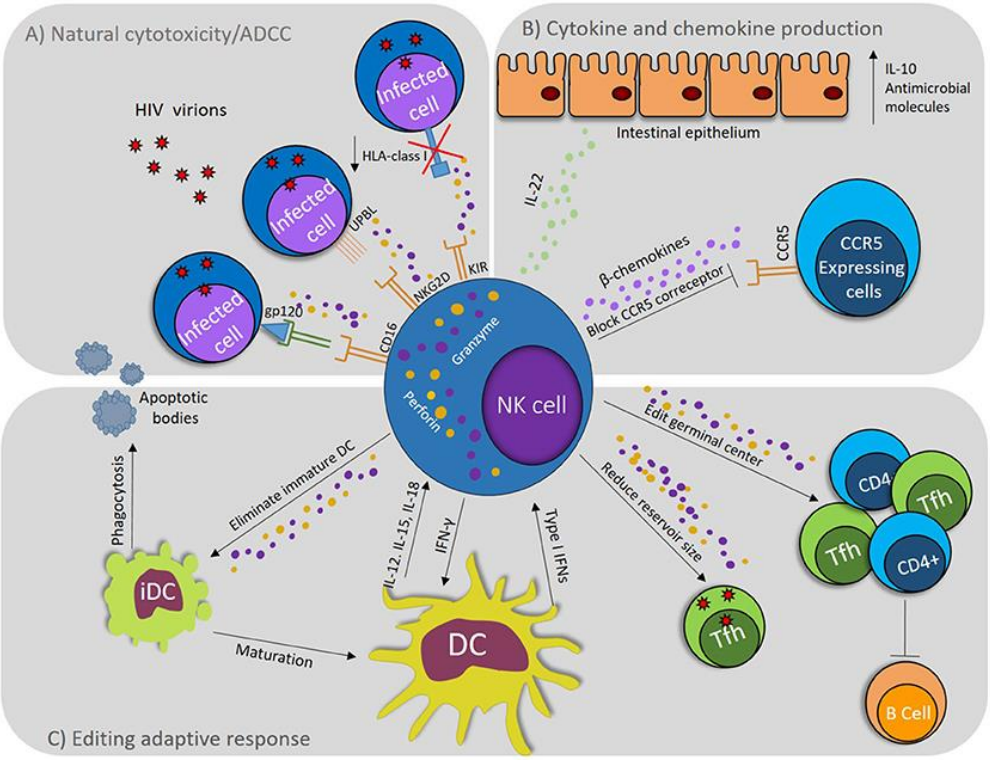

Figure 12. The role of NK cells during HIV-1 infection (Flórez-Álvarez et al., 2018)

1.16 HIV, HPV AND MICROBIOTA: PARTNER IN CRIME? THE EFFECTS OF THE INTESTINAL FLORA MODULATION ON HPV INFECTION

Both the innate and adaptive immune responses are essential for the recognition and elimination of HPV, but in most cases patients with immunodeficiency have a greater risk of disease progression. Despite the beneficial impact of cART, its role on immune recovery of HPV-related lesions remains controversial. To date, HIV-1 infected women exhibit a higher prevalence of HPV infection, and an increase in microbiome diversity; a reduction in *Lactobacillus spp* and the vaginal dysbiosis were deeply related to persistent HPV infection, cervicovaginal dysplasia and the development of cervical cancer (Audirac-Chalifouret al., 2016; Kroon, et al., 2018; Wakabayashi et al., 2019). As known, alterations in the composition of the microbiome can modify local innate immunity, favoring the production of pro-inflammatory cytokines and reducing the epithelium barrier function (Crakes et al., 2019; Takiishi et al., 2017). Furthermore, in the vaginal compartment, the microbiome allows to

maintain an acid pH (4.5), produce peptides with antimicrobial properties and hinder the colonization by other pathogenic microorganisms (Audirac-Chalifouret al., 2016; Kroon, et al., 2018).

Notably, HIV-1 infected individuals almost invariably show alterations of the normal composition of the gut microbiota (Zevin et al., 2016), and evidence from literature highlight that a balanced microbiota could exert a role in the clearance of HPV infection, preventing the risk of HPV related carcinogenesis (Serrano-Villar et al., 2017). It is well established that in HIV-1 positive patients, gut dysbiosis is associated with impairment of the local immune system (Vujkovic-Cvijin et al., 2013; Dillon et al., 2014; Brenchley et al., 2006) and in this scenario any corrective action may contribute to reduce the persistence of HPV infection and the risk of the onset of epithelial dysplasia.

Despite the beneficial effect of cART in HIV-1 infection, a large set of data suggest that long-term cART does not completely reverse the damage inflicted by chronic HIV-1 replication. This is exemplified by the persistence of T cell activation and inflammation and the lasting depletion and/ or retained dysfunction of innate immune subsets (Hearps et al., 2012). This immune activation is mainly associated to the damage of the intestinal epithelium and an increase in microbial translocation. However, the relationship between persistent immune activation and HPV infection on cART-mediated immune reconstitution remains unclear until to date.

Because microbiome can play an important role in the clearance of HPV infection, several studies have investigated the potential effectiveness of oral bacteriotherapy to restore bacterial flora composition and to dampen the chronic immune activation found in virologically suppressed HIV-1 infected patients (Verhoeven et al., 2013; Ceccarelli et al., 2018; d'Ettorre et al., 2017).

2. AIM OF THE STUDY

According to the World Health Organization (WHO) estimates, cancer ranks as one of the main leading cause of death worldwide and was responsible for an estimated 10 million deaths in 2020 (Sung et al., 2021). Although the age-related increase in cancer risk is well-documented, potentially modifiable risk factors, as smoking, alcohol consumption, poor diet, lack of physical activity, obesity or exposure to chemicals and other substances may not be underestimated.

In this context, more than 5 percent of all cancers are directly or indirectly attributable to HPV infections that play significant roles in the multistage carcinogenic process. Among all HPV-related cancers, a high proportion is accounted by squamous cell carcinoma of the anal canal (SCCA), whose risk of developing is extremely high among HIV-1 infected subjects, especially MSM, despite the availability of antiretroviral therapy.

Indeed, people living with HIV-1 still face increased rates of HPV infection and anal cancer and this might be associated to the HIV-1 related immunosuppression that reduces the ability to control HPV oncogenic processes and this abnormal immune response can regulate both the development and progression of cancer. Of note, HPV early proteins can inhibit specific components of the innate immune response; E6 and E7 can block type I IFNs signaling and decrease the expression of multiple IFN stimulate genes. It is also becoming increasingly clear that type III IFN, and IL28R receptor exert distinct and non-redundant functions compared to type I IFN, especially in mucosal tissues. Despite the established antiviral and antiproliferative activity of type I and III IFN at anal mucosal level, to date their role during HPV infection has still been scarcely studied. In parallel, HPV proteins are also able to alter NK cell activity (Bere et al., 2014). The latter might represent an evasion mechanism employed by HPV to support anogenital cancer progression. However, limited data is available on the role of innate immune response (e.g. IFN and NK response) in determining the course of HPV anal infection, especially among a high-risk population as HIV-1 infected MSM.

Beside the HPV and HIV fields, emerging evidence supports that microbiota might have an impact on host immunity and might amplify or mitigate carcinogenesis. Indeed, an altered composition and function of the so-called HIV-1 associated microbiota might explain the altered course of HPV anal disease during HIV-1 infection. It is also well established that the modulation of microbiota composition with oral bacteriotherapy, might contribute to the HPV clearance and HPV related dysplasia regression. In addition, oral bacteriotherapy supplementation seems to be involved in the modulation of innate immunity pathways, especially IFN and NK response, as well as in the reduction of immune activation levels detected among HIV-1 infected subjects (Pinacchio et al., 2018; d'Ettorre et al., 2017).

Therefore, we hypothesized that HPV, through evasion strategies adopted to overcome the host immune defense, might modulate levels of different type I and III IFN genes, as well as of NK cells in the anal mucosa of HIV-1 MSM patients.

In particular, my PhD project has been divided into 3 main areas of research (phases), as reported in Figure 13:

1) Assessment of virus parameters (HPV prevalence, HPV genotypes, rates of persistent HPV infection) and anal HPV cytological abnormalities; Evaluation of gene expression level of type I (IFN α, IFN β and IFN ε, and the receptor IFNAR1 and IFNAR2), type III (IFN-λ 1, 2 and 3 subtypes and IL-28R) in anal cells of HIV-1 positive MSM patients (n=110).
2) Analysis of the frequencies of several NK cell subsets ($CD56^{+}$, $CD16^{+}$, $CD56^{dim}$, $CD56^{bright}$ and NKT cells) by multiparametric flow cytometry in anal samples obtained from a subgroup of HIV-1 positive MSM (n=8) with ascertained HPV anal infection and anal dysplasia. These patients underwent HRA, from which anal biopsies were obtained from abnormal areas and from normal mucosa. This analysis was conducted in collaboration with the Department of Experimental Medicine of Sapienza, University of Rome.
3) Analysis of the role of gut microbial composition in relation to anal HPV clearance and anal HPV related dysplasia in a sub-group of HPV-HIV-1 infected MSM receiving oral

bacteriotherapy supplementation for six months. The effect of the modulation of intestinal microbiota were also analyzed in relation to anal IFN transcript levels and the frequencies of intra-epithelial NK cell subsets. To address the role oral bacteriotherapy supplementation on immune activation status of HPV-HIV-1 infected subjects, the frequencies of CD4+ and CD8+ T lymphocytes expressing CD38 and HLADR were evaluated after oral bacteriotherapy supplementation.

Overall, this study aimed to identify immunological markers of HPV persistence and precancerous mucosal lesions progression, and to explore the effectiveness of oral bacteriotherapy to restore a healthy microenvironment in anal mucosal tissues of MSM individuals.

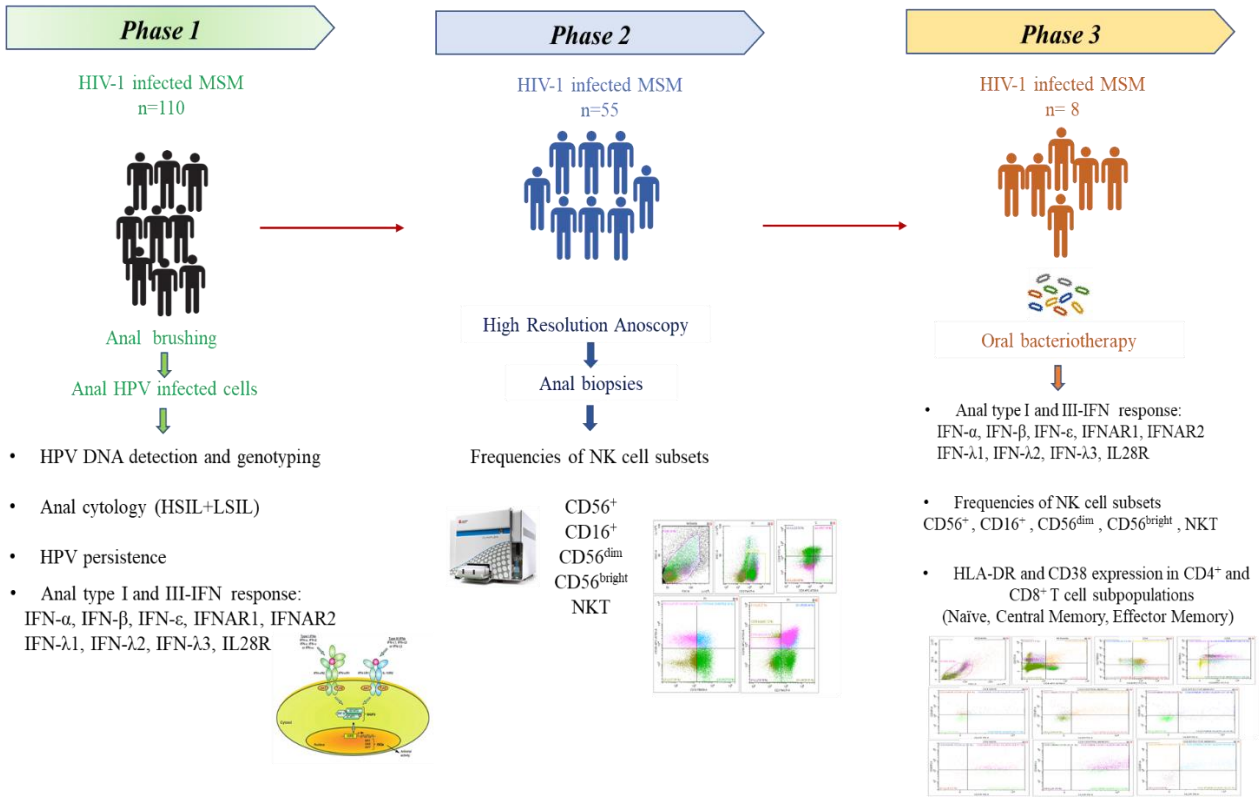

Figure 13. Study design

3. MATERIAL AND METHODS

3.1 STUDY POPULATION

This study included 110 HIV-1 infected MSM successfully cART treated, consecutively attending the proctology clinic of Policlinico Umberto I Hospital, Rome, and the HIV Outpatient Clinic of the Department of Infectious Diseases of Sapienza, University of Rome. All the subjects enrolled in this study met the following criteria: 1) confirmed HIV infection, 2) age ≥ 18 years old, 3) being on stable and effective cART for at least 12 months. The Exclusion criteria were 1) abnormal anal cytology, 2) a history of AIN, and 3) being already diagnosed or treated for HPV/anal condyloma. The study was approved by the Ethics Committee of Sapienza, University of Rome and a written informed consent was obtained from all patients prior to enrolment.

The main immuno-virological parameters for HIV infection were recorded for each participant, as nadir $CD4^+$ T lymphocyte count, $CD4^+$ T cell count and plasma HIV RNA at enrolment and at the end of the study, years of exposure to HIV, type and years of exposure to cART. Past or present sexually transmitted infections were also investigated, with attention to documented HPV infection or warts, focusing on any treatments performed and their outcome. In addition, HPV vaccination status was also considered.

Each patient underwent a careful remote and near pathological history, focusing on conditions favouring the persistence of HPV infection and the progression of dysplastic lesions, such as taking immunosuppressant drugs. The following lifestyle and sexual habits of the participants were also investigated: sexual identity, age of first sexual intercourse, number of partners throughout life and over the past six months, presence of receptive anal intercourse, education, occupation. In addition, voluptuous habits were also considered, as alcohol, smoking and drug use. Finally, the occurrence of present or previous anal pathology and anal symptomatology, as

tenesmus, burning, itching, pain, bleeding, and their relationship with respect to receptive intercourse, was also recorded.

3.2 LABORATORY PROCEDURES

All patients underwent proctological examination and brushing of the anal canal with a Dacron Cytobrush (2X), which allows the collection of epithelial cells required for cytological analysis and HPV-DNA detection. The brushing was inserted into the anal canal up to the anorectal junction (approximately 4.0 – 7.0 cm proximal to the anal margin) and rotated three times clockwise and three times anticlockwise to scrape along the anal wall and to collect a large number of epithelial cells.

This procedure was repeated three times for each patient during each study visit: the first brushing was used for anal cytological examination, the second was used for HPV DNA research and the third one to evaluate IFN gene expression in anal cells.

The brushing samples were then suspended in 1 ml of phosphate-buffered-saline (PBS) and anal cells collected were centrifuged at low speed (8000 rpm) for 10 minutes at room temperature.

The supernatant was removed, and the samples were then stored at -20°C or -80°C for subsequent anal cytology evaluation and DNA and RNA extraction.

3.2.1 CYTOLOGICAL EXAMINATION

Cytological analysis was performed using a cytology slide. The biological sample obtained from the anal brushing was distributed on a slide and fixed within few seconds after the sample collection. This preparation was treated with the Papanicolaou stain, a multichromatic (multicolored) cytological staining technique widely used in cytology (Gill, 2013). This

procedure consists in a combination of a nuclear stain (hematoxylin) and two counterstains (OG-6 and EA-50). OG-6 recognizes and stains keratin, while EA-50 (a double stain, eosin and blue) stains the cytoplasm of squamous epithelial cells, nucleoli, and red blood cells. The so-obtained slide was then observed under an optical microscope. The results were described following the 2014 Bethesda Classification (Nayar et al., 2015):

• NILM: negative for intraepithelial or malignant lesions

• ASCUS: atypical squamous cells of indeterminate meaning

• ASC-H: atypical squamous cells that do not allow to exclude HSIL

• LSIL: low grade intraepithelial squamous cell lesions

• HSIL: high grade intraepithelial squamous cell lesions

• SCC: squamous cell carcinoma.

3.2.2 TOTAL HPV DNA EXTRACTION

HPV DNA extraction was performed using a QIAamp Blood and Tissue kit (Qiagen, Milano). Prior to freezing, 200 µl of ATL (Animal Tissue Lysis) buffer containing SDS (Sodium Dodecyl Sulfate, concentration > 0.5%) and EDTA (Ethylenediaminetetraacetic acid, concentration > 8 mM), were added to anal samples to allow lysis of the cell pellet. To carry out the DNA extraction from anal cells, anal samples were thawed and treated with 20 µl of proteinase K (600 mAU / ml), a broad-spectrum serine protease commonly used to purify nucleic acid preparations by digesting proteins that could interfere with the extraction process; samples were then incubated in a thermostatic bath at 56° C for 10 minutes, to allow proteins lysis.

Subsequently, samples were lysed by treatment with 200 µL of lysis buffer (Buffer AL), in highly denaturing conditions, allowing the isolation of an intact viral DNA. To obtain an efficient lithic action, it is essential to shake the mixture vigorously to produce a homogeneous

solution and then incubate for 10 minutes at 56° C. 200 µL of 96-100% ethanol was then added to allow the precipitation of nucleic acids.

The lysate was then transferred to special columns (QIamp spin columns), containing silica gel filters with high affinity for nucleic acids. The saline conditions and the pH of the lysate ensure that proteins and other contaminants are not absorbed by the filter. The nucleic acids binding to the membrane contained in the columns is guaranteed by a one-minute centrifuge at 8000 rpm, which allows the components of the mixture not absorbed by the filter to flow into the discharge tube. Subsequently, two washes with two different buffers (AW1 buffer: 20 mM NaCl, 2 mM Tris-HCl, pH 7.5 and 57% ethanol; AW2 buffer: 20 mM NaCl, 2 mM Tris-HCl, pH 7.5 and 70% ethanol) were carried out to increase the purity of the extracted DNA and to ensure complete removal of any contaminants. The purified viral DNA was finally eluted with 200 µL of a specific AE buffer (10mM Tris-HCL, 0.5mM EDTA, pH 9) which allows the release of DNA from the membrane and prevents its degradation.

3.2.3 DETERMINATION OF THE EXTRACTED DNA AMOUNT

To determine the amount of total DNA in each sample, 5 µl of extracted DNA were mixed with small quantities of Bromophenol blue containing glycerol (used both to make the sample heavy and to exactly define sample location along the run, given its typical blue colour) and then loaded into a 1% agarose gel in a TAE buffer (40 mM tris-Acetate and 1 mM EDTA) containing Ethidium bromide (1µg/ml) to visualize DNA. In addition, 5 µl of a marker (at a concentration of 0.25 µg/µl) were loaded near the samples, whose bands, at a known wavelength and quantity, were compared with the intensity and wavelength of the extracted genomic DNA bands. The gel was subjected to an electrophoresis assay (150 Volts for 35-40 minutes) and observed through a UV transilluminator.

3.2.4 AMPLIFICATION OF THE HLA GENE

The PCR technique exploits a DNA polymerase, an enzyme able to synthesize new DNA fragments (typically a DNA sequence of 200-800 bases) in the presence of specific primers that act as attachment sites on the template.

Before amplification of HPV genome, a PCR was carried out to amplify a highly conserved small internal region of a cellular gene coding for the human leucocyte antigen (HLA) complex. This allows to ascertain the samples amplifiability and, therefore, the absence of any inhibitors that could interfere in this reaction.

The sequences of the primers used in the HLA PCR were the following:

HLA1: 5'-GTCCTGCAGGTGTAAACTTGTACCAG-3 '

HLA2: 5'-CACGGATCCGGTAGCAGCGGAGAGTTG-3 '

A positive control for the HLA gene (CTRL +), that is a DNA sample previously tested as positive, and a negative control (CTRL-), consisting only of the reaction mixture and sterile bidistilled H_2O, were added to the reaction to evaluate the correct outcome of the amplification, excluding false negative or false positive samples.

The HLA PCR products were then run on a 2% agarose gel in TAE buffer (40 mM tris-Acetate and 1 mM EDTA) containing ethidium bromide (1 µg / ml). 10 µl of each amplification sample, the negative and positive controls, and the known molecular weight marker, which allowed to identify the amplified fragments of the expected size, were loaded into the gel (always mixed with Blue of chromophenol). The gel was subjected to electrophoresis (150 Volts for 35-40 minutes) and observed through UV transilluminator. A 200-bp molecular ladder was used to estimate the amplicon size.

3.2.5 PCR FOR THE L1 REGION

The HPV identification has been performed through PCR using a pair of degenerated primers able to amplify nearly all mucosal HPV genotypes: MY09 / MY11. These primers allowed the amplification of a 450 bp fragment of the late L1 region of HPV.

The oligonucleotide sequences were the following:

MY09: 5'-GCMCAGGGWCATAAYAATGG-3 '

MY11: 5'-CGTCCMARRGGAWCATGATC-3 '

The cycles that outline the amplification of L1 are the same as those found for HLA (n=35).

The PCR products were detected by ethidium bromide staining after electrophoretic migration through a 2% agarose gels.

3.2.6 HPV GENOTYPING

PCR products corresponding to proper fragments were purified with QIAquick PCR purification kit, according to Qiagen protocol. DNA sequencing was performed by an automatic DNA sequencer (Applied Biosystems, model 370A), according to the manufacturer's specifications (Amplicycle Kit, Applied Biosystems). Sequence similarity was determined by BLAST and ClustalW programs. Positive samples were classified as multiple infections when L1 sequence shows mixed chromatograms (Verteramo et al., 2006).

3.2.5 TOTAL RNA EXTRACTION

Anal samples collected from HIV-1 infected MSM were centrifuged at 1200 RPM for 20 min and the pellet immediately resuspended in 200 µl of guanidinium thiocyanate (TRIZOL), a chaotropic agent that denatures the intracellular proteins and inhibits the RNase activity;

guanidinium thiocyanate is also capable of cell and virus particle lysis. Total RNA was extracted using the RNA Mini Prep Plus (Zymo Research, USA).

Briefly, 200 µL of ethanol (95-100%) were added on anal pellets and mixed well. The mixture was transferred to a Zymo-Spin™ IIICG Column Collection Tube and centrifuged at 1000 RPM for 1 minute; then, 400 µl RNA Prep Buffer were added to the column and centrifuged at 1000 RPM for 1 minute; Samples were treated with DNase I: 5 µl of DNase I and 75 µl of DNA Digestion Buffer were added directly to the mix into the column and incubate at room temperature for 15 minutes; then, 700 µl RNA Wash Buffer were added to the samples and centrifuged at 1000 RPM for 1 minute. 400 µl RNA Wash Buffer were added and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Lastly, to elute the RNA, 100 µl DNase/RNase-Free Water were added directly to the column matrix and centrifuged at 1000 RPM for 1 minute.

3.2.6 RETROTRANSCRIPTION (RT)

Retro-transcription (RT) is the enzyme-mediated synthesis of a DNA molecule from a single strand RNA" template (ssRNA) through the DNA polymerase-dependent RNA enzyme, or reverse transcriptase. Samples were reverse transcribed using the “High-Capacity kit cDNA Reverse Transcription” (Applied Biosystems, Foster City, California, USA).

The single sample mix, with a final volume of 60 µl, contains:

- 12 µl RT Buffer 10x,
- 4.8 µl dNTP Mix (100 mM), 1
- 2 µl Ex-R 10x,
- 6 µl MultiScribe ™ RT,
- 25.2 µl Water
- 60 µl of the extracted sample

3.2.7 TaqMan Real Time PCR for gene expression analysis

Gene expression analysis was performed using Real-time PCR assays using the LightCycler480 instrument (Roche, Basel, Switzerland). It is known that Real Time PCR allows the quantification of a target sequence within a heterogeneous mixture of DNA or cDNA molecules, based on the principle of a direct relationship between the amount of starting nucleic acid and the corresponding PCR product. In this study, the TaqMan method was used for the quantitative analysis. Amplified products were marked with "primers" and “probes” following the FRET (Fluorence Resonance Energy Transfer) principle, or however, mechanisms that involve the emission of fluorescence, and involve a “Fluorophore” and a non-fluorescent “Quencher”.

The Real Time PCR reactions were performed in 96-well plates (Roche, Basel, Switzerland). Primers and probes for each gene were added to the Probes Master Mix (Roche; Basel, Switzerland) at 500 and 250 nM, respectively, in a final volume of 20 µl. The housekeeping gene β-glucuronidase was used as an internal control.

The primers and probe sequences used for each gene were the following:

GUS: Forward 5’-TCTGTCAAGGGCAGTAACCTG-3’

Reverse 5’-GCCCACGACTTTGTTTTCTG-3’

Probe 5’-6FAM-TCAAGTTGGAAGTGCGTCTTTTGGATGC-TAM-3’

IFN α: Hs. PT.58.24294810.g

IFN β: Hs. PT.58.39481063.g

IFN ε: Hs. PT.58.4812867.g

IFNAR1: Hs. PT.58.25402720.g

IFNAR2: Hs. PT.58.1621113.g

ISG15: Forward 5'-TGGCGGGCAACGAATT-3'

Reverse 5'-GGGTGATCTGCGCCTTCA-3'

Probe 5'-FAM-TGAGCAGCTCCATGTC-TAMRA-3'

IRF1: Hs.PT.58.26847423

EGFR: Hs.PT.58.15419889

IFN λ1: Hs.PT. 56a.21113836.g

IFN λ2: Hs.PT. 56a.38564463.g

IFN λ3: Forward 5'-ATATGGTGCAGGGTGTGAAG-3'

Reverse 5'-GACGCTGAAGGTTCTGGAG-3'

Probe 5'-/56-FAM/CCACCGCTG/ZEN/ACACTGACCCA/3IABkFQ/-3'

IL28R: Forward 5'-CCCAAGGGTAAGAGCTTCGAT-3'

Reverse 5'-CCTTCATATTTTACTGACATGGACAAG-3'

Probe 5'-6-FAM-CAGCCGGGCCAACCTCTGACC-TAMRA-3

The Light Cycler 480 instrument (Roche, Basel, Switzerland) has been set up to perform the following cycles: 50° C for 2 minutes; 95° C for 10 minutes, followed by 45 cycles at 95° C for 15 seconds, and 60° C for 1 minute.

All the primers/probe sets were purchased from PrimeTime® Std qPCR Assay (IDT, Coralville, Iowa, USA).

The mRNA expression values were calculated by the comparative Ct method, which uses the expression levels of the housekeeping GUS gene (β-glucuronidase) to normalize data.

The amount of mRNA is provided by the $2^{-\Delta CT}$ formula, where ΔCt is the difference in threshold cycles between the gene considered and the reference gene.

3.2.8 PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATION

Twenty millilitres of whole blood were collected from a subgroup of HPV-HIV-1 positive MSM by venipuncture in Vacutainer tubes containing EDTA (BD Biosciences, San Jose, California, USA). Peripheral Blood Mononuclear cells (PBMC) were isolated from peripheral blood and the plasma was separated by centrifuging at 1800 RPM for 10 minutes at room temperature. Subsequently, PBMCs obtained by density gradient centrifugation (Lympholyte H; Cedarlane Labs, Hornby, Ontario, Canada) were centrifuged at 1800 RPM for 10 minutes and stored with Fetal Bovin Serum (FBS) and 10% of Dimethyl sulfoxide (DMSO) for immunophenotyping.

3.2.9 ISOLATION OF ANAL INTRAEPITHELIAL LYMPHOCYTES

Anal biopsies obtained through HRA from a subgroup of HPV-HIV-1 positive MSM were collected in RPMI 1640 medium with 10% FBS and overnight treated with 200 µL of RNAlater Storage Solution (Sigma-Aldrich, Milan, Italy) that stabilizes and protects cellular RNA in intact, unfrozen tissues. Anal biopsies were, then, digested by 2 h incubation at 37 °C in 1 mg/ml Collagenase-Dispase solution (Sigma-Aldrich, Milan, Italy), leading to the isolation of Intraepithelial Lymphocytes (IEL).

3.2.10 CITOFLUORIMETRIC ASSAYS

PBMC and IEL were collected and aliquoted with RPMI 1640 medium and 10% FBS and then washed by centrifugation. The following antihuman monoclonal Antibodies (mAbs) were added: CD3-PerCP, CD4-APC-750, CD8-FITCH, CD16-Vio Blue, CD56 Alexa Fluor 700. T- and NK- cells subpopulations were identified according to the following phenotypic combinations:

- Total lymphocytes (CD3+)
- CD4+ T lymphocytes (CD3+/CD4+)
- CD8+ T lymphocytes (CD3+/CD8+)

- CD4+/CD8+ T lymphocytes (CD3+/CD4+/CD8+)
- NK T lymphocytes (CD3+/CD56+)
- NK cells (CD3-/CD56+)
- CD56dim NK cells (CD56+/CD16+)
- CD56bright NK cells (CD56++/CD16-)

One aliquot of PBMC has been also collected to evaluate the immune activation level on CD4+ and CD8+ T lymphocytes; the following mAbs were added: CD3-PerCP, CD4-APC-Vio770, CD8-FITC, CD45RO-PEVio770, CD27-VioBlue, CD38-APC, and HLA-DR-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). The expression of markers of immune activation (CD38, HLADR) on naïve (CD27+CD45RO−), central memory (TCM- CD27+CD45RO+), and effector memory (TEM- CD27−CD45 RO+) CD4 and CD8 T cells was evaluated by multi-parametric flow cytometry. Gating strategies for analysis of NK cell subsets and T cell immune activation levels were represented in Figure 14 Panel A and Panel B, respectively.

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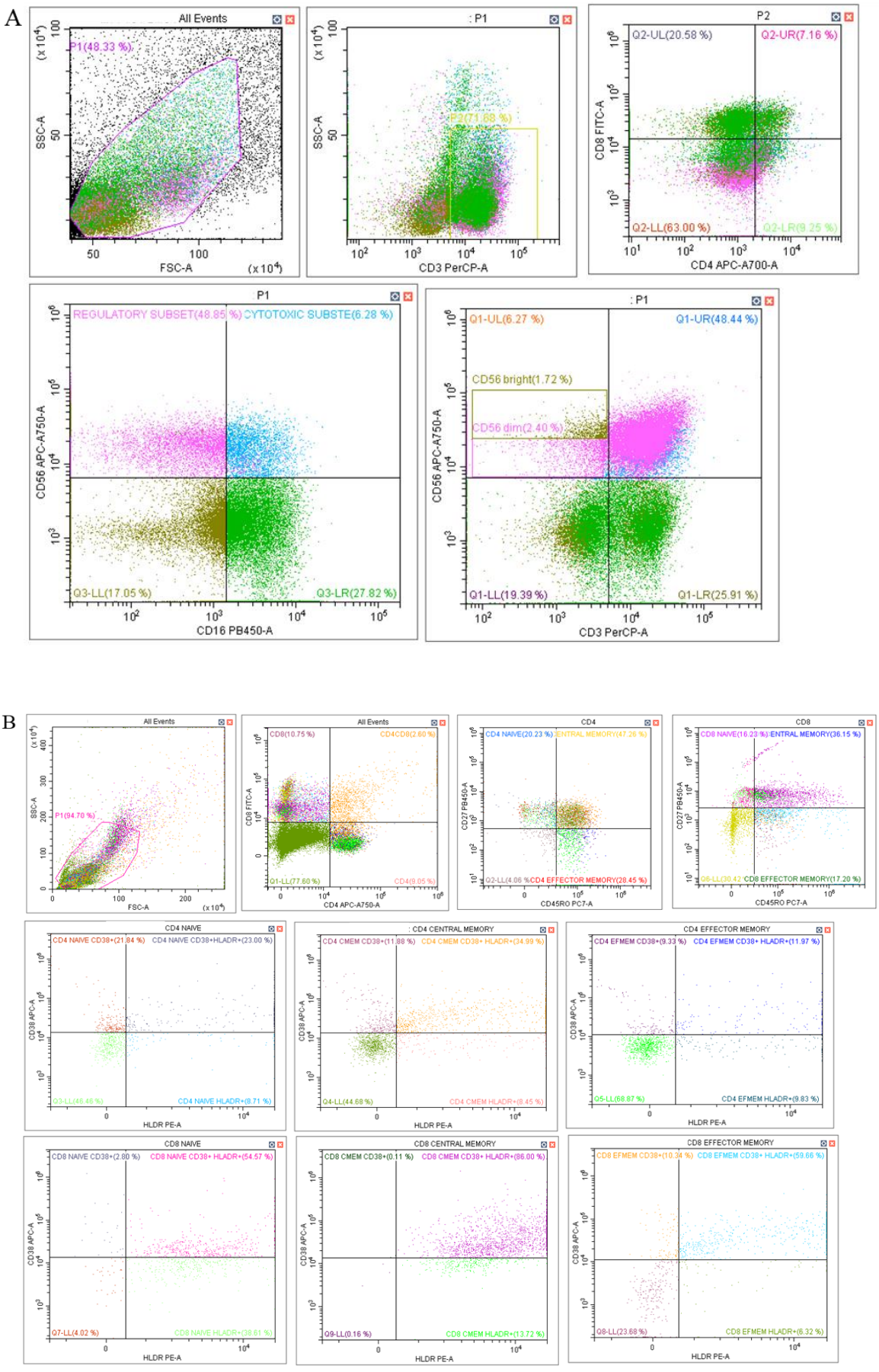

Figure 14 (Panel A, B). Gating strategies.

3.3 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 20.0, and data were presented as median (IQR = interquartile range, 25th and 75th percentile) for continuous variable and as simple frequencies (n) and proportions (or percentages) for dichotomous variable (yes/no or 0/1), unless otherwise stated. Differences between HPV positive and HPV negative MSM, HR and LR HPV patients were assessed using the Mann–Whitney U test. Comparison of data obtained before and after probiotic supplementation were performed using Wilcoxon signed-rank test. Parameters that were statistical significance with univariate analysis were used in the logistic regression analysis to estimate adjusted odds ratio (OR) and 95% confidence intervals (95% CI) for the risk factors associated with the development of HPV, including the confounding factors (age, gender, etc.). A p value less than 0.05 was considered statistically significant.

4. RESULTS

4.1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF HIV-1 INFECTED MSM

The present study included 110 long-term cART treated HIV-1-positive MSM, attending the Proctology Clinic of the Policlinico Umberto I and the Department of Infectious Diseases at Sapienza, University of Rome.

The demographic and clinical characteristics of HIV-1 infected patients are summarized in Table 5.

Briefly, HIV-1 positive population had a median age of 47.4 (range: 41–52.75) years. The median nadir $CD4^+$ count was 226 (range: 50-396) cell/mm^3 and the CD4+ T-cell counts at enrolment ranged between 585 and 897 cell/mm^3 blood, with a median value of 700 cell/mm^3. All participants were virologically suppressed (HIV RNA <37 copies/ml) for at least 12 months. The median years of exposure to HIV-1 was 10 (range: 4.25-18) years. All patients enrolled were negative for Cytomegalovirus or Epstein–Barr virus IgM and had no hepatitis B virus or hepatitis C virus coinfection; no other opportunistic infections or sexually transmitted diseases (STDs) were recorded in the study population.

Table 5. Demographic and clinical characteristics of cART treated HIV-1 positive MSM

Item[a]	**HIV-1-positive MSM (n=110)**
Age (years)	47.4 (41–52.75)
nadir CD4+ T cells (cell/mm^3)	226 (50-396)
CD4+ T cell at enrolment (cell/mm^3)	700 (585-897)
HIV-1 RNA (copies/ mL)[b]	<37
Years HIV-1 diagnosis (years)	10 (4.25-18)
Years of treatment	12 (7-18.5)

[a] Data are expressed as median (range).

[b] HIV-1 viral load was determined by Versant HIV-1 RNA kPCR assay (Siemens Healthineers, Tarrytown, NY, USA) which has a detection limit of 37 copies/mL

4.2 Anal HPV prevalence and HPV genotype distribution in HIV-1 infected MSM

Since several HPV genotypes show a greater association with cancer development, the definition of the HPV genotype, LR and HR, represents a crucial information in the clinical history of high-risk patient, as HIV-1 infected MSM. The prevalence of anal HPV genotypes among the total study participants were reported in Figure 15. Overall, HPV DNA was detected in 79.1% (87/110) of anal samples collected from HIV-1 infected MSM, 1.8% of which showed multiple HPV infections. In particular, HR anal HPV infection was found in 43/87 patients (49.4%), and LR-HPV infection was detected in 44.9% (n=39) of HPV positive study participants (Figure 15). In 5.7% (5/87) of the positive anal samples the HPV genotype and/or the associated oncogenic risk has not been determined.

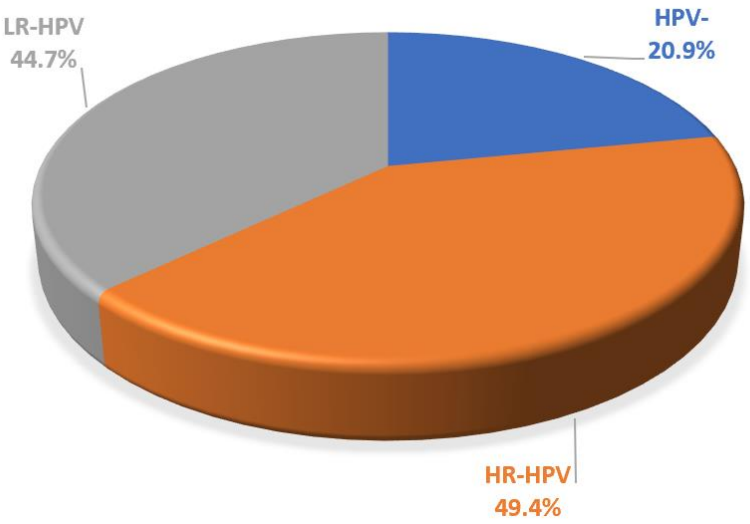

Figure 15. Anal HPV prevalence, HR and LR genotypes distribution in HIV-1 infected MSM

Moreover, a detailed analysis of the prevalence of individual HPV genotypes has been performed among all samples identified to be positive for HPV DNA. As reported in Figure 16, among HR-HPV genotypes, HPV16 was the most prevalent (11%, 9/82); HPV45, HPV53 and HPV83 were the second HPV common types (5%, 4/82 for each genotype); among others, 4% of HPV positive patients were affected by HPV58 and HPV85 (3/82 for each HPV genotype) and the frequency of HPV18, HPV31, HPV54 and HPV97 was 2% (2/82 for each HPV genotype). All the other HR-HPV genotypes (HPV38, HPV66, HPV68, HPV81, HPV82, HPV102, HPV107, HPV120) were detected only in 1% of HPV-positive patients (Figure 16). Among LR-HPV positive patients, the most common LR-HPV genotype was HPV6 (27%, 22/82), followed by HPV11(6%, 5/82) HPV62 and HPV72 (4%, 3/82). Only 1% of patients was infected with HPV42 (1/82), HPV44 (1/82), HPV61 (1/82) and HPV71 (1/82) (Figure 16).

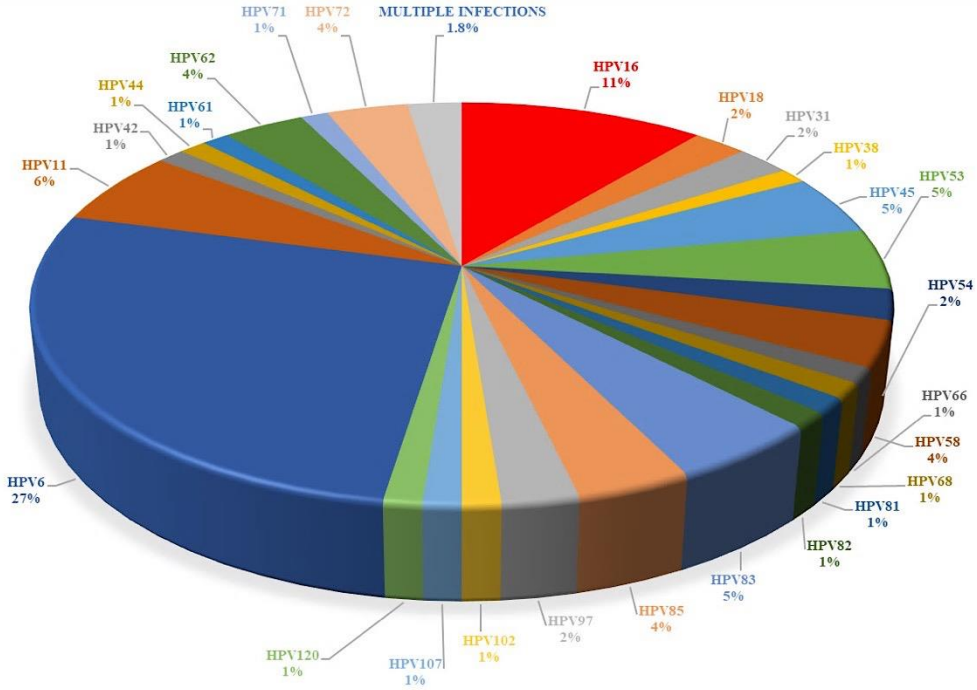

Figure 16. Overall prevalence of anal HPV genotypes (HR-HPV and LR-HPV).

The total number of HR HPV and LR HPV was calculated as a percentage of the total number of HPV-HIV-1 infected MSM. HR-HPV: HPV16, HPV18, HPV31, HPV38, HPV45, HPV53, HPV54, HPV66, HPV58, HPV68, HPV82, HPV82, HPV83, HPV85, HPV97, HPV102, HPV107, HPV120; LR-HPV: HPV6, HPV11, HPV42, HPV44, HPV61, HPV62, HPV71, HPV72.

4.3 ANAL CYTOLOGICAL GRADES AND PREVALENCE

In order to assess an association between HPV infection and anal squamous cell cancer, anal swabs were also collected to perform cytology analysis. Prevalence of anal intra-epithelial abnormalities in HPV-HIV-1 infected MSM was reported in Figure 17**.** Overall, cytology was adequate in 101/110 (91.8%) patients and was unsatisfactory only for nine participants (8.2%); 42/101 (41.6%) anal samples were negative for intra-epithelial abnormalities (NO SIL). By contrast, the prevalence of SIL (LSIL or HSIL) detected on anal swabs of HIV-1 infected MSM was 58.4% (59/101). Because HSIL was detected in 2.0% of anal lesions screened (2/101), it was pooled together with LSIL results. Among the HPV positive MSM, 45/87 (51.7%) showed

a SIL (LSIL or HSIL); among these 45 patients, the HR-HPV infection rate was 42.2% (19/45), accounting for 44.2% of patients with an HR-HPV-positive anal sample.

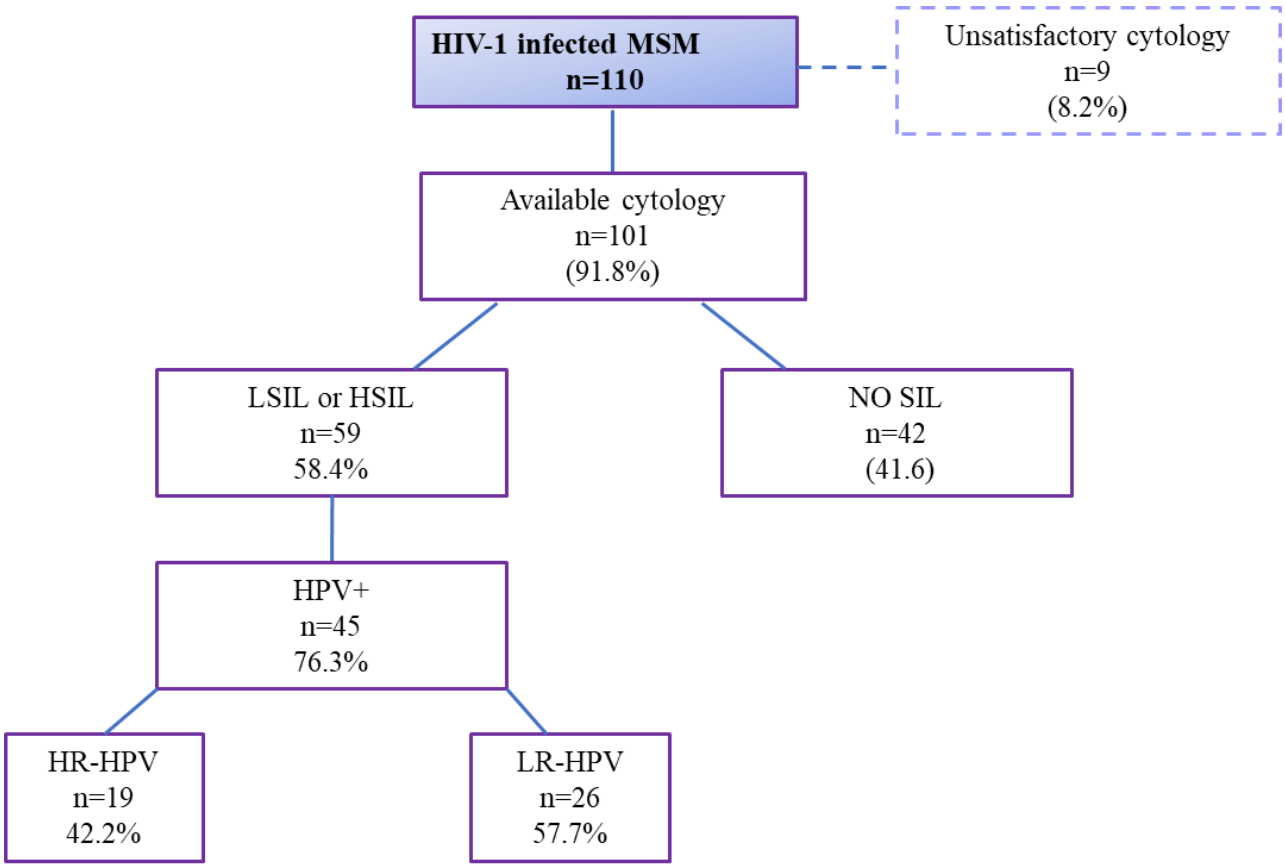

Figure 17. Anal cytology results among HIV-1 infected MSM

4.4 PERSISTENT OR CLEARED HPV INFECTION IN HIV-1 INFECTED MSM PATIENTS

Local immunosuppression in the anogenital mucosa can promote HPV persistence, leading to an increased chance of acquiring abnormalities and developing cancers at the site of viral infection (Frazer, 2009;). Also, mucosal immune dysfunction may cause the development of a latent HPV reactivation (Maglennon et al., 2014). Thus, a subgroup of MSM HIV-1 infected patients (29/87-33.3%) underwent anal brushing after one year from the first proctologic visit,

in order to evaluate if HPV infection was associated with a persistent infection. This analysis showed that 31% (9/29) of the patients analysed, which were HPV positive at the first study visit, cleared spontaneously the infection (Figure 18). By contrast, 69% (20/29) of HPV infected patients remained HPV-positive; in particular 11/20 (55%) maintained the same HPV genotype (persistent infection) while 9/20 patients (45%) were infected with a different HPV genotype (Figure 18).

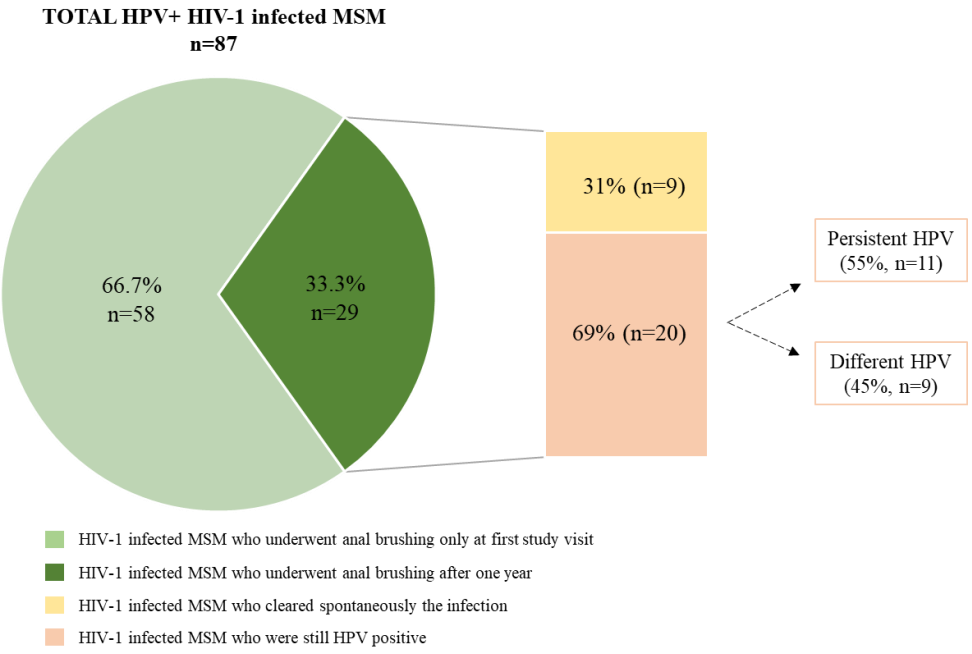

Figure 18. Prevalence of persistent or cleared HPV infection in HIV-1 infected MSM after one year from enrolment. A subgroup of HIV-1 infected patients MSM (29/87-33.3%) underwent anal brushing after one year from the first study visit (dark green square). 31% (9/29) of these patients, cleared spontaneously the HPV infection (yellow square). 69% (20/29) were still HPV-positive (orange square): among them, 11/20 (55%) displayed persistent HPV infection (same HPV genotype) and 9/20 patients (45%) were infected with a different HPV genotype.

4.5 RISK FACTORS ASSOCIATED WITH HPV INFECTION AND PERSISTENCE

Although an increasing interest in understanding the burden of HPV infection in HIV-1 infected MSM has recently arisen, the predictive risk factors associated with the development of anal HPV infection remained not clearly identified. Therefore, HIV-1 infected MSM were asked to participate in a face-to-face interview with clinicians to investigate several parameters as risk factors including education, occupation, lifestyle, use of substances in the last 90 days, and sexual behaviours. Among 110 patients, 50 individuals (45.5%) agreed to complete the lifestyle questionnaire. A multivariate logistic regression analysis was performed to assess the associations between these variables and the HPV infection status (Table 6). Results indicated that HIV-1 infected MSM, who reported having a secondary education were less likely to be positive for HPV infection as compared to those patients who reported having less than a high school diploma [age-adjusted OR=0.0 (95% CI: 0.1-0.2), $p<0.001$] (Table 6). Moreover, a higher likelihood of contracting HPV infection was observed for MSM who reported being employed (73%) [age-adjusted OR=0.1 (95% CI: 0.1-0.3), $p<0.001$]. In this group of patients, tobacco smoking was a risk factor associated to a higher prevalence of HPV infection compared to not-smoking [age-adjusted OR=9.6 (95% CI: 1.2-76), $p<0.001$]. Notably, patients who identified themselves as MSM were more than 5 times more likely to be infected with any anal HPV type [age-adjusted OR=5.5 (95%CI: 1.2-26), $p=0.031$] as compared to patients who reported having bisexual experiences (Table 6). By contrast, all the other parameters considered (age, lifestyle, Cannabis use, drug use, age of first sexual intercourse and number of partners in the last six months) were not associated with the HPV infection development (Supplementary material, Table S6).

Table 6. Prevalence and Correlates Associated with Anal HPV Infection

Parameters	**Class**	**HPV+ N=37 n (%)**	**HPV- N=13 n (%)**	**OR-adjusted (CI 95%)**	**p-value[a]**
Education	Primary	7(18.9)	1(7.7)	0.8(0.2-7.5)	0.827
	Secondary	13(35.1)	5(38.5)	0.0(0.1-0.2)	**<0.001**
	Tertiary	17(46)	7(53.8)	0.4(0.1-1.3)	0.117
Occupation	Unemployed	10(27)	0(0)	5.0(0.3-89)	0.279
	Employed	27 (73)	13(100)	0.1(0.1-0.3)	**<0.001**
Smoking	Yes	17(46)	4(30.8)	9.6(1.2-76)	**0.031**
	No	20(54)	9(69.2)	0.3(0.1-2.2)	0.216
Sexual identity	MSN	33(89.2)	11(84.6)	5.5(1.2-26)	**0.031**
	BISEX	4(10.8)	2(15.4)	0.1(0.1-0.4)	**0.003**

[a] $p<0.05$ were considered as statistically significant

Considering the risk associated to the acquisition of an HR HPV genotype with respect to LR HPV (Table 7), the multivariate analysis showed that MSM with an age ranging from 25 to 50 years were more than 3 times more likely to be infected with an HR-HPV genotype as compared to younger and/or older patients [age-adjusted OR= 3.4(95% CI:1.2-9.2), p=0.015]. Moreover, a higher likelihood of contracting an HR-HPV infection was observed for MSM who reported having a tertiary education (70.6%) [age-adjusted OR=4.1 (95% CI: 1.2-14), p<0.001]. In addition, MSM who reported being smokers displayed an 8.7 times higher likelihood of contracting an HR-HPV genotype [age-adjusted OR= 8.7 (95% CI:1(1.7-41) p=0.007], as compared to patients using cannabis (70.6%) [age-adjusted OR= 3.6 (95% CI:1(1.2-10) p=0.02] (Table 7). No association with occupation, lifestyle, drug use, sexual identity, first sexual intercourse, number of partners in the last six months and the likelihood to contract an HR- or LR-HPV infection were recorded (Supplementary materials, Table S7).

Table 7. Prevalence and Correlates Associated with HR and LR Anal HPV Infection

Parameters	Class	HR-HPV N=17 n (%)	LR-HPV N=20 n (%)	OR-adjusted (CI 95%)	p-value[a]
Age	<25	0(0)	0(0)	0.3(0.2-3.7)	0.264
	25 to 50	15(88.2)	19(95)	3.4(1.2-9.2)	**0.015**
	>50	2 (11.8)	1(5)	0.5(0.2-1.2)	0.136
Education	Primary	1(5.9)	3 (15)	0.6(0.2-3.7)	0.577
	Secondary	4(23.5)	8(40)	0.8(0.2-2.4)	0.635
	Tertiary	12(70.6)	9(45)	4.1(1.2-14)	**0.026**
Substance use					
Cannabis	Yes	5(29.4)	2(10)	3.9(0.6-16)	0.186
	No	12(70.6)	18(90)	3.6(1.2-10)	**0.021**
Tobacco	Yes	10(58.8)	7(35)	8.7(1.7-41)	**0.007**
	No	7(41.2)	13(65)	0.2(0.1-0.6)	**<0.001**

[a] $p<0.05$ were considered as statistically significant

Among all the variables considered in this analysis, as age, education, occupation, lifestyle, Cannabis use, tobacco smoking, drug use, sexual identity, first sexual intercourse and number of partners in the last six months, age adjusted models showed that HIV-1 infected patients who reported having had their first sexual intercourse when they were younger than 17 years old showed a lower risk of developing SIL than those who had the first anal intercourse when they were adult [age-adjusted OR=0.3; (95%CI: 0.1-0.8), $p<0.011$] (Table 8). Finally, tobacco smoking could be considered as a dependent risk factor associated to the SIL development in HIV-1 infected MSM [age-adjusted OR=12.60; (95%CI: 2.6-61), $p<0.001$] (Table 8). However, no further associations were found between the SIL development and the other variables considered (Supplementary materials, Table S8).

Table 8. Prevalence and Correlates Associated with SIL and NO SIL during anal HPV Infection

Parameters	**Class**	**SIL N= 31 n (%)**	**NO SIL N= 19 n (%)**	**OR-adjusted (CI 95%)**	**p-value[a]**
Lifestyle	Sedentary	11(35.5)	1(5.3)	34(4.3-278)	**<0.001**
	Not sedentary	20(64.5)	18(94.7)	0.3(0.2-0.8)	**0.032**
Substance use					
Cannabis	Yes	6(19.3)	4(21)	8.2(1.6-41)	**0.021**
	No	25(80.7)	15(79)	0.1(0.2-0.6)	**0.010**
Smoking	Yes	11(35.5)	12(63.2)	12.6(2.6-61)	**<0.001**
	No	20(64.5)	7(36.8)	0.2(0.1-0.4)	**<0.001**
Sexual behaviour					
Sexual identity	MSM	26(83.9)	18(94.7)	8(2.3-27)	**<0.001**
	BISEX	5(16.1)	1(5.3)	1.4(0.1-23)	0.812
First sexual intercourse (age)	≤17	5(16.1)	5(26.3)	1.7(0.6-5.5)	**<0.001**
	>17	26(83.9)	14(73.7)	0.3(0.1-0.8)	**0.018**
Partner last 6 months (n)	≤10	22(71)	14(73.7)	4(1.3-12)	0.125
	>10	9(29)	5(26.3)	6.1(0.6-60)	0.124

[a] $p<0.05$ were considered as statistically significant

Considering that different co-factors could play a role in the establishment of a persistent HPV infection, increasing the risk of development and progression of anal lesions, a multivariate logistic regression analysis was carried out to identify several risk factors associated with the persistence of HPV infection detected in the sub-group of HIV-1 infected MSM that underwent the follow-up visit after one year. Results showed no variables that could be significantly associated with HPV persistence or clearance in HIV-1 infected MSM (age, education, occupation, Cannabis use, tobacco smoking, drug use, sexual identity, first sexual intercourse and number of partners in the last six months), and this could be due to the small sample size of patients included in this analysis (Supplementary materials Table S9). The only life-style

feature that appeared significant in the analysis was physical activity that was associated with HPV clearance [age-adjusted OR=0.1; (95%CI: 0.1-0.2), p<0.001].

4.7 IFN-I and III SUBTYPES EXPRESSION IN ANAL CELLS OF HIV-1 INFECTED MSM

4.7.1 DIFFERENCES IN THE EPRESSION OF TYPE I AND III IFNS BETWEEN HPV POSITIVE AND NEGATIVE PATIENTS

Since IFN induction was demonstrated to be a crucial target of HPV oncoproteins by *in vitro* studies (Beglin et al., 2009) and given that HPV-infected cervical cells mounted an attenuated IFN response (Cannella et al, 2014), there is an urgent need to better the profile of the type I and III IFN response during anal HPV infection. The mRNA expression of the following genes, IFN-α, IFN-β and IFN-ε, IFNAR1, IFNAR2, IFN-λ1, IFN-λ2, IFN-λ3 and IL28R, has been investigated in anal cells of HPV positive and HPV negative MSM patients. As depicted in Figure 19, a decreased mucosal expression of IFN-β (Figure 19, Panel B), IFN-ε (Figure 19, Panel C), IFNAR1 and IFNAR2 (Figure 19, Panel D and E) was recorded in HPV positive anal samples compared to those negative for HPV-DNA ($p < 0.01$ for all genes). As far as the analysis of type III IFN response is concerned, no differences in the expression of IFN-λ1, IFN-λ2 and IL28R-mRNAs were recorded between HPV positive and negative patients (Figure 20, Panel A; $p > 0.05$, Panel D; $p > 0.05$). By contrast, IFN-λ3 mRNA levels were reduced in HPV positive patients compared to those without HPV infection (Figure 20, Panel C; $p < 0.01$).

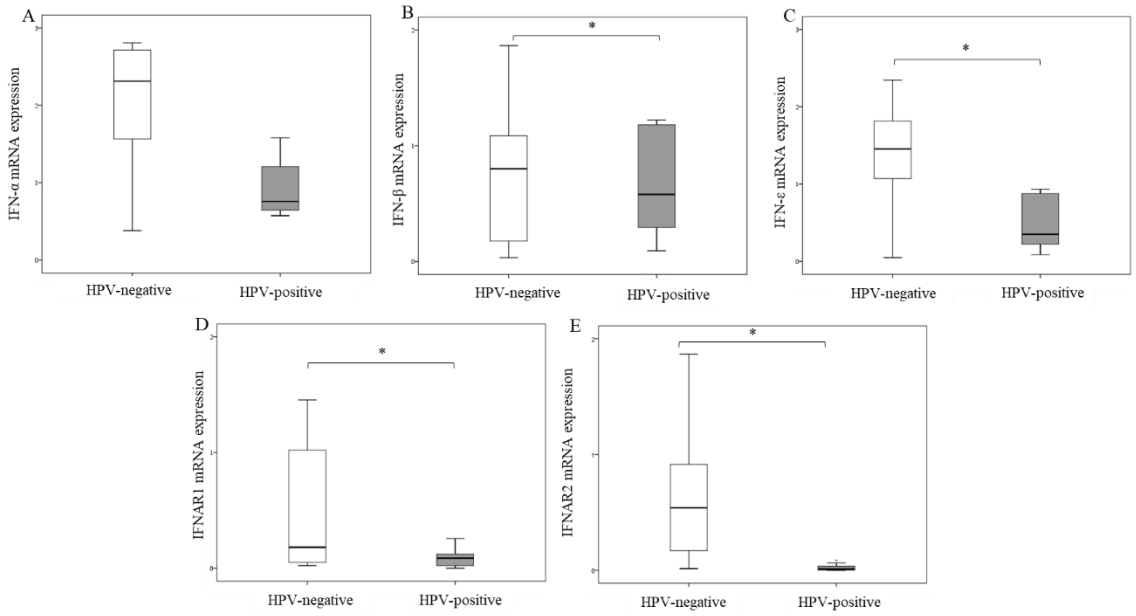

Figure 19 (Panel A-E). Type I-IFN mRNA expression levels in anal cells of HPV positive and negative HIV-1 infected MSM. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analyzed using the Mann–Whitney U test (* $p<0.05$).

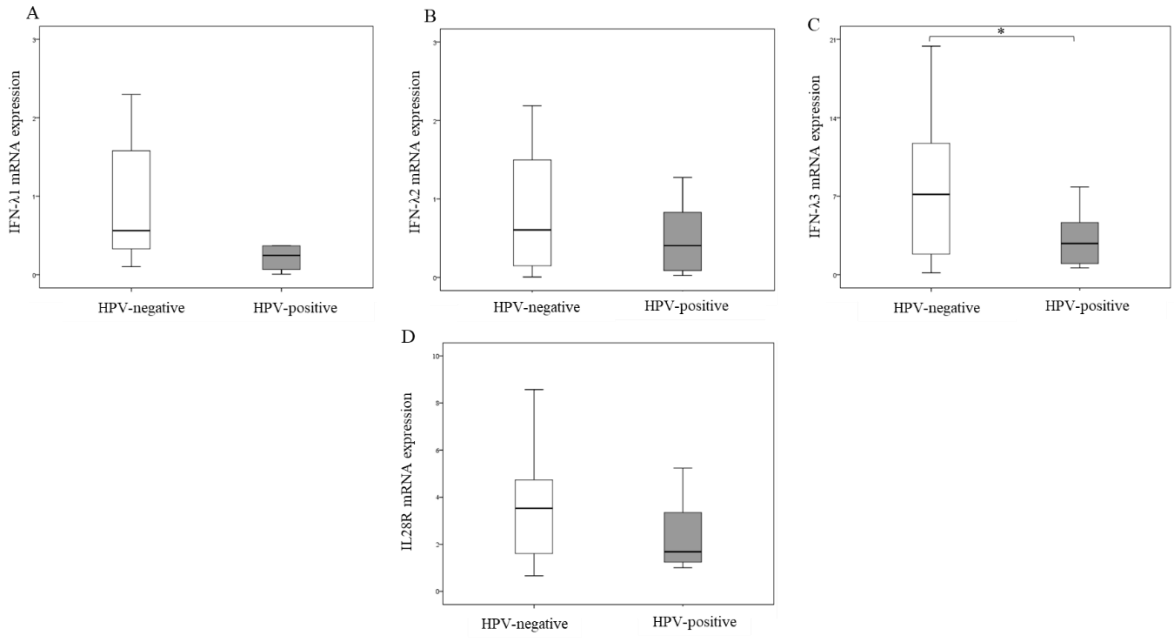

Figure 20 (Panel A-D). IFN-λ 1-3 and IL28R mRNA expression levels in anal cells of HPV positive and negative HIV-1 infected MSM. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT*. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

Having observed a deregulation in the expression of IFNs in anal samples of HPV infected patients, we next compared their mRNA levels in relation to the oncogenic potential of the different HPV genotypes (Figure 21 and Figure 22). Our results showed that type I IFN genes, IFN-β (Figure 21, Panel B), IFN-ε (Figure 21, Panel C), IFNAR1 and IFNAR2 (Figure 21, Panel D and E) were downregulated in anal cells of HR HPV positive patients compared to LR HPV positive patients and HPV negative MSM (HR vs LR vs negative: IFN-α, p=0.523; IFN-β, p<0.01; FN-ε, p=0.03; IFNAR1, p<0.01; IFNAR2, p<0.01). In addition, we showed that HR-HPV positive patients had lower IFN-λ2 and IFN-λ3 levels in anal cells compared to LR-HPV positive patients and to those without HPV (Figure 22, Panel B and C; HR vs LR vs negative: p <0.01).

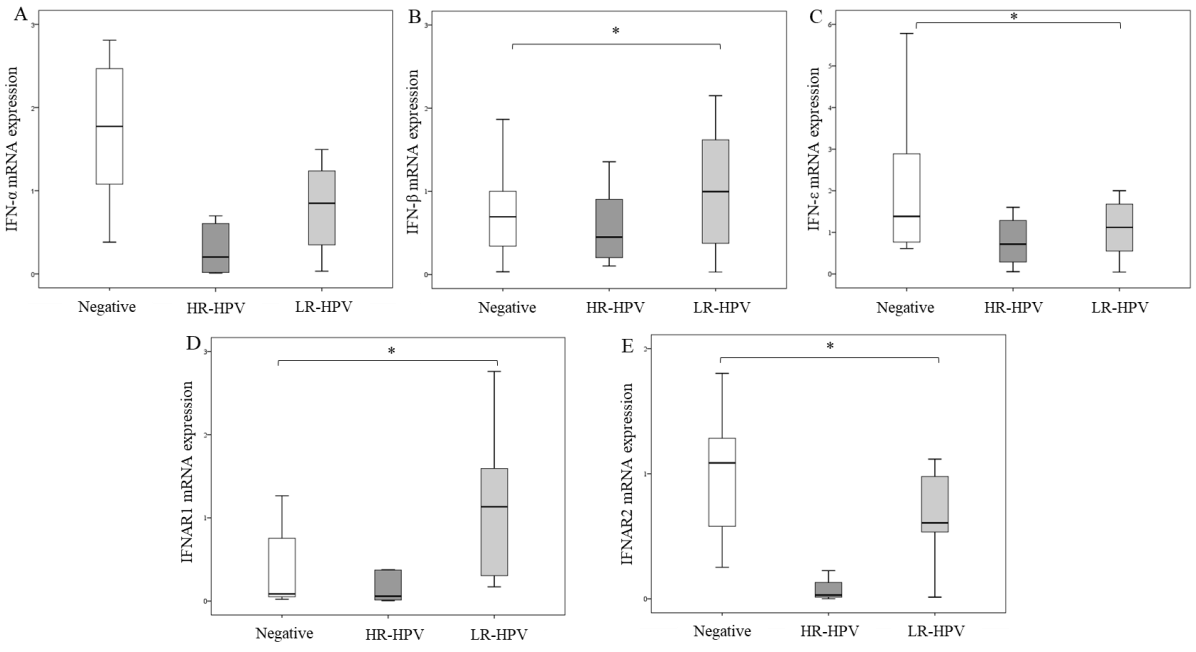

Figure 21 (Panel A-E). Type I-IFN mRNA expression levels in anal cells of HR-HPV, LR-HPV positive and HPV negative HIV-1 infected MSM. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analysed using the Mann–Whitney U test (* p<0.05).

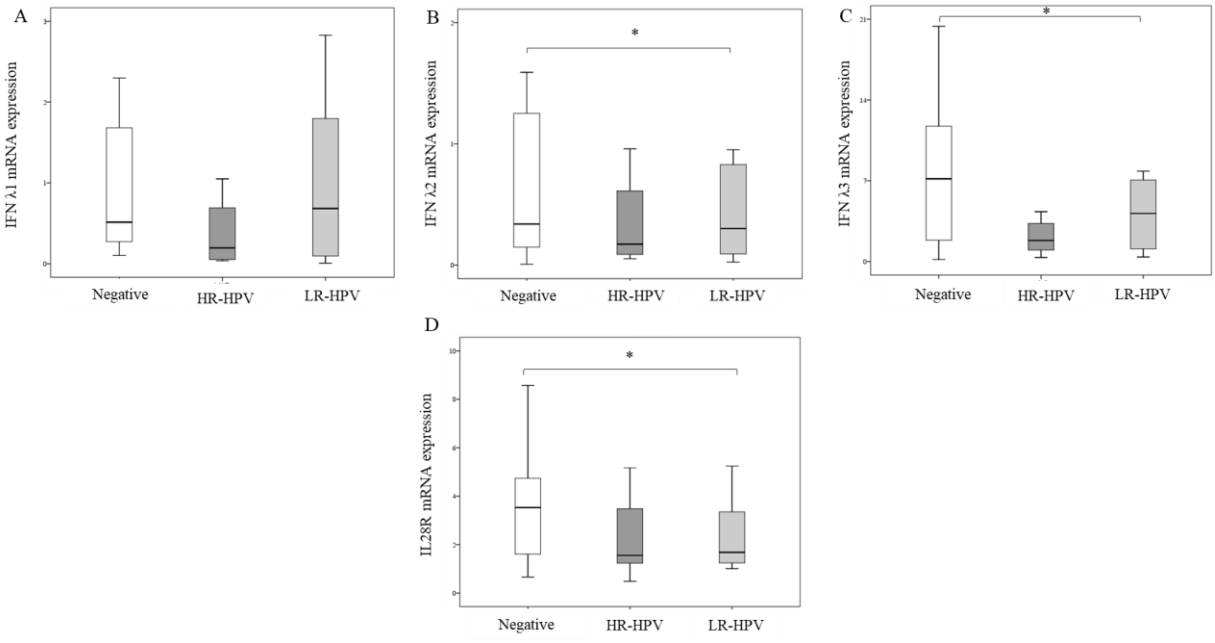

Figure 22 (Panel A-C). IFN-λ 1-3 and IL28R mRNA expression levels in anal cells of HR-HPV, LR-HPV positive and HPV negative HIV-1 infected MSM. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

4.7.2 TYPE I AND III IFN GENE EXPRESSION IN HIV-1 INFECTED MSM ACCORDING TO ANAL CYTOLOGY

To characterize the role of IFN response during anal SIL, as well as its possible contribution in the lesion progression, we compared type I (IFN-α, IFN-β, IFN-ε, IFNAR1 and IFNAR2) and type III IFN (IFN-λ1, IFN-λ2, IFN-λ3, IL28R) gene expression levels stratifying HPV positive patients according to the anal cytology results. A difference in the transcript expression of IFN-ε was recorded among HPV positive patients that developed a SIL (HSIL+LSIL) compared to those who showed no anal lesions (Figure 23, Panel C; $p=0.019$). A trend toward a general increase in the expression of IFN-α and IFNAR1 was also observed in HPV positive patients with anal SIL. However, no statistically significant differences were observed for the other type I IFN genes analysed (Figure 23, Panel A, B, D, E; $p>0.05$).

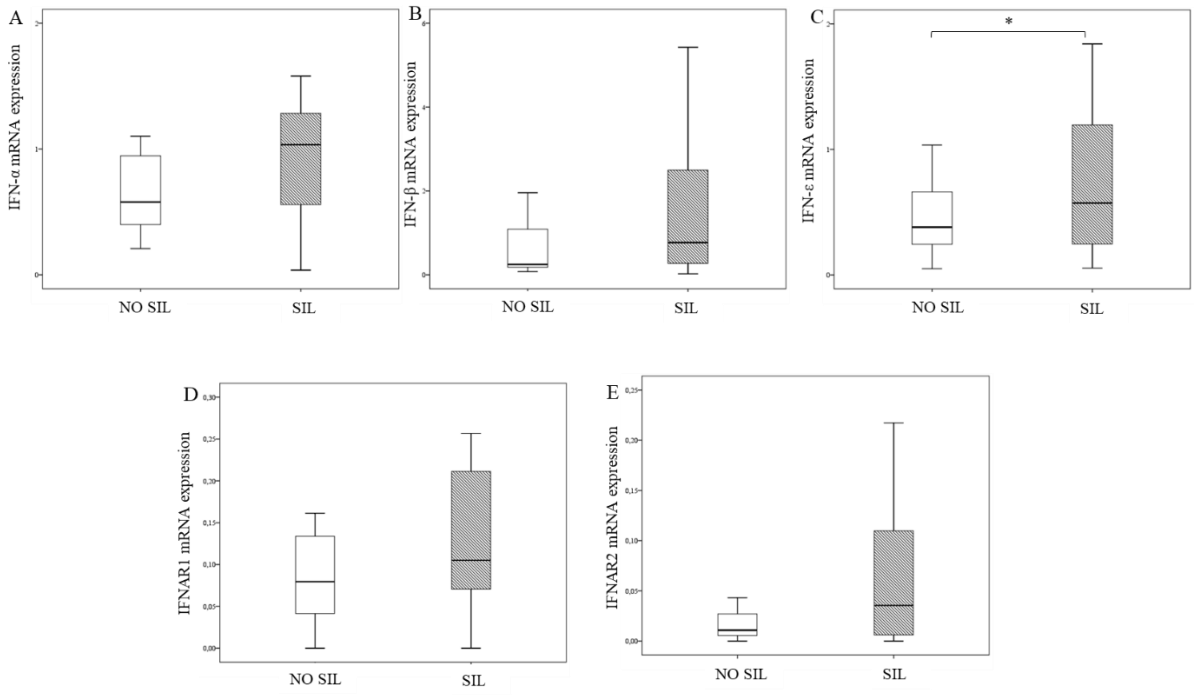

Figure 23 (Panel A-E). Type I-IFN mRNA expression levels in anal cells of HPV positive HIV-1 infected MSM with and without SIL. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analyzed using the Mann–Whitney U test (* $p<0.05$).

Concerning type III IFN, the expression of IFN-λ 3 was different between patients that showed a SIL compared to patients who showed no anal lesions (Figure 24, Panel C, $p<0.01$), while no statistically significant differences were recorded for the other genes (Figure 24, Panel A and B). Moreover, we found differential IL28R mRNA levels among HPV positive MSM who exhibited an anal SIL, as compared to those without SIL (Figure 24, Panel D; $p=0.04$)

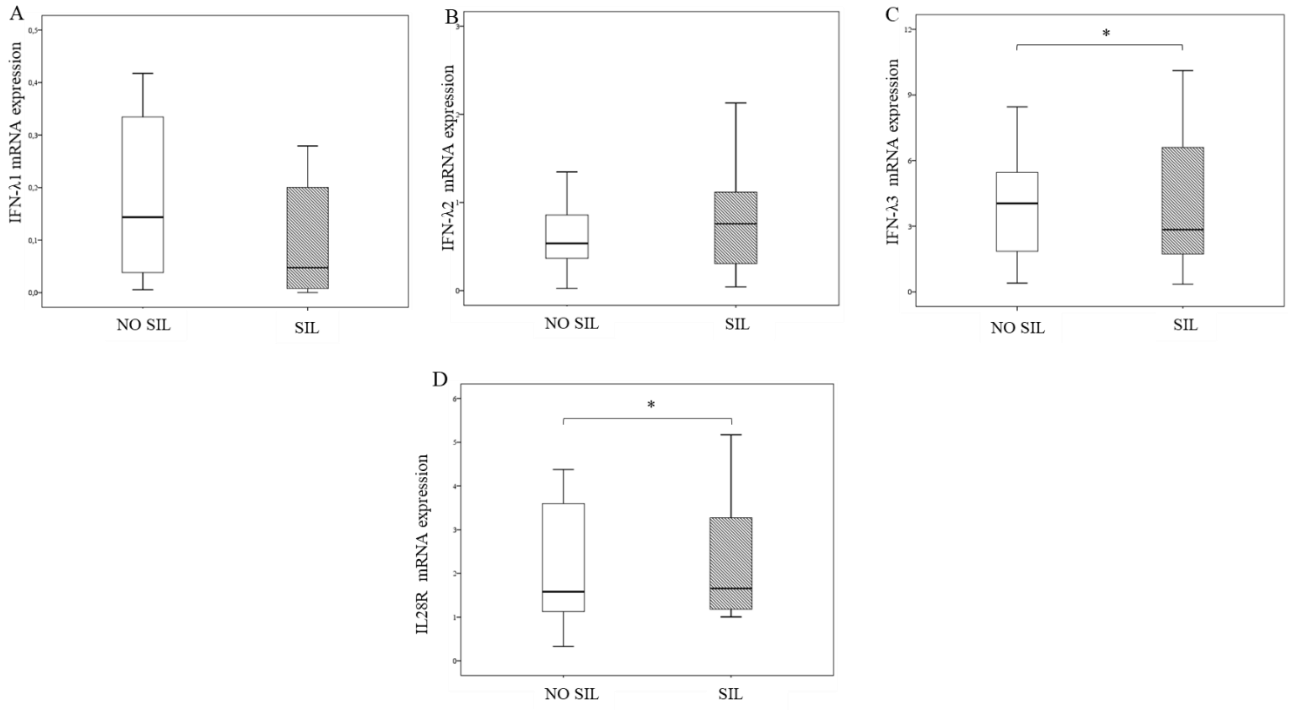

Figure 24 (Panel A-D). **IFN-λ 1-3 and IL28R mRNA expression levels in anal cells of HPV positive HIV-1 infected MSM with SIL and with no dysplasia.** Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analyzed using the Mann–Whitney U test (* $p<0.05$).

4.7.2 EVALUATION OF ANAL TYPE I AND III IFN EXPRESSION DURING PERSISTENT HPV INFECTION

It is known that innate immune escape mechanisms employed by HPV are related to the viral persistence and might be responsible for increased risk of anal cell proliferation and cancer development. Thus, we compared type I and type III IFN response between a subgroup of MSM who displayed a persistent HPV infection for at least one year and with those who spontaneously cleared the viral infection. As reported in Figure 23, HPV persistence was associated with a reduction in IFN-β (Figure 25, Panel B), IFN-ε (Figure 25, Panel C), IFNAR1 (Figure 25, Panel D) and IFNAR2 (Figure 25, Panel E) transcript levels, compared to the type I IFN gene expression levels detected in patients who cleared the HPV infection (Figure 25, $p<0.01$ for all genes).

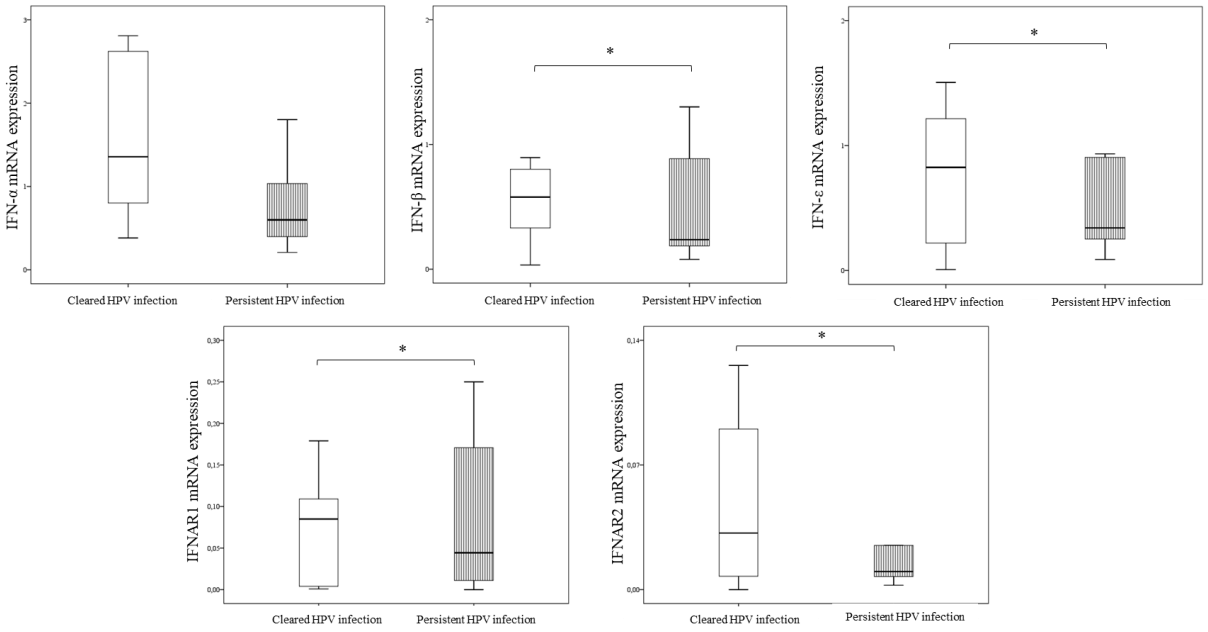

Figure 25 (Panel A-E). Type I-IFN mRNA expression in anal cells of HIV-1 infected MSM during persistent and cleared HPV infection
Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

Moreover, as depicted in Figure 24, IFN-λ1 (Figure 26, Panel A), IFN-λ3 (Figure 26, Panel C), and IL28R (Figure 26, Panel D) transcript levels were lower in HPV-positive patients who had a persistent HPV infection compared to those who cleared the viral infection (Figure 26, Panel A-C; $p<0.01$ for all genes). The coordinate activation of type III IFN response in favouring HPV persistence was confirmed by the presence of positive correlations between IL28R mRNA levels and those of IFN λ subtypes (IFN-λ1 vs IL28R: $r=0.413$, $p=0.01$; IFN-λ2 vs IL28R: $r=0.668$, $p<0.01$; IFN-λ3 vs IL28R: $r=0.777$, $p<0.01$).

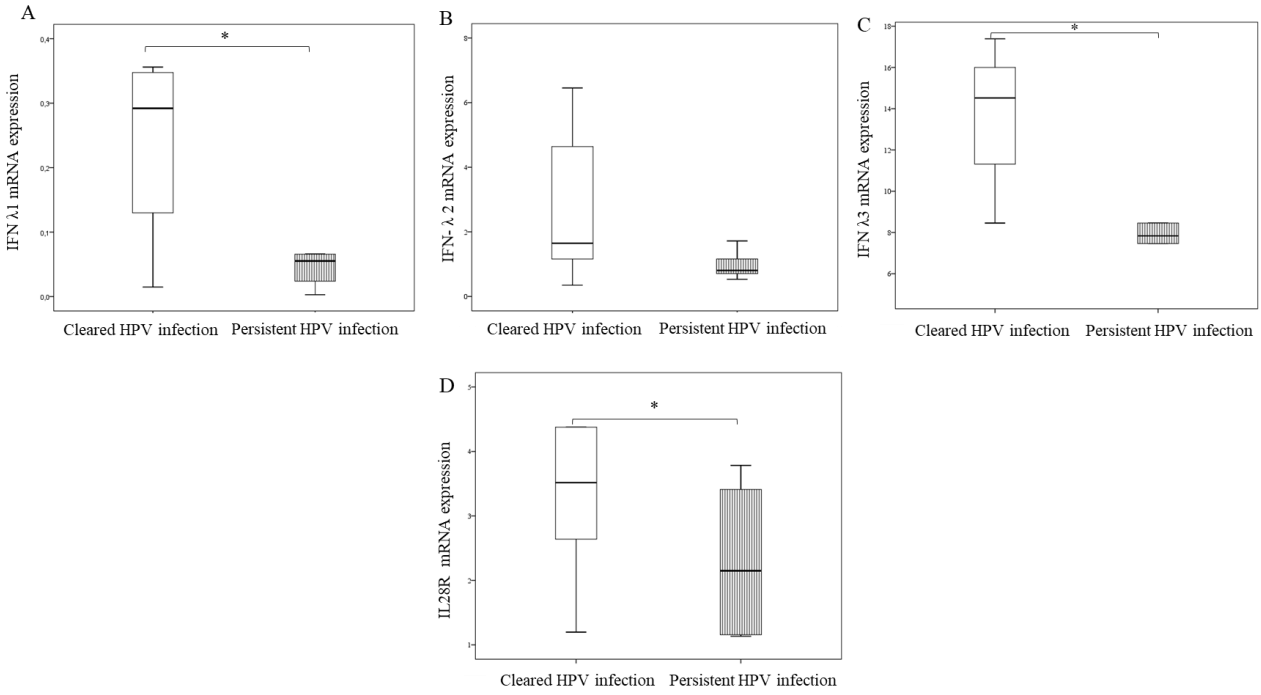

Figure 26 (Panel A-D). IFN-λ 1-3 and IL28R mRNA expression in anal cells of HIV-1 infected MSM during persistent and cleared HPV infection. Gene expression levels were determined as the mRNA copies relative to the constitutively expressed GUS gene and were expressed as 2^-ΔCT. Results are presented as the median (horizontal bar within boxes), 25-75 percentile (upper and lower margin boxes) and range (horizontal bar above or below boxes) of the relative number of copies. Data were analyzed using the Mann–Whitney U test (* $p<0.05$).

Of note, a multivariate logistic analysis showed that HPV positive patients who displayed reduced levels of IFN-β and IFN-ε were 10 times more likely to develop a persistent infection compared to those in who have cleared HPV [IFN-β age-adjusted OR=10.1 (95%CI: 1.4-19), p=0.022; IFN-ε age-adjusted OR=10.1 (95%CI: 1.4-71), p=0.023; Figure 27]. On the other hand, increased levels of IFNAR1 were associated to the development of a persistent anal HPV infection [age-adjusted OR=0.1; (95%CI: 0-0.4), p=0.011; Figure 27]. No other associations were recorded between IFN-α, IFNAR2, IFN-λ1, IFN-λ2 and IFN- λ3 levels (Figure 27, $p<0.05$, Figure 28, $p<0.05$) and the development of persistence of HPV infection.

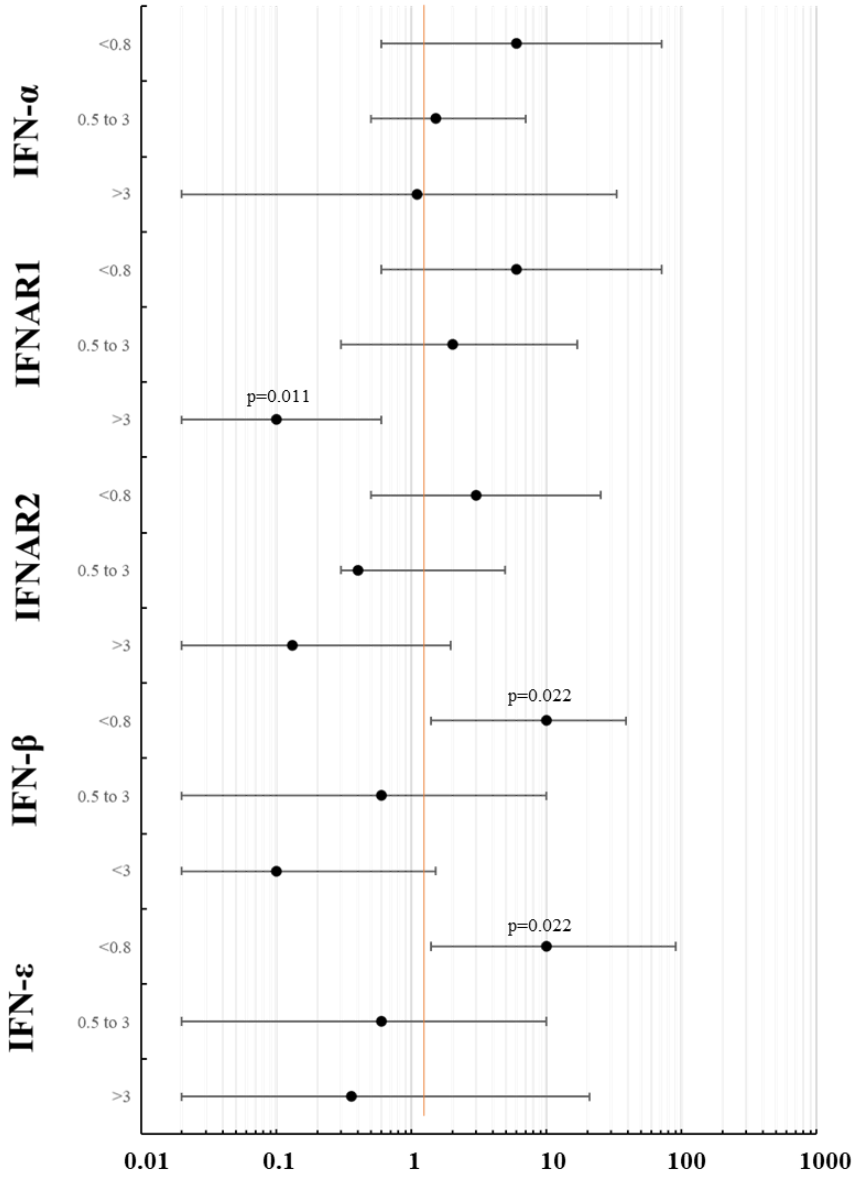

Figure 27. Multivariate regression analysis for type I IFN levels and persistent HPV infection. The Forest Plot shows odds ratio values and 95% confidence intervals. P value <0.05 was considered statistically significant.

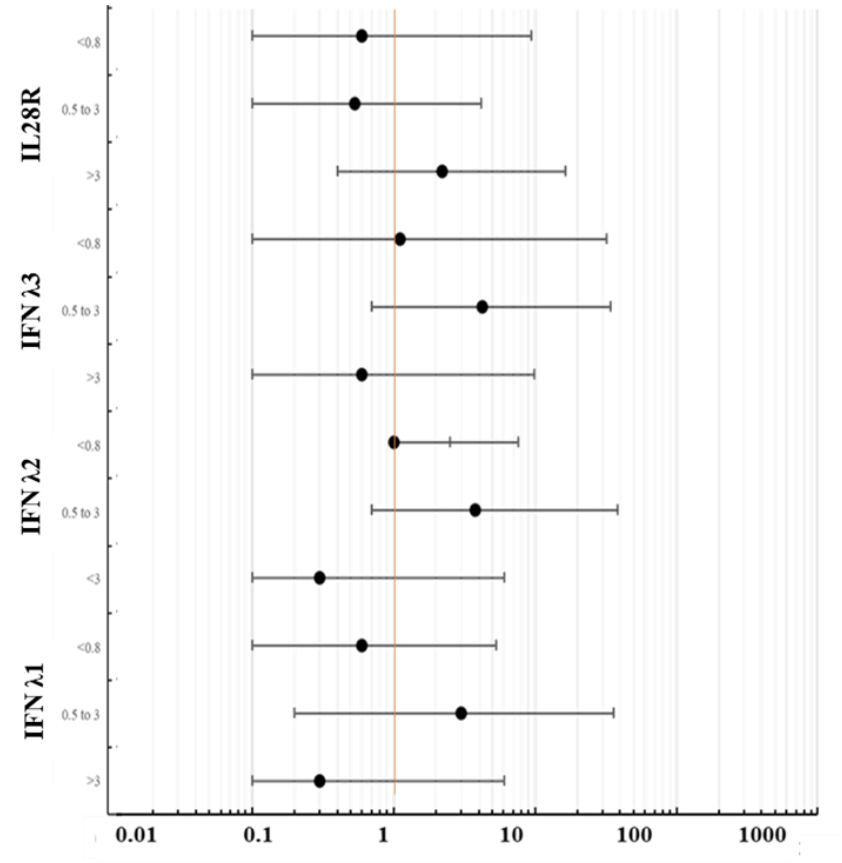

Figure 28. Multivariate regression analysis for type III IFN levels and persistent HPV infection. The Forest Plot shows odds ratio values and 95% confidence intervals. p value <0.05 was considered statistically significant.

4.8 EVALUATION OF NATURAL KILLER CELLS FREQUENCIES IN THE ANAL MUCOSA OF HPV-HIV INFECTED MSM

It has been reported that NK cell activity is impaired during persistent HPV infection (Garcia-Iglesias et al., 2009; Renoux et al., 2011). Nevertheless, there is no data on the profile of NK cells in anal HPV infected tissues.

Here, we evaluated the frequencies of $CD56^{+}$, $CD16^{+}$, $CD56^{dim}$, $CD56^{bright}$ NK cells NKT cells in anal biopsy samples collected from a subgroup (n=8/110) of HPV infected MSM. The biopsy samples were collected close to anal mucosal areas defined as normal (Healthy mucosa-HM) or dysplastic (LSIL) based on the evidence provided by the histological examination. The data obtained from this analysis were compared to an observational reference cohort of 47 HIV-1

infected MSM in follow-up at the Department of Infectious Diseases of the Policlinico Umberto I, which data were considered as a reference values for the data obtained from the HPV-HIV-1 positive MSM enrolled in this study.

The demographical and clinical characteristics of the subgroup of HPV infected MSM were reported in Table 9. Among HPV-HIV-1 infected MSM, 62.5% (5/8) of subjects showed an HR-HPV genotypes [HPV18: 3 (37.5%), HPV31(12.5%) and 45(12.5%)], as reported in the Table 9 and Figure 29. Moreover, the digital examination of the anal canal and the last tract of the rectum revealed no suspected lesions related to anal or rectal neoplasia in any of the participants at enrolment. However, all HPV-HIV-1 infected participants showed the presence of atypical cells in the anal cytology, compatible with a LSIL (100%) (Table 9).

Table 7. Demographic and clinical characteristics of cART treated HPV-HIV-1 positive MSM

Item[a]	**HPV-HIV-1-positive MSM (n=8)**
Age	50.1 (38.5-57.7)
nadir CD4+ T cells (cell/mm^3)	470 (271–850)
CD4+ T cell at enrolment (cell/mm^3)	897 (838-1817)
HIV-1 RNA (copies/ mL)[b]	<37
Years HIV diagnosis (years)	12 (7-18.5)
Years of treatment	11.5 (6.2-18.5)
Diabetes [n (%)]	1(12.5)
Opportunistic infections [n (%)]	0(0)
Smokers [n (%)]	3 (37.5)
Previous anal condylomatosis [n (%)]	3 (12.5%)
Anal conylomatosis at enrolment [n (%)]	2 (25%)
HPV vaccination [n (%)]	0(0)
HR-HPV infection [n (%)]	5 (62.5)
LR-HPV infection [n (%)]	3 (37.5)
LSIL [n (%)]	8 (100)

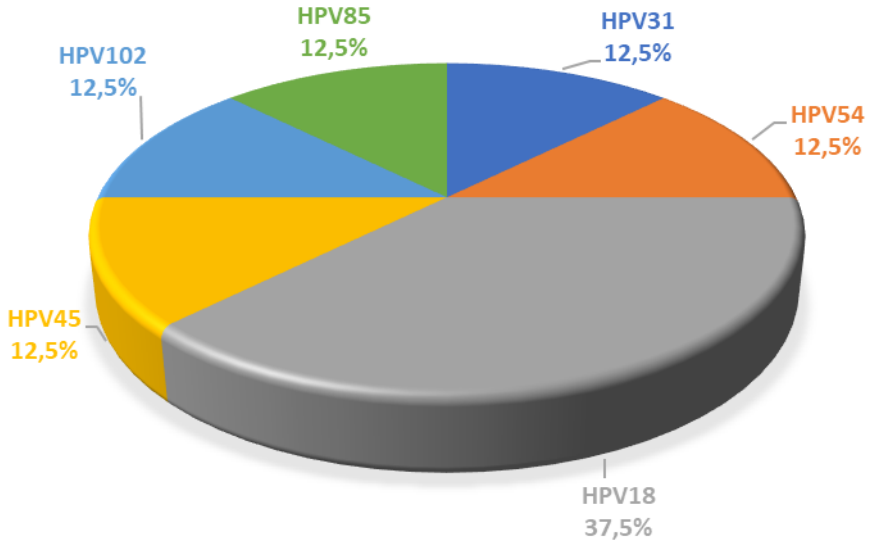

Figure 29. Prevalence of anal HPV genotypes in a subgroup of HPV-HIV-1 positive MSM (n=8)

A trend toward an increase in the frequencies of $CD56^{+}$, $CD16^{+}$, $CD56^{dim}$ NK cells was observed in the normal mucosa of the HPV positive subjects as compared to the reference population (Figure 30, Panel A and B, $p > 0.05$). Also, the $CD56^{dim}$ $CD56^{bright}$ NK cells and NKT cells frequencies detected in the healthy mucosa were similar between HPV positive MSM patients and the reference population (Figure 30, Panel C and D, $p > 0.05$).

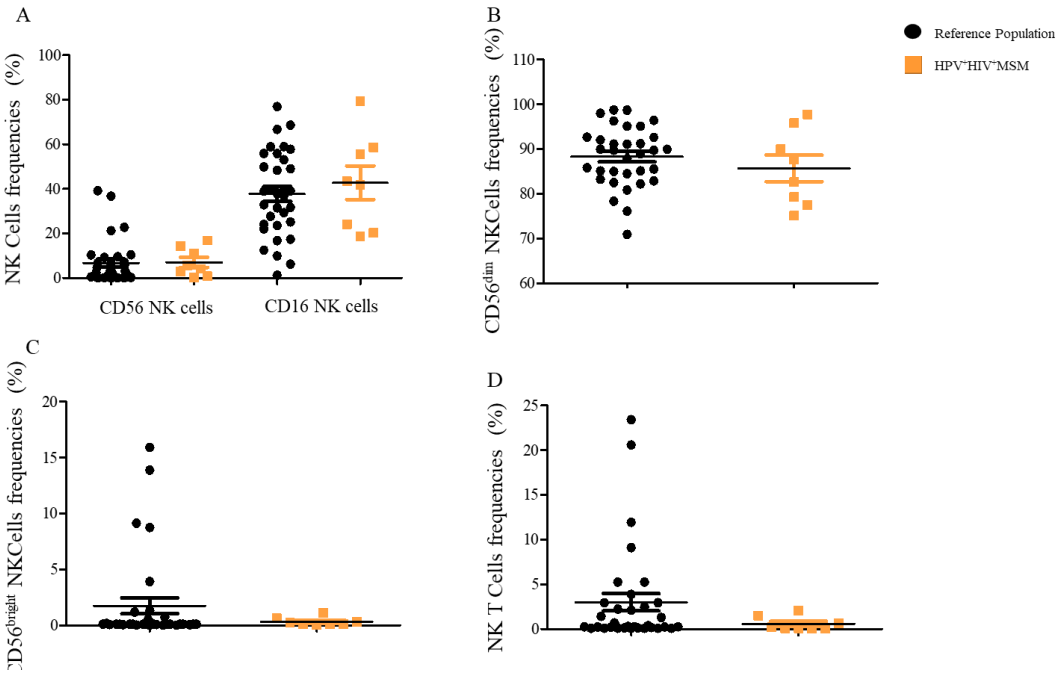

Figure 30 (Panel A- D). Frequencies of CD56^{+} and CD16^{+}, CD56 dim, CD56 bright NK cells and NK T lymphocytes in the normal mucosa of HPV-HIV-1 infected MSM and the reference population before oral bacteriotherapy intake. Data were analysed using the Mann–Whitney U test (* $p<0.05$)

As far as an anti-tumour activity of NK cells is concerned, CD56^{dim} NK cells levels increased in LSIL lesions (Figure 31, Panel D; $p<0.01$), while no differences were recorded in the frequencies of CD56^{+} (Figure 31, Panel A), CD16^{+} (Figure 31, Panel B), CD56^{bright} (Figure 31, Panel C) NK cells and NKT cells (Figure 31, Panel E) in the LSILs detected in the anal mucosa of the HPV+ HIV-1 infected MSM as compared to the reference population (Figure 31, Panel A-E; $p>0.05$).

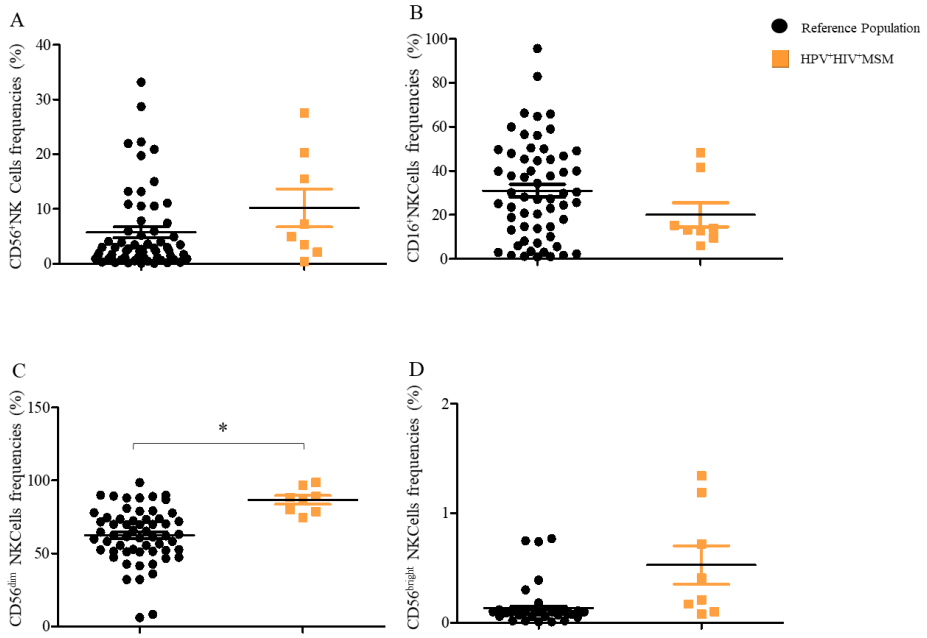

Figure 31 (Panel A-D). Frequencies of CD56^{+} and CD16^{+}, CD56^{dim} and CD56^{bright} NK cells in the LSIL of HPV-HIV-1 infected MSM and the reference population before oral bacteriotherapy intake. Data were analysed using the Mann–Whitney U test (* p<0.05)

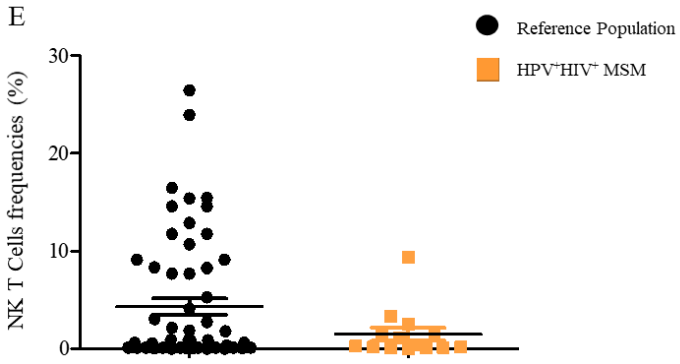

Figure 31 (Panel E). Frequencies of NKT lymphocytes in the LSIL of HPV-HIV-1 infected MSM and the reference population before oral bacteriotherapy intake. Data were analysed using the Mann–Whitney U test (* p<0.05)

Afterwards, the frequencies of CD56^{+}, CD16^{+}, CD56^{dim}, CD56^{bright} NK cells and NKT cells were compared between the normal (HM) and LSIL mucosa obtained from the anal compartment. As reported in Figure 31, a reduction in the frequencies of CD16^{+} NK cells was found in the dysplastic mucosa (LSIL) as compared to the normal anal mucosa (Figure 32, Panel B; p=0.029); while, the levels of CD56^{+} (Figure 32, Panel A), CD56^{dim} (Figure 32, Panel C), CD56^{bright} (Figure 32, Panel D)

NK cells and NKT cells (Figure 32, Panel E) were similar between LSIL and normal anal mucosa of HPV positive patients (Figure 32, Panel A-E; p>0.05).

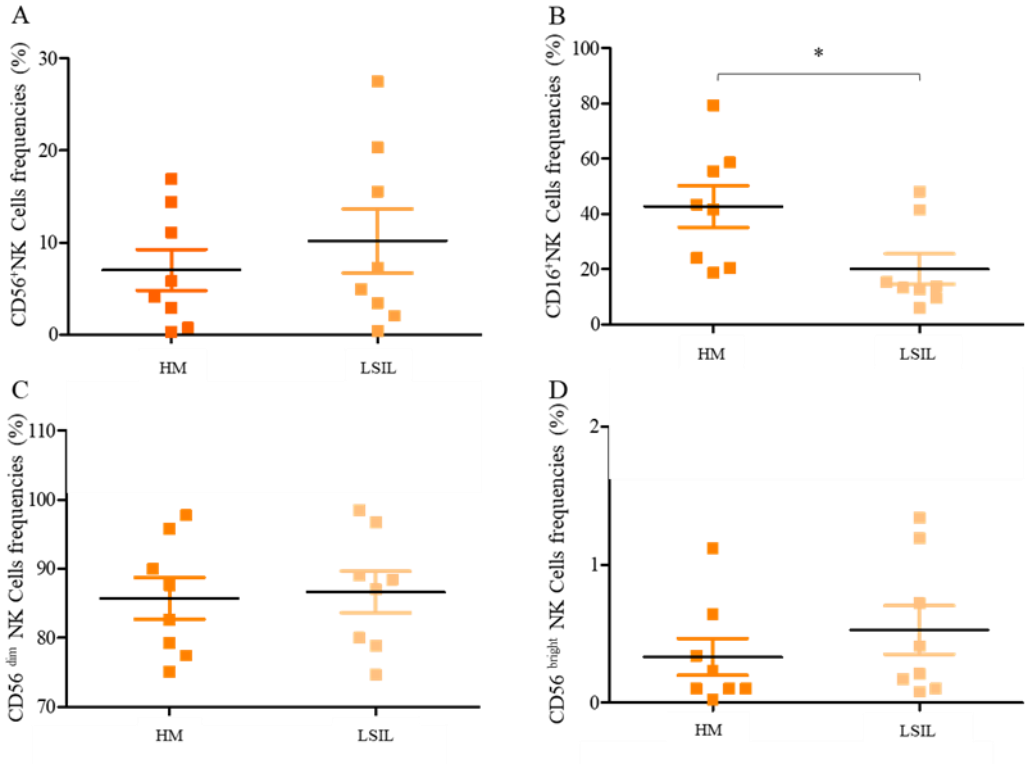

Figure 32 (Panel A- D). Frequencies of $CD56^{+}$, $CD16^{+}$, $CD56^{dim}$, and $CD56^{bright}$ NK cells in the anal HM and LSIL of HPV-HIV-1 infected MSM). Data were analysed using the Mann–Whitney U test (* p<0.05)

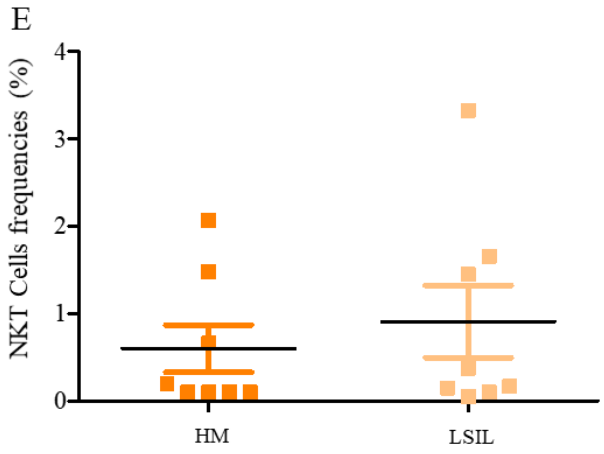

Figure 32 (Panel E). Frequencies of NK T lymphocytes in the anal HM and LSIL of HPV-HIV-1 infected MSM. Data were analysed using the Mann–Whitney U test (* p<0.05).

4.9 ROLE OF PROBIOTIC SUPPLEMENTATION IN THE ANAL MICROENVIRONMENT DURING HPV INFECTION

Having observed a high prevalence of persistent HPV infection among HIV-1 infected MSM and considering that recent studies supported that a balanced microbiota might exert a key role in the clearance of HPV infection (Serrano-Villar et al., 2017), we assessed whether oral bacteriotherapy supplementation to cART might promote the clearance of HPV infection and the regression of HPV-related dysplastic lesions in the subgroup of HPV positive MSM that have been previously analysed for anal NK cell frequencies (n=8/110). The effects of oral bacteriotherapy on mucosal type I and III IFN mRNA levels, and NK cells frequencies were evaluated. Because the relationship between persistent immune activation and HPV persistence, we also examined CD4 and CD8 T lymphocytes immune activation levels in blood of MSM receiving for 6 months oral bacteriotherapy. In particular, 4 patients (50%) received the probiotic formulation (probiotic group) for six months and the other ones received a placebo formulation, containing maltose and silicon dioxide as inactive substances and administered as well as the oral bacteriotherapy supplementation (placebo group).

All MSM patients analysed had anal mucosal lesions attributable to the presence of LSILs. HRA results showed that these patients had a clinical regression of anal lesions and condylomas after six months of oral bacteriotherapy, except for one patient who developed a new anal lesion. By contrast, we observed the appearance of new anal intraepithelial lesions in the placebo group during the study observational period (Figure 33).

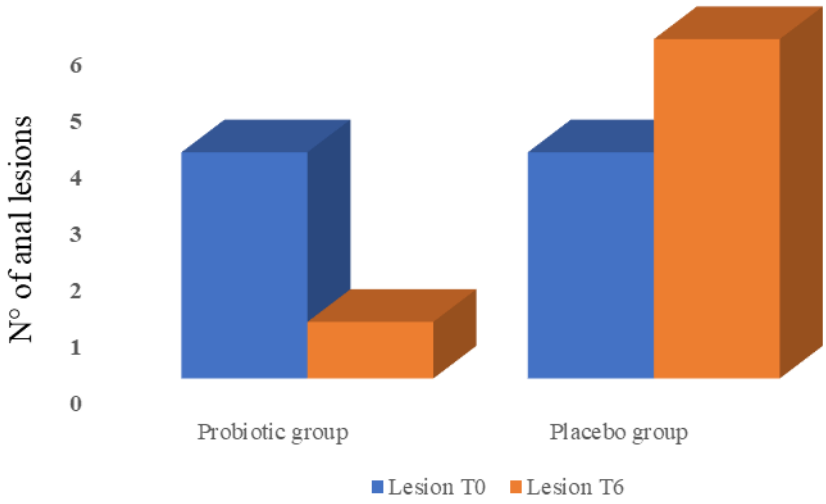

Figure 33. Impact of oral bacteriotherapy on the anal intraepithelial lesions.

Probiotic group patients displayed 4 LSIL at T0 (n=4) (blue box) only one patient displayed LSIL a T6 (orange box). In the placebo group, all patients displayed LSIL at T0 (blue box), and at T6 the regression of 3 lesions and 5 new lesions were recorded in the anal mucosa (orange box).

As far the impact of oral bacteriotherapy on HPV infection was concerned, one patient (Pt3) cleared the anal HPV infection after 6 months of probiotic intake, while Pt1 and Pt2 had an infection with a different HPV genotype at T6 (Table 10).

No changes in the type of HPV genotype were found in two patients (Pt6 and 7) belonging to the placebo group, while Pt 8 developed a new HPV 16 infection. Because to the low HPV-DNA levels detected in the anal samples, HPV analysis was not performed for patients 4 and 5 at T6 (Table 10).

Table 8. HPV genotypes before and after oral bacteriotherapy in HIV-1 infected MSM

		HPV T0	HPV T6
Probiotic group	**Pt1**	HPV31	HPV58
	Pt2	HPV54	HPV66
	Pt3	HPV18	Negative
	Pt4	HPV45	NA
Placebo group	**Pt5**	HPV31	NA
	Pt6	HPV18	HPV18
	Pt7	HPV102	HPV102
	Pt8	HPV85	HPV16

4.9.1 ROLE OF ORAL BACTERIOTHERAPY SUPPLEMENTATION ON TYPE I AND III IFN SIGNATURE IN THE ANAL COMPARTMENT

Having observed a potential beneficial role of the oral bacteriotherapy on the outcome of anal lesions, we next evaluated whether the oral bacteriotherapy might change mucosal levels of type I and III IFN.

In Figure 34 (Panel A-I) the levels of type I and III IFN and IFN related genes found before (T0) and after oral bacteriotherapy supplementation (T6) in anal cells are reported. In particular, both IFNAR1 (Figure 34, Panel D, $p=0.04$) and IFN λ3 (Figure 34, Panel H, $p<0.01$) mRNA levels resulted significantly increased at T6. A trend toward an increase in anal IFN-β (Figure 34, Panel B), IFN-ε (Figure 34, Panel C) and IL28R (Figure 34, panel I) levels was also observed after oral bacteriotherapy as compared to the placebo group (Figure 34). On the other hand, a trend toward a reduction in the transcript levels of IFN-α (Figure 34, Panel A), IFNAR2 (Figure 34, Panel E), IFN λ1 (Figure 34, Panel D) and IFN λ2 (Figure 34, Panel G) has been recorded after oral bacteriotherapy supplementation (T6).

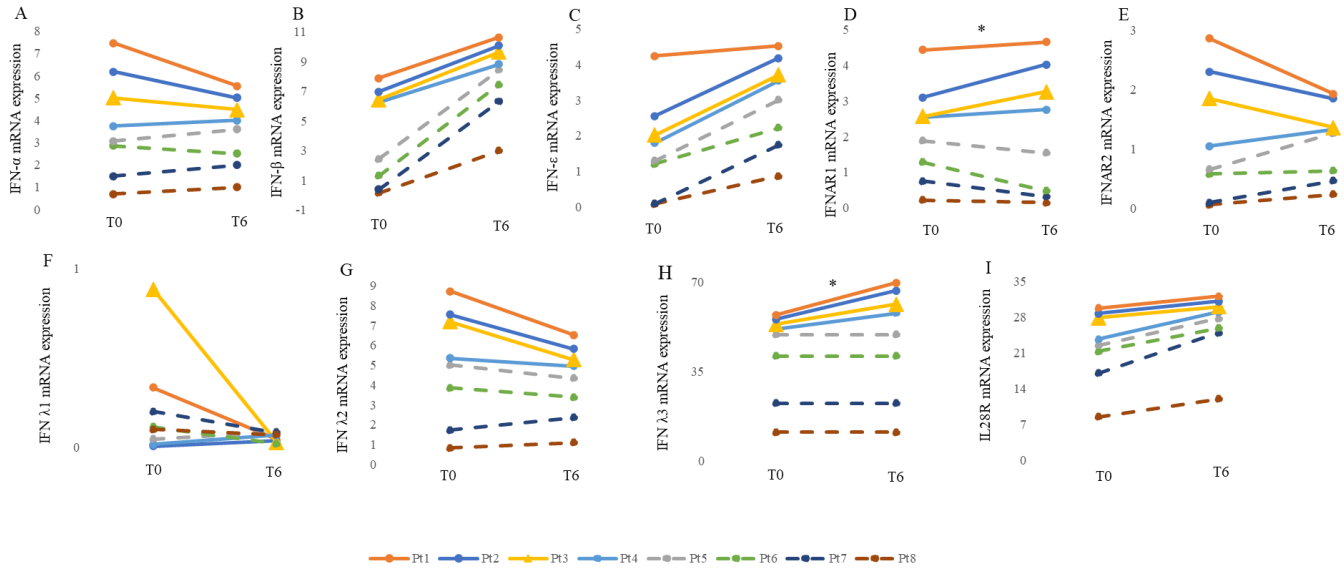

Figure 34 (Panel A-I). Type I and III IFN signature in anal cells of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Individual type I and III IFN expression before (T0) and after (T6) oral bacteriotherapy supplementation. Each patient is representeby a line. Continuous lines represent oral bacteriotherapy group and dotted lines refer to placebo group; ▲ indicates Pt3 who cleared the HPV infection. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

4.9.2 EVALUATION OF NATURAL KILLER CELLS FREQUENCIES IN THE ANAL MUCOSA OF HPV-HIV INFECTED PATIENTS BEFORE AND AFTER PROBIOTIC SUPPLEMENTATION

To examine the effect of oral bacteriotherapy on the frequencies NK cells subsets in anal tissues of HPV infected MSM, we compared the levels of $CD56^{+}$ and $CD16^{+}$, $CD56^{dim}$, $CD56^{bright}$ NK cells and NKT cells between LSIL and normal mucosa. This analysis was performed before (T0) and after (T6) oral bacteriotherapy supplementation. As reported in Figure 35, a decrease in the frequencies of $CD56^{+}$ NK cells (Figure 35, Panel A) and an increase of $CD16^{+}$ NK cells (Figure 35, Panel B) and NKT cells (Figure 35, Panel E) were detected in the anal LSIL at T6 (Figure 35, Panel A-E; $p<0.05$). An increase in the frequencies of $CD16^{+}$ (Figure 36, Panel B), $CD56^{dim}$ (Figure 36, Panel C), $CD56^{bright}$ (Figure 36, Panel D) NK cells and NKT cells (Figure 36, Panel E) were also recorded in the normal anal mucosa after probiotic supplementation (Figure 36, Panel A-E; $p<0.05$). In addition,

MSM belonging to the placebo group displayed a trend toward an increase in the frequencies of $CD56^{+}$, $CD16^{+}$, $CD56^{bright}$ NK cells and NKT cells in the dysplastic (Figure 34, Panel A-E) and healthy mucosa at T6 (Figure 35, Panel A-E).

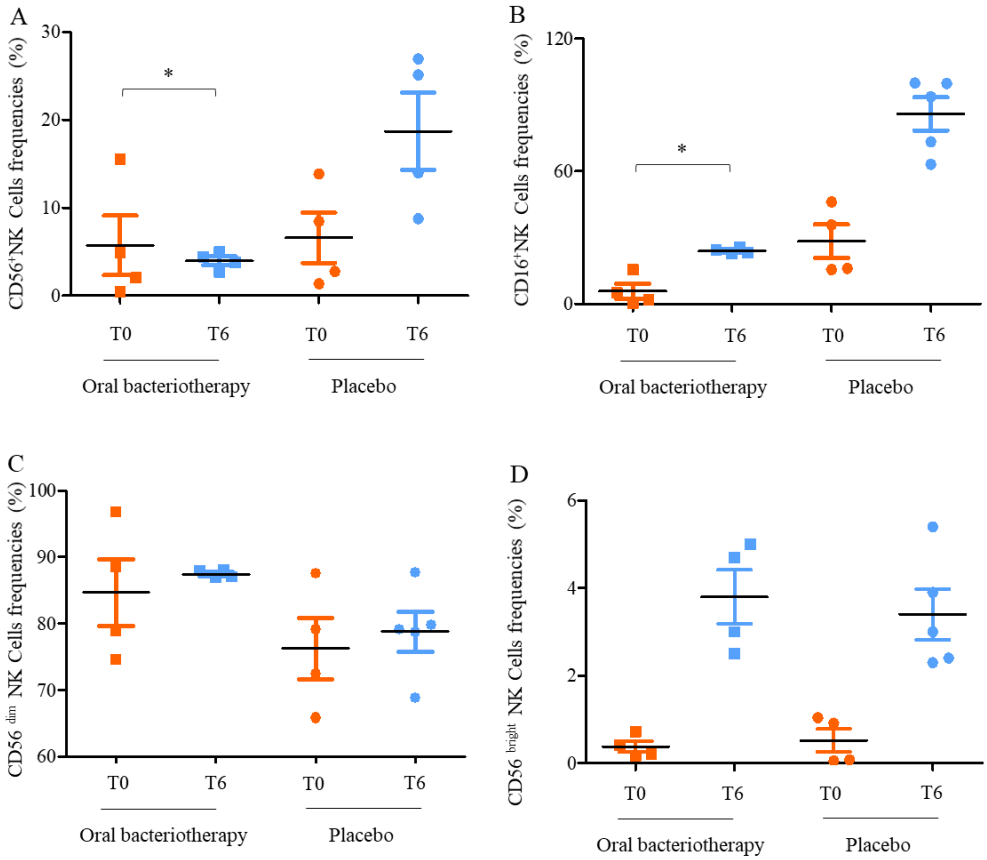

Figure 35 (Panel A-D). Frequencies of $CD56^{+}$ and $CD16^{+}$, $CD56^{dim}$, $CD56^{bright}$ NK cells in LSIL of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

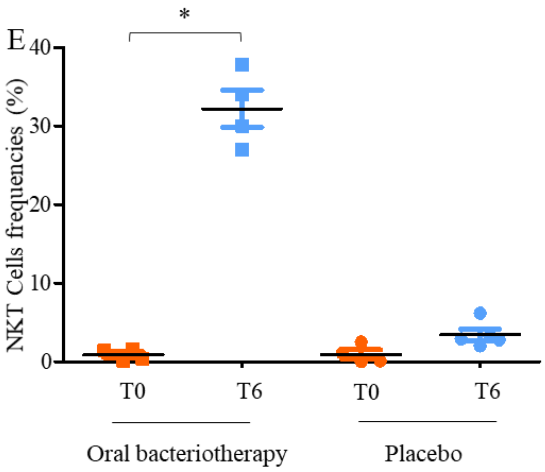

Figure 35 (Panel E). Frequencies of $CD56^{+}$ and $CD16^{+}$, $CD56^{dim}$, $CD56^{bright}$ NK cells in LSIL of HPV-HIV-1 infected MSM before and after oral bacteriotherapy intake supplementation. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

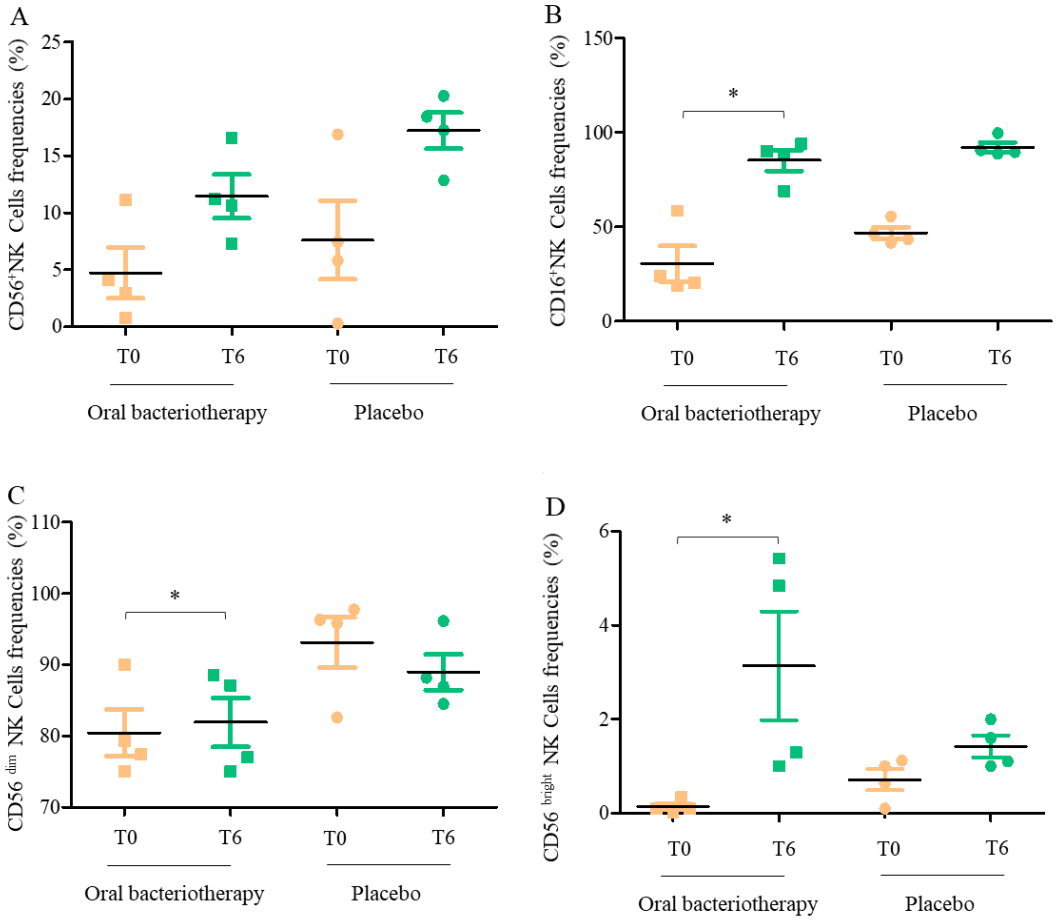

Figure 36 (Panel A-D). Frequencies of CD56^{+} and CD16^{+}, CD56^{dim}, CD56^{bright} NK cells in HM of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Data were analysed using the Mann–Whitney U test (* p<0.05).

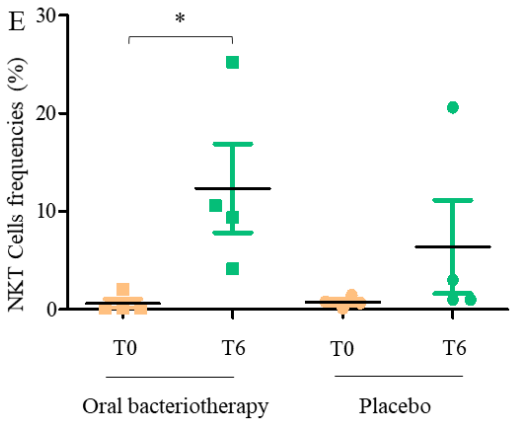

Figure 36 (Panel E). Frequencies of NK T lymphocytes in HM of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Data were analysed using the Mann–Whitney U test (* p<0.05).

4.9.3 EFFECTS OF ORAL BACTERIOTHERAPY ON THE PERIPHERAL IMMUNE ACTIVATION LEVELS

Several studies performed in HIV-1 positive patients have shown that persistent T cell immune activation associated with HIV-1 infection can predispose to HPV persistence (Strickler et al., 2005). Thus, we assessed the effects of oral bacteriotherapy supplementation on the blood frequencies of immune activated CD38 and HLADR (single and double) $CD4^+$ and $CD8^+$ T cell subsets (naïve, central and effector memory) in HPV positive MSM. As reported in Figure 37, the blood frequencies of $CD38^+$ and $HLADR^+$ CD4 T cell subsets, especially those with central and effector memory phenotype, were lower after oral bacteriotherapy supplementation (T6) compared to baseline (T0) (Figure 37, Panel A-D; $p<0.05$). A reduction in the levels of $CD38^+$ $HLADR^+$ CD8+ T cell subsets (effector memory phenotype) was also observed at T6 (Figure 38, Panel A-D; $p<0.05$).

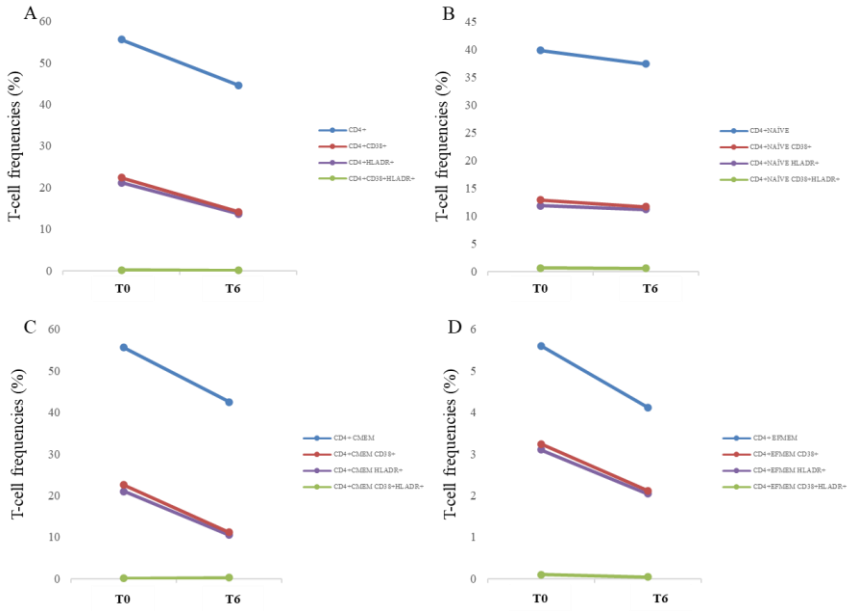

Figure 37 (Panel A-D). Frequencies of $CD4^+$ T cell subsets (naïve, CMEM and EFEM) expressing CD38 and HLADR (single and both) in PBMC of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

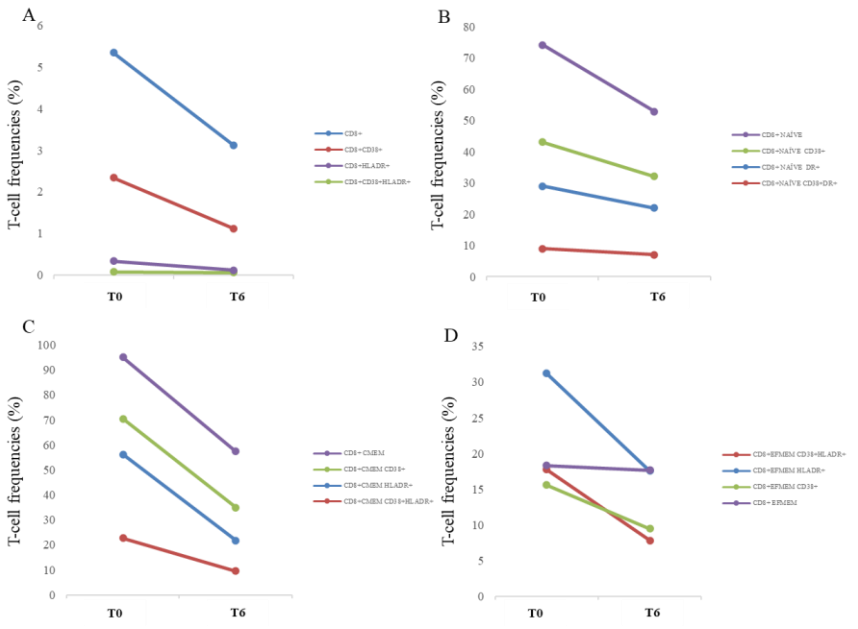

Figure 38 (Panel A-D). Frequencies of CD8$^+$ T cell subsets (naïve, CMEM and EFEM) expressing CD38 and HLADR (single and both) in PBMC of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation. Data were analysed using the Mann–Whitney U test (* $p<0.05$).

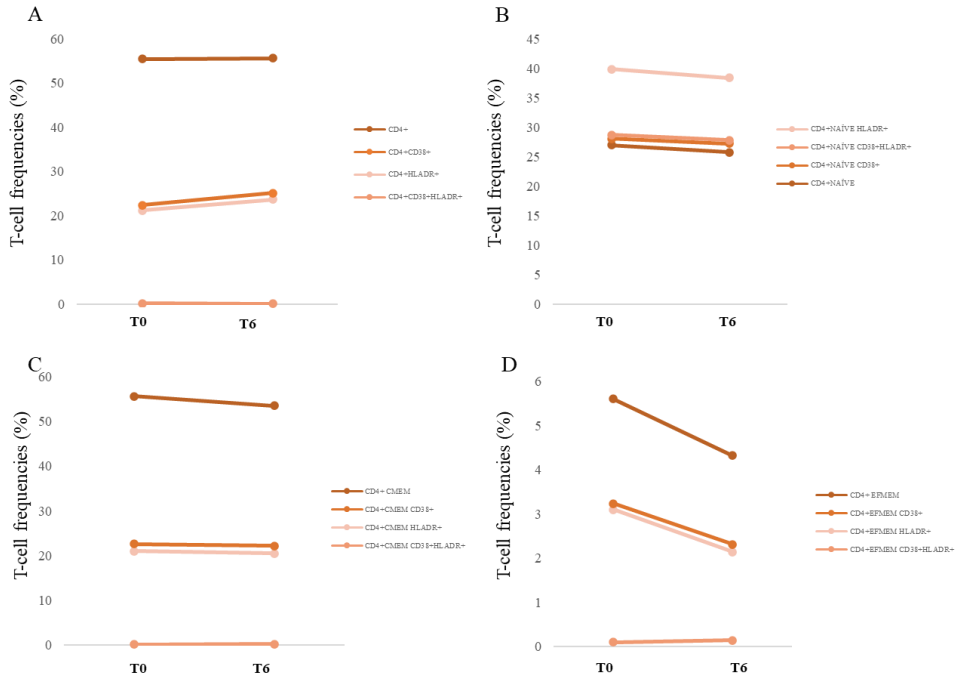

Figure 39 (Panel A- D). Frequencies of CD4$^+$ T cell subsets (naïve, CMEM and EFEM) expressing CD38 and HLADR (single and both) in PBMC of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation (placebo group). Data were analysed using the Mann–Whitney U test (* $p<0.05$).

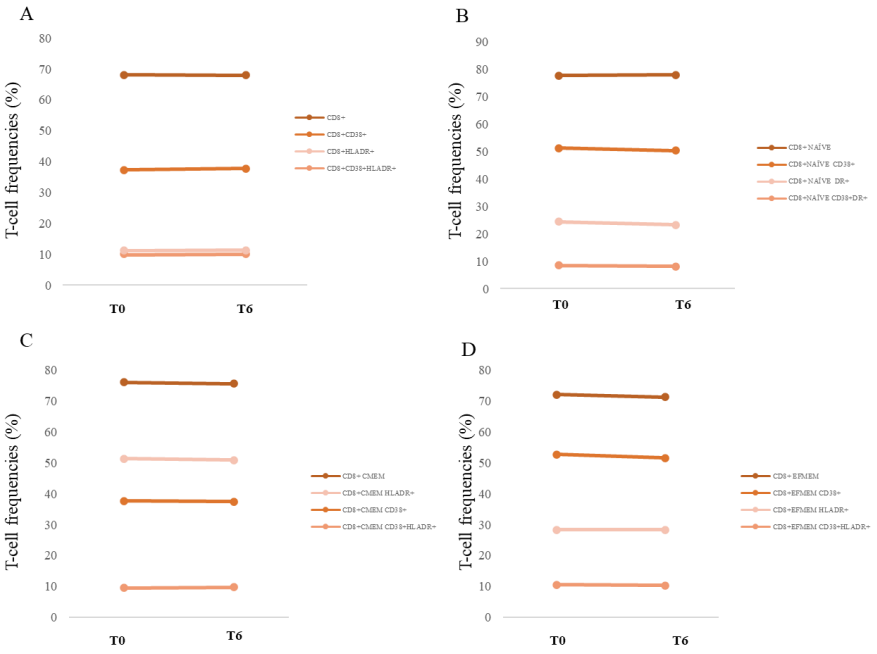

Figure 40 (Panel A-D). Frequencies of CD8$^+$ T cell subsets (naïve, CMEM and EFEM) expressing CD38 and HLADR (single and both) in PBMC of HPV-HIV-1 infected MSM before and after oral bacteriotherapy supplementation (placebo group). Data were analysed using the Mann–Whitney U test (* $p<0.05$).

5. DISCUSSION

HPV is defined as the most common sexually transmitted infection worldwide, that causes approximately 5% of all cancers in men and 10% of all cancers in women (Lehtinen et al., 2013). Of note, infection by high-risk HPV types is necessary but not sufficient for progression to cancer (Bosch et al., 2002); in fact, most HPV infections do not lead to cytological anomalies or cancer but are cleared by the immune system within 12-18 months. However, a small percentage of HPV infection can persist in the ano-genital compartment and promote the development of low and high-grade lesions, which may regress or progress to an invasive carcinoma. The ability of HPV to escape the host immune response and establish a persistent infection could be in part due to an impaired IFN activation observed in HPV infected individuals. Therefore, failure of the host defences to clear persistent HPV infections can lead to the development of ano-genital cancer even after several decades.

Compared to other HPV-associated cancers, including cervical cancer, the pathogenesis of anal cancer is still poorly understood, and this is partly due to the lack of immortalized HPV-positive anal epithelial cell lines that can be exploited to comprehend the progression of anal cancer (Wechsler et al., 2018). In this context, HPV-related intraepithelial squamous lesions of the anal canal are thought to behave likewise to HPV-related cervical lesions because of similarities in the epithelium but considerably less is known about the natural history of anal HPV infections (Palefsky et al., 1998).

The incidence of anal cancer has been increasing over the years in patients at high risk for HPV persistent infection, such as women with previous cervical lesions, and in immunosuppressed patients as HIV-1 infected individuals.

To date, little is known about how the local immune response affects the natural history of HPV infection in HIV-1 infected patients and which are the most important immunological factors (inflammatory cytokines, IFNs, cell mediated immunity) involved in the control of HPV infection and clearance. Considering the evasion strategies employed by HPV to overcome the host immune

response, we hypothesized that HPV might modulate type I and III IFN pathways and alter NK cell subsets frequencies in the anal mucosa of HIV-1 infected MSM. To clarify these issues, in this PhD thesis, we aimed to characterize the clinical, epidemiological, and immunological effects of HPV infection in the anal compartment, to identify potential immune markers that might contribute to persistent infection and precancerous mucosal lesions progression. A clinical plus of this project was the focus on the role of oral bacteriotherapy and its effectiveness in restoring an "healthy" immunological microenvironment in the anal mucosal tissues of HPV-HIV-1 infected MSM.

Therefore, this study was articulated into the following three main research phases: i) assessment of HPV parameters (prevalence, genotypes, rates of persistent HPV infection) and evaluation of type I and III IFN gene expression levels (Phase I); ii) evaluation of NK cell subsets frequencies in anal biopsies obtained through HRA from a subgroup of HIV-1 positive MSM with ascertained HPV anal infection and anal dysplasia (Phase II); iii) analysis of the role of oral bacteriotherapy on IFN transcript levels, on the frequencies of intra-epithelial NK cell subsets and on T immune cell activation levels in HPV-HIV-1 infected subjects.

Overall, data emerging from my PhD thesis showed an overall prevalence of about 80% of HPV infection in HIV-1 infected MSM. These results were in line with data obtained in previous studies, suggesting that anal HPV infection prevalence in MSM range from 70% to 90% (Marra et al., 2019; Garbuglia et al., 2015). Interestingly, HR-HPV was found in a half of the MSM, with a higher prevalence of HPV16, suggesting that this HPV genotype may be tightly linked to severity of anal disease. In this regard, a previous meta-analysis study showed a lower HPV16 prevalence among HIV-1 negative compared to HIV-1 positive MSM, independently from the results obtained on the anal cytology (Machalek et al., 2012). To this extent, the prevalence of HPV16 in MSM appears to be caused by the combined effects of HIV-1 infection and sexual behaviours, as shown by an increased prevalence of HPV16 in these subjects compared to other male populations; thus, HIV-1 infected MSM need clinical priorities for male anal cancer prevention (Marra et al., 2019; Mooij et al., 2016; Sudenga et al., 2017; Darwich et al., 2013). Nevertheless, the risen prevalence of anal HR-

HPV infection observed is well-known to be increased by anal sexual intercourse and worsened by HIV-1 related immunosuppression, so that sexual preference and HIV status are potential determinants of anal cancer risk in men. Moreover, the diagnosis of multiple infections can also contribute to this high risk of HPV infection in MSM; according to our analysis, about 2% of HPV positive patients displayed multiple HPV infections; by contrast, findings on the literature evidenced an elevated prevalence of multiple HPV infections, reaching 80% of HPV-infected cases, with a large heterogeneity of concurrent HPV type combinations (Donà et al., 2012; Garbuglia et al., 2015). The low rate of multiple HPV infection detected in our study might be due to the limited sensitivity of the methodology applied to identify multiple infections and/or the lack of interactions between different anal HPV genotypes in this group of HIV-1 infected MSM (Hasanzadeh et al., 2019).

The higher HPV prevalence in MSM is consistent with the higher rate of LSIL detected in anal cytology: indeed, the majority of HIV-1 positive MSM with SIL enrolled in this study were HPV positive and 42% of them had an HR-HPV genotype; however, the number of abnormal reports related to ASCUS was scarce and this could be due to the presence of anucleate squamous cells, degenerative changes and obscuring materials that could make cytological reading difficult. Moreover, anal low-grade SIL, although not a direct cancer precursor, is related to the precancer and cancer progression (Bennets et al., 2015). Nevertheless, HPV prevalence was slightly higher in studies including the evaluation of anal cytopathology, suggesting that clinical studies focused on screening program might overestimates the rates of HIV-1 positive MSM with existing anal HPV-related disease, since people participating in these screening campaigns considered themselves to be at greater risk of sexually transmitted infections and may not be representative of the general population. Hence, it is of great importance to identify HIV-1 infected patients at a higher risk of progression to HSIL and cancer, not only considering clinical parameters and the HIV status (Mooij et al., 2016) but also behavioural variables, in order to potentially identify predictors of HPV infection susceptibility and severe lesion progression. Indeed, we found that smoke, age and sexual behaviours represented important risk factors for HR-HPV and anal SIL occurrence in HIV-1 infected MSM.

The most consistently risk determinants for HPV infection are history of receptive anal intercourse, a high number of partners as well as non-intercourse anal sexual practices (Wong et al.,2020), opposite to the frequency of condom use during homosexual behaviours associated to a lower risk of acquiring HPV.

In our virological analysis, we found that about 70% of MSM remained HPV positive after one year from the first study visit: more than half MSM displayed the same HPV genotype, while 45% of patients were infected with a different HPV genotype. These findings confirmed that the HPV clearance rarely occurs in MSM and that specific sexual behaviours were associated with HPV persistence (Moscicki et al., 2014). Nevertheless, HPV clearance frequencies observed in my PhD study were lower than the rates reported in other previous studies (Darwich et al., 2013; Hernandez et al., 2014). It must be also considered that the small number of patients, who completed this study's follow-up stage compared to the total study population, might have limited the identification of crucial risk factors associated to HPV persistence.

Since persistent HPV infections can progress to cancer, it is critical to better understand the innate immunity mechanisms that control the early viral clearance. Then, for the first time to our knowledge, we analysed the expression of type I (IFN α, IFN β and IFN ε, and the receptor IFNAR1 and IFNAR2), the IFN-λ pathways (IFN-λ 1, 2, 3 and IL-28R, the IFN-lambda receptors) in the anal cells of MSM; results obtained were analysed and correlated with the HPV genotype, viral persistence, and cytological and histological grade of anal lesions. In particular, we showed that there was a general decline in the mucosal expression of all type I and III IFN subtypes in response to anal HPV infections, confirming the overall weakening of the IFN innate immune response found in HPV positive patients (Pierangeli et al., 2011; Cannella et al., 2014). Indeed, previous studies reported that, despite an inter-individual variability, an overall reduction in the activation of IFN response during HR-HPV, as well as in the expression of IFN induced genes was ascertained in HPV infected women (Pierangeli et al., 2011). In agreement, the capability of HPV to control type I IFN pathway in immune cells was reported (Garcia-Pifieres etal., 2006); both E6 and E7 HPV16 proteins were also shown to

inhibit type I IFN induction in transfected primary keratinocytes (Nees et al., 2001). At the same time, HR-HPV efficiently inhibits the type I IFN response in the presence of increased expression levels of the viral oncoprotein, E6 and E7 (Barnard et al., 1999; Stanley, 2012; Hong et al., 2011). These data were in accordance with the reduction in the expression of type I IFN components in the anal compartment of MSM, particularly in those with an HR-HPV infection, that represented about 50% of the study population. Thus, all these findings suggest that HPV can modulate cytokine production (e.g. IFN), impairing pro-inflammatory response, as a possible strategy to evade the host immune response, ultimately resulting in development of mucosal lesions in patients in which this immunomodulation is successful (Amador-Molina et al., 2013).

Furthermore, the importance of the induction of the more recently identified type III IFN during viral infections is well documented in different mucosal compartments (Kotenko et al., 2017), but data regarding its antiviral and antiproliferative activity in anal HPV infection is limited. Therefore, the expression of the type III IFN subtypes and their receptor was examined in cells collected from anal brushings of HIV-1 positive MSM patients. A reduction in the IFN-λs expression level was detected in HPV-HIV-1 infected MSM, especially in those patients with an HR-HPV genotype. In addition, an elevated expression level of IL28R-mRNA (the specific chain of the IFN-λ receptor) in patients with LR-HPV infection was correlated with levels of IFN- λ subtypes. In agreement, we previously found a coordinated expression of several IFN λ-related genes in cervical samples from LR- and HR-HPV positive women that suggest an activation of type III IFN response in the course of LR-HPV infection (Cannella et al., 2014). Consistently, the lack of type III IFN response in anal cells during HR-HPV infections might favour the HPV oncogenic properties.

Furthermore, a decreasing trend of type III IFNs expression was observed in the presence of SIL (HSIL+LSIL). These findings suggested that type III IFNs might exert antiviral activity against HPV but also had antitumor effects in targeted cells (Lasfar et al., 2011; Liu et al., 2013). On the light of the aforementioned considerations, it is possible to hypothesize that HPV hindrance on type III IFNs might be one of the main strategies used by HPV to favour viral persistence and promote cancer

development in the anal mucosal tissues of HIV-1 infected MSM. Thus, this data confirmed that the HPV infected cells are not able to activate a robust antiviral innate immune response, potentially facilitating HPV persistence in the anal compartment. Moreover, it is also established that the main target of HPV infection is represented by keratinocytes, that even under resting conditions, secrete an array of cytokines to sustain immune surveillance (Tay et al., 2013; Nestle et al., 2009; Goldschmidt et al., 2006; Feldmeyer et al., 2007).

Our study showed that both type I and III IFN responses were decreased in HPV positive patients who exhibited, for at least one-year, persistent HPV infections. In this group of HIV-1 infected MSM, about 70% of patients monitored, displayed a persistent anal HPV infection, in accordance with a persistence rate of 75% recorded in a longitudinal cohort study performed in northern Italy in HIV-1 infected MSM (Parisi et al., 2011). Notably, type I IFNs are downregulated in keratinocytes persistently infected with HPV16 (Nees et al., 2001) and in HPV positive cervical carcinoma cell lines (Bachmann et al., 2002), and IFN-γ expression is decreased in cervical carcinoma tissue (de Gruijl et al., 1999; Gey et al., 2003). *In vitro* studies have shown that in keratinocytes persistently immortalized by plasmid HPV-16 genomes, exposure to a low-dose of IFN transiently stimulates viral early gene expression, augments initial plasmid amplification, and increases viral plasmid copy numbers by activating IFN-stimulated regulatory factor (IRF)-1 (Lace et al., (2009). In turn, HPV oncogenes can modulate IFN response pathways (Bodily J. et al., 2011; Muto et al., 2011). However, evidence illustrating the inhibitory effects of HPV on IFNs expression emerged only from studies performed in cell lines or during cervical HPV infection. (Tay et al., 2013; Nestle et al., 2009; Goldschmidt et al., 2006; Feldmeyer et al., 2007; Nees et al., 2001; Bachmann et al., 2002; de Gruijl et al., 1999; Gey et al., 2003). Also, this inhibition or modification of host gene expression patterns, including innate immune response genes, driven by HPV oncogenes, contribute to the establishment of a persistent HPV infection (Bodily et al., 2011). It is also known that the risk of re-infections with a different HPV genotype may be linked to factors as lifestyle or type of sexual intercourse (Kost et al., 2017; Nyitray et al., 2010). Our analysis showed a relationship between type I IFN expression

levels and the likelihood of developing a persistent HPV infection. Notably, large *in vivo* studies with patient's follow-up have the potential to identify which aspects of the IFN pathways are more deregulated and eventually address them with immune-modulating therapies.

Besides the modulation of type I and III IFN genes, interestingly, HR-HPV, as HPV16, HPV18, or HPV31, downregulate the constitutive expression of several IFN-stimulated genes in keratinocytes, contributing to the progression of HPV related lesions, but the underlying mechanisms are not well understood (Nees et al., 2001; Karstensen et al., 2006). In particular, HPV could interfere with the expression of IRF1, by inhibiting its tumour suppressor role and with ISG15 transcription, enhancing the risk of progression to anal cancer (Werness et al., 1990; Pierangeli et al., 2011.).

In this complex scenario, the suppression of immune cells in the local mucosal environment might be an additional major reason of HPV persistence in the anogenital tract. The evaluation of the frequencies of NK cells subsets performed in my PhD thesis revealed that HPV-HIV co-infected MSM displayed a reduction in the frequencies of $CD16^{+}$ NK cells in the anal dysplastic lesion as compared to the normal anal mucosa while, similar levels of other NK cell subsets, as $CD56^{+}$, $CD56^{dim}$, $CD56^{bright}$ and NKT cells were recorded between LSIL and normal anal mucosa. It has been reported that NK cells are important biological barriers that emerge at an early stage in HPV-infected lesions (Renoux et al., 2011). HPV can activate the loss of NK cells membrane receptors thus leading to their malfunction and contributing in this manner to cancer development (Garcia-Iglesias et al., 2009). However, only limited studies have been focused on the analysis of NK cells distribution and function in HPV-infected mucosal tissues until to date. It is important to underline that CD16 is a vital NK membrane protein that can induce HPV-VLP (Virus-Like Particles) endocytosis followed by degranulation and cytokine secretion (IFN-γ and TNF) (Renoux et al., 2011; Holder et al., 2018.), thus resulting on NK cells ADCC activation (Pal et al., 2018). Based on our results, the reduction in the anogenital NK cells frequencies might fail in the elimination of the HPV virions from the anal infected cells. This condition might be due to an inadequate IL-2 production. This interleukin can

positively regulate NK cell cytotoxic function and their downstream differentiation (Mirjačić Martinović et al., 2017).

Moreover, HIV-1 infection can certainly provide a strong effect on both phenotype and function of NK cells, causing the activation and expansion of the whole pool of NK cells (Mavilio et al., 2005): in particular, pro-inflammatory NKCD56^{bright} populations were reduced, while the cytolytic CD56^{dim}CD16^{pos} NK cell and dysfunctional CD56^{neg}CD16^{pos} NK cells were increased in HIV-1 infected people compared to healthy subjects. Additionally, HIV-1 can contribute to the expression of NK cytotoxicity receptors (NCR), as NKp30, NKp44 and NKp46 (De Maria et al., 2003; Terra et al., 2016). Interestingly, Bere et al. have compared *in vitro* functional responses of NK and T cells in genital warts and blood of HPV/HIV-1 infected women, showing reduced frequencies of CD56^{dim} NK cells in both blood and warts, as compared to HIV-1 negative women (Bere et al., 2014.). To this extent, HPV-associated wart regression also depends on CD4 T cell infiltration levels that reached a peak during the resolution stages of HPV-related lesions (Nicholls et al., 2001). Several studies have reported a reduction in the frequencies of CD56^{+} NK cells during HIV-1 infections (Goodier et al., 2003; Mavilio et al., 2005; Lucia et al., 1997), as well as an enrichment of CD56^{-} CD16^{+} NK cells frequencies that was associated with reduced frequencies of CD56^{dim} NK cells (Bere et al., 2014). Although these findings, the mechanism describing HPV-associated genital lesions resolution during CD4 T cell lymphopenia, occurring during chronic HIV-1 disease, has not been yet described. Thus, it is reasonable that defects in the NK activation might underlie the failure of HPV-HIV-1 infected MSM to clear HPV infection and the related lesions.

Aside from the relationship between mucosal innate immunity and HPV/HIV-1 infections, emerging evidence support that microbiota might amplify or mitigate HPV carcinogenesis (Garrett, 2015; Tjalsma et al., 2012). Indeed, an alteration of the microbiota composition and function associated to HIV-1 infection (Harper, 2016; Serrano-Villar et al., 2016) might explain the altered course of anal disease during HIV-1 infection. Keeping in mind the role of HPV in regulating the immune response in HIV-1 positive subjects and considering the lack of therapeutic strategies able to prevent the

development of HPV infection and/or promote the clearance of the infection, another aim of this study was to evaluate the effects of oral bacteriotherapy on the HPV clearance, resolution of HPV-related lesions, and innate immune response pathways in a subgroup of HIV-1 infected patients. Interestingly, no HIV-1 patients undertaking oral bacteriotherapy developed new condylomas or warts in the mucosal anal compartment. Notably, despite there are no similar direct evidence for anal condylomatosis in literature, the spontaneous regression of multiple large condylomas is not frequent among HIV-1 infected patients. The oral bacteriotherapy might exert a beneficial role in the clearance of HPV related lesions, as also previously shown by our group (Ceccarelli et al., 2018). Based on previous findings of gynecological studies that supported the relationship between probiotic supplementation and the regression of HPV related lesions, it is possible to hypothesize that oral bacteriotherapy could also exert a positive role on anal microenvironment of HIV-1 infected subjects. As a matter of fact, the effects of several oral bacteriotherapy formulations on HPV associated alterations have been only investigated in the female genital tract. In particular, a prospective controlled pilot study by Verhoeven et al. indicated that women with HPV associated low-grade squamous intraepithelial lesions treated with probiotic supplementation showed a higher chance of clearing HPV infection and higher rates of clearance of HPV associated cytological abnormalities (Verhoeven et al., 2013). Similarly, Ceccarelli et al., showed that a 4-month course of supplementation with a high concentration multistrain probiotic formulation administered orally and by rectal instillation boosted the clearance of the anal condylomas in a 56-year-old HIV-1 infected man with multiple anal condylomas and positivity for anal HPV 18 (Ceccarelli et al., 2018).

It has been reported that HIV-1 infected MSM undergoing screening for HPV-related cancer displayed specific fecal and mucosal bacteria that are able to predict the existence of precancerous anal lesions (Serrano-Villar, et al., 2017); in particular, at mucosal level, *Campylobacter* was found to be predictive of LSIL, suggesting that colonization by this pathogenic genus might promote carcinogenesis (Serrano-Villar, et al., 2017). A member of the Lachnospiraceae family, *Catenibacterium*, which is a component of the HIV-1 associated microbiota (Vázquez-Castellanos et

al., 2015), was also considered a biomarker of HSIL development (Serrano-Villar, et al., 2017). Faeces collected from HIV-1 infected subjects with HSIL showed enrichment for *Pseudomonas aeruginosa*, which might influence HIV/HPV disease via induction of the kynurenine pathway (Favre et al., 2010). By contrast, depletion of *Bifidobacterium* was detected in faeces of HIV-1 infected subjects with AIN, indicating that loss of this taxa could promote HPV disease (Sivan et al., 2015). All these findings support that several bacteria taxa might increase during HPV-HIV-1 coinfection as a compensatory response to a chronic perturbation. Indeed, it is widely accepted that the gut microbiota represents a key factor for immune homeostasis, and it is associated with T cell immune activation status that is known to characterize HIV-1 infection. This evidence supports that therapeutic interventions in HIV-1 positive patients are needed to restore the integrity of the gut immune system. In this context, it has been found that the use of probiotics can recover gut barrier functions, remodel the microbiome, and decrease bacterial translocation and pro-inflammatory cytokine production, improving immune functions (Carter et al., 2016).

Our study investigated the immunomodulatory effect of six-month of oral bacteriotherapy supplementation in cART experienced HPV positive subjects, focusing on NK cells frequencies, type I and III IFN signature, and T cell immune activation levels. Specifically, oral bacteriotherapy showed the ability to modulate CD16+ NK cells frequencies in the dysplastic mucosa (LSIL) compared to the normal anal mucosa and to reduce several NK cell subsets frequencies ($CD56^{dim}$, $CD56^{bright}$ NK cells) in the normal anal mucosa. In this regard, several bacterial strains have been reported to be associated with modulation of NK cell activity (Aziz et al., 2016). A meta-analysis of randomized control trials highlighted a favourable association between probiotic supplementation and NK cell activity (Gui et al., 2020). Remarkably, there is an extensive interest in the bacteria strain specificity of immune modulation by probiotics; it has been demonstrated that Lactobacilli and Bifidobacteria, which are the most common species used in probiotic formulations (Isolauri, et al., 2004), can increase NK cell activity (Dong et al., 2011). For instance, probiotic supplementation has been shown to increase concentrations of IgA, the number of NK cells number, and NK cell activity in young adults (Olivares

et al., 2006). In elderly adults, *Bifidobacterium lactus* HN019 therapy increased phagocytosis of monocytes, polymorphonuclear cells, and NK cell tumoricidal activity (Gill et al., 2001; Chiang et al., 2000).

The analysis performed in this study indicated that anal mucosal levels of the type I and III IFN signatures were modulated by oral bacteriotherapy supplementation. These findings were supported by our previous studies performed with the same probiotic formulation, that showed the ability of oral bacteriotherapy to reduce distinct biomarkers of inflammation in the gut and blood compartment of HIV-1 infected patients (Pinacchio et al., 2018; d'Ettorre et al., 2017). Moreover, induction of type I IFN has been shown to be a mediator of protective effects of commensal and probiotic bacteria (McFarland, et al., 2011). However, to date, the effects of oral bacteriotherapy on the IFN response has not been yet well documented during anal HPV infection in the HIV-1 positive MSM.

Because HIV-1 infection is characterized by a persistent T cell immune activation (d'Ettorre, et al., 2017.), that might contribute to HPV persistence and anal dysplasia development, we performed the evaluation of blood T cell immune activation level in the subgroup of HIV-1 infected subjects treated with probiotic supplementation. As demonstrated by flow cytometry analysis, a reduction in the frequencies of CD4+ and CD8+ T-cell subsets, expressing CD38+ and/or HLA-DR+ were recorded in the peripheral blood after the probiotic intervention.

It is known that the impact of probiotic mediated immune recovery during HPV infection in HIV-1 infected MSM remains controversial: some studies showed reduction of lesion severity and rate of progression, as well as increased lesion regression following cART (Heard et al., 1998; Heard et al., 2002; Minkoff et al., 2001), others reports have shown that cervical dysplasia can persist despite cART-mediated CD4+ naive and memory T cells recovery (Lillo et al., 2001; Schuman P et al., 2003; Ahdieh-Grant et al., 2004). The relationship between persistent immune activation and HPV infection on cART-mediated immune reconstitution was supported by the hypothesis that HR-HPV infection was associated with increased immune activation in blood, independently of anogenital dysplasia (Papasavvas et al., 2016). Likewise, HR oncogenic HPV genotype infection was related to increased

immune activation and T cell exhaustion in cART-suppressed HIV-1-infected women (Papasavvas et al., 2016). Other studies have showed that HR-HPV related cervical dysplasia is linked to a local increase in immune cells density (Papasavvas et al., 2016). Interestingly, our results indicated that oral bacteriotherapy can promote the reduction of blood T cell immune activation in patients coinfected with HPV and HIV-1. Hence, our observations highlighted the beneficial role of oral bacteriotherapy on reducing T cell immunoactivation, providing the first evidence of a relationship between T cell immunoactivation and the clearance of anal HPV infection in HIV-1 infected MSM.

In conclusion, this study has allowed to define the role of anal HPV infection in HIV-1 positive MSM. We showed that HR-HPV infection might deeply inhibit the innate immune response by the reduction of IFN expression and the alteration of NK cells frequencies. These negative effects of HPV on innate immunity might favour the development of high-grade HPV-related lesions. However, further studies are needed to better characterize the role of NK cells in HPV-related anal lesions among HIV-positive MSM. Moreover, this PhD study shed light on oral bacterial therapy as potential strategy for anal HPV infection treatment. This approach appears to be able to modulate the innate response and reduce the rate of T cell immune activation observed in HPV/HIV-1 co-infected subjects. Therefore, we might hypothesize that the modulation of gut microbial composition might favor the clearance of anal HPV and the recovery of a "healthy" anal microenvironment. Altogether, these results should be considered with caution since they are preliminary data on the effects of this specific probiotic formulation on type I and III IFN response, NK cells frequencies and T cell activation in HIV-1 infected MSM. In addition, the effects of oral probiotic supplementation were evaluated on a small group of HPV/HIV-1 coinfected MSM and this could potentially limit the statistical power of this analysis.

Further evaluations will be performed on HIV-1 negative subjects, to better characterize the role of HIV-1 in the persistence of HPV infections and to define immunological factors involved in the significant spread of high-grade HPV lesions and anal cancer in HIV-1 infected MSM.

6. Supplementary materials

Table S6. Prevalence and Correlates Associated with Anal HPV Infection

Parameters	**Class**	**HPV+ N=37 n (%)**	**HPV- N=13 n (%)**	**OR-adjusted (CI 95%)**	**p-value[a]**
Age	<25	0(0)	1(7.7)	0.2(0.2-3.4)	0.265
	25 to 50	27(73)	9(69.2)	1.1(0.3-3.2)	0.971
	>50	10(27)	3(23.1)	0.5(0.2-1.3)	0.107
Lifestyle	Sedentary	8(21.7)	2(15.4)	0.9(0.2-3.8)	0.884
	Not sedentary	29(85.3)	11(84.6)	1.1(0.3-4.5)	0.883
Substance use					
Cannabis	Yes	7(18.9)	7(53.8)	0.6(0.2-3.2)	0.499
	No	30(81.1)	6 (46.2)	0.9(0.2-7.5)	0.332
Drugs	Yes	37(100)	13(100)	5.0(0.2-255)	0.430
	No	0(0)	0(0)	0.95(0.1-10.1)	0.429
Sexual behaviour					
Type of sexual intercourse	Anal	37(100)	13(100)	0.6(0.1-0.5)	0.641
First sexual intercourse (age)	≤17	8(21.6)	2 (30.8)	1(0.2-10)	0.975
	>17	29 (78.4)	9(69.2)	2.1(0.4-8.3)	0.692
Partner last 6 months (n)	≤10	28(75.7)	9(69.2)	1(0.2-4.1)	0.693
	>10	9 (24.3)	4(30.8)	2.1(0.4-19)	0.524

[a] $p<0.05$ were considered as statistically significant

Table S7. Prevalence and Correlates Associated with HR and LR Anal HPV Infection

Parameters	**Class**	**HR-HPV N=17 n (%)**	**LR-HPV N=20 n (%)**	**OR-adjusted (CI 95%)**	**p-value[a]**
Occupation	Unemployed	1(5.9)	4(20)	3.0(0.7-12.7)	0.129
	Employed	16(94.1)	16(80)	1.0(0.4-2.8)	0.976
Lifestyle	Sedentary	2(11.8)	6(30)	1.7(0.6-4.4)	0.284
	Not sedentary	15(88.2)	14(70)	0.8(0.2-2.6)	0.631
Substance use					
Drugs	Yes	17(100)	20(100)	1.3(0.3-56)	0.965
	No	0(0)	0(0)	0.95(0.1-47)	0.967
Sexual behaviour					
Sexual identity	MSM	16(94.1)	17(85)	1.6(0.6-4.4)	0.328
	BISEX	1(5.9)	3(15)	1.0(0.12-6.8)	0.938
Type of sexual intercourse	Anal	17(100)	20(100)	1.7(0.6-6.6)	0.409
Partner last 6 months (n)	≤10	14(82.4)	13(65)	0.5(0.2-2)	0.294
	>10	3(17.6)	7(35)	2.5(0.5-15)	0.309

[a] $p<0.05$ were considered as statistically significant

Table S8. Prevalence and Correlates Associated with SIL and NO SIL during anal HPV Infection

Parameters	**Class**	**SIL N= 31 n (%)**	**NO SIL N= 19 n (%)**	**OR-adjusted (CI 95%)**	**p-value[a]**
Age	<25	0(0)	0(0)	2.2(0.8-56)	0.631
	25 to 50	16(51.6)	19(100)	0.9(0.4-2.1)	0.761
	>50	12(38.7)	0(0)	2.0(0.7-4.9)	0.171
Occupation	Unemployed	3(9.7)	2(10.5)	3.9(0.7-21.6)	0.116
	Employed	28(90.3)	17(89.5)	0.3(0.1-1.4)	0.115
Substance use					
Drugs	Yes	31(100)	19(100)	1.3(0.2-72)	0.870
	No	0(0)	0(0)	0.75(0.1-37.2)	0.871
Sexual behaviour					
Partner last 6 months (n)	≤10	22(71)	14(73.7)	4(1.3-12)	0.125
	>10	9(29)	5(26.3)	6.1(0.6-60)	0.124

[a] $p<0.05$ were considered as statistically significant

Table S9. Prevalence and Correlates Associated with persistent and cleared anal HPV Infection

Parameters	Class	Persistent HPV N= 20 n (%)	Cleared HPV N= 9 n (%)	OR-adjusted (CI 95%)	p-value[a]
Age	<25	1(5)	0(0)	0.29(0.1-20)	0.622
	25 to 50	10 (50)	5(55.6)	1.6(0.2-9.3)	0.582
	>50	9(45)	4(44.4)	0.6(0.1-3.2)	0.581
Education	Primary	5(25)	0(9)	1.3(0.1-24)	0.879
	Secondary	9(45)	5(25)	0.6(0.1-3.1)	0.528
	Tertiary	6(30)	4(44.4)	0.2(0.0-1.2)	0.065
Occupation	Unemployed	7(35)	0(0)	1.7(0.1-34.1)	0.705
	Employed	13(65)	9(100)	0.7(0.1-2.8)	0.566
Substance use					
Cannabis	Yes	2(10)	1 (11.1)	1.8(0.19-16)	0.606
	No	18(90)	8 (88.9)	5.7(0.4-70)	0.181
Smoking	Yes	4(44.4)	1(11.1)	1.8(0.2-16)	0.738
	No	16(55.6)	8(88.9)	0.6(0.1-5)	0.606
Drugs	Yes	20(100)	9(100)	2.7(0.2-150)	0.623
	No	0(0)	0(0)	0.4(0.1-20.1)	0.623
Sexual behaviour					
Sexual identity	MSM	17(85)	8(88.9)	0.2(0.1-1.5)	0.136
	BISEX	3(15)	1(11.1)	0.2(0.1-3)	0.280
Type of sexual intercourse	Anal	20(100)	9(43)	1.4(0.5-0.18)	0.211
First sexual intercourse (age)	≤17	5(25)	2(10)	0.6(0.1-8)	0.439
	>17	15(75)	7(90)	2.6(0.2-32)	0.506
Partner last 6 months (n)	≤10	17(85)	7(90)	1(0.2-10)	0.975
	>10	3(25)	2(10)	2.1(0.4-19)	0.524

[a] $p<0.05$ were considered as statistically significant

7. REFERENCES

- **Abramowitz L, Benabderrahmane D, Ravaud P, Walker F, Rioux C, Jestin C, et al.** Anal squamous intraepithelial lesions and condyloma in HIV-infected heterosexual men, homosexual men and women: prevalence and associated factors. *AIDS.* 2007 Jul 11;21(11):1457-65. doi: 10.1097/QAD.0b013e3281c61201.
- **Ahdieh-Grant L, Li R, Levine AM, Massad LS, Strickler HD, Minkoff H, et al.** Highly active antiretroviral therapy and cervical squamous intraepithelial lesions in human immunodeficiency virus-positive women. *J Natl Cancer Inst.* 2004 Jul 21;96(14):1070-6. doi: 10.1093/jnci/djh192.
- **Alter G, Altfeld M.** NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med*. 2009;265(1):29-42. doi:10.1111/j.1365-2796.2008.02045.x
- **Amador-Molina A, Hernández-Valencia JF, Lamoyi E, Contreras-Paredes A, Lizano M.** Role of innate immunity against human papillomavirus (HPV) infections and effect of adjuvants in promoting specific immune response. *Viruses.* 2013 Oct 28;5(11):2624-42. doi: 10.3390/v5112624.
- **Androphy EJ, Hubbert NL, Schiller JT, Lowy DR.** Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. *EMBO J.* 1987 Apr;6(4):989-92.
- **Arbyn M, de Sanjosé S, Saraiya M, Sideri M, Palefsky J, Lacey C, et al.** EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. *Int J Cancer.* 2012 Nov 1;131(9):1969-82. doi: 10.1002/ijc.27650.
- **Au-Yeung N, Horvath CM.** Transcriptional and chromatin regulation in interferon and innate antiviral gene expression. *Cytokine Growth Factor Rev.* 2018 Dec;44:11-17. doi: 10.1016/j.cytogfr.2018.10.003.

- **Audirac-Chalifour A, Torres-Poveda K, Bahena-Román M, Téllez-Sosa J, Martínez-Barnetche J, Cortina-Ceballos B, et al.** Cervical Microbiome and Cytokine Profile at Various Stages of Cervical Cancer: A Pilot Study. *PLoS One.* 2016 Apr 26;11(4):e0153274. doi: 10.1371/journal.pone.0153274.
- **Aziz N, Bonavida B.** Activation of Natural Killer Cells by Probiotics. *For Immunopathol Dis Therap.* 2016;7(1-2):41-55. doi: 10.1615/ForumImmunDisTher.2016017095.
- **Bachmann A, Hanke B, Zawatzky R, Soto U, van Riggelen J, zur Hausen H, et al.** Disturbance of tumor necrosis factor alpha-mediated beta interferon signaling in cervical carcinoma cells. *J Virol.* 2002 Jan;76(1):280-91. doi: 10.1128/jvi.76.1.280-291.2002.
- **Bald T, Krummel MF, Smyth MJ, Barry KC.** The NK cell-cancer cycle: advances and new challenges in NK cell-based immunotherapies. *Nat Immunol.* 2020 Aug;21(8):835-847. doi: 10.1038/s41590-020-0728-z.
- **Barbosa MS, Schlegel R.** The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. *Oncogene.* 1989 Dec;4(12):1529-32.
- **Barnard P, McMillan NA.** The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. *Virology.* 1999 Jul 5;259(2):305-13. doi: 10.1006/viro.1999.9771.
- **Barnard P, Payne E, McMillan NA.** The human papillomavirus E7 protein is able to inhibit the antiviral and anti-growth functions of interferon-alpha. *Virology* 2000; 277(2): 411-9.
- **Barrow AD, Martin CJ, Colonna M.** The Natural Cytotoxicity Receptors in Health and Disease. *Front Immunol*. 2019;10:909. doi:10.3389/fimmu.2019.00909
- **Bartlett NW, Buttigieg K, Kotenko SV, Smith GL.** Murine interferon lambdas (type III interferons) exhibit potent antiviral activity in vivo in a poxvirus infection model. *J Gen Virol.* 2005 Jun;86(Pt 6):1589-1596. doi: 10.1099/vir.0.80904-0.

- **Becker KA, Florin L, Sapp C, Maul GG, Sapp M.** Nuclear localization but not PML protein is required for incorporation of the papillomavirus minor capsid protein L2 into virus-like particles. *J Virol.* 2004 Feb;78(3):1121-8. doi: 10.1128/jvi.78.3.1121-1128.2004.
- **Beglin M, Melar-New M, Laimins L.** Human papillomaviruses and the interferon response. *J Interferon Cytokine Res.* 2009 Sep;29(9):629-35. doi: 10.1089/jir.2009.0075.
- **Bennetts LE, Wagner M, Giuliano AR, Palefsky JM, Steben M, Weiss TW.** Associations of Anogenital Low-Risk Human Papillomavirus Infection With Cancer and Acquisition of HIV. *Sex Transm Dis.* 2015 Oct;42(10):541-4. doi: 10.1097/OLQ.0000000000000319.
- **Bere A, Tayib S, Kriek JM, Masson L, Jaumdally SZ, Barnabas SL, et al.** Altered phenotype and function of NK cells infiltrating human papillomavirus (HPV)-associated genital warts during HIV infection. *Clin Immunol.* 2014 Feb;150(2):210-9. doi: 10.1016/j.clim.2013.12.005.
- **Bergvall M, Melendy T, Archambault J.** The E1 proteins. *Virology.* 2013 Oct;445(1-2):35-56. doi: 10.1016/j.virol.2013.07.020.
- **Bernard HU, Calleja-Macias IE, Dunn ST.** Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer.* 2006 Mar 1;118(5):1071-6. doi: 10.1002/ijc.21655
- **Berti FCB, Lombardi Pereira AP, Martelossi Cebinelli GC, Trugilo KP, de Oliveira KB.** The role of interleukin 10 in human papilloma virus infection and progression to cervical carcinoma. *Cytokine Growth Factor Rev.* 2017;34:1-13. doi: 10.1016/j.cytogfr.2017.03.002.
- **Bertisch B, Franceschi S, Lise M, Vernazza P, Keiser O, Schöni-Affolter F et al.** Risk factors for anal cancer in persons infected with HIV: a nested case-control study in the Swiss HIV Cohort Study. *Am J Epidemiol.* 2013 Sep 15; 178(6):877-84
- **Bekisz J, Sato Y, Johnson C, Husain SR, Puri RK, Zoon KC.** Immunomodulatory effects of interferons in malignancies. *J Interferon Cytokine Res.* 2013 Apr;33(4):154-61. doi: 10.1089/jir.2012.0167.

- **Björkström NK, Ljunggren HG, Michaëlsson J.** Emerging insights into natural killer cells in human peripheral tissues. *Nat Rev Immunol.* 2016 Apr 28;16(5):310-20. doi: 10.1038/nri.2016.34.
- **Blasius AL, Beutler B.** Intracellular toll-like receptors. *Immunity*. 2010 Mar 26;32(3):305-15. doi: 10.1016/j.immuni.2010.03.012.
- **Boasso A.** Type I Interferon at the Interface of Antiviral Immunity and Immune Regulation: The Curious Case of HIV-1. *Scientifica (Cairo)*.2013;2013:580968. doi: 10.1155/2013/580968.
- **Bodily J, Laimins LA.** Persistence of human papillomavirus infection: keys to malignant progression. *Trends Microbiol.* 2011 Jan;19(1):33-9. doi: 10.1016/j.tim.2010.10.002.
- **Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV.** The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol*. 2002 Apr;55(4):244-65. doi: 10.1136/jcp.55.4.244.
- **Bosch FX, Broker TR, Forman D, Moscicki AB, Gillison ML, Doorbar J et al.** authors of ICO Monograph Comprehensive Control of HPV Infections and Related Diseases Vaccine Volume 30, Supplement 5, 2012. Comprehensive control of human papillomavirus infections and related diseases. *Vaccine.* 2013 Dec 31;31 Suppl 7(Suppl 7):H1-31. doi: 10.1016/j.vaccine.2013.10.003.
- **Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S et al.** Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med.* 2006 Dec;12(12):1365-71.
- **Buck CB, Thompson CD, Pang YY, Lowy DR, Schiller JT.** Maturation of papillomavirus capsids. *J Virol.* 2005 Mar;79(5):2839-46. doi: 10.1128/JVI.79.5.2839-2846.2005.
- **Burd EM.** Human Papillomavirus and Cervical Cancer. *Clin Microbiol Rev.* 2003 Jan; 16(1): 1–17. doi: 10.1128/CMR.16.1.1-17.2003

- **Burgos J, Curran A, Tallada N, Guelar A, Navarro J, Landolfi S et al.** Risk of progression to high-grade anal intraepithelial neoplasia in HIV-infected MSM. *AIDS.* 2015 Mar 27;29(6):695-702. doi: 10.1097/QAD.0000000000000603.
- **Caberg JH, Hubert PM, Begon DY, Herfs MF, Roncarati PJ, Boniver JJ et al.** Silencing of E7 oncogene restores functional E-cadherin expression in human papillomavirus 16-transformed keratinocytes. *Carcinogenesis.* 2008 Jul;29(7):1441-7. doi: 10.1093/carcin/bgn145.
- **Campbell KS, Hasegawa J.** Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol.* 2013 Sep;132(3):536-544. doi: 10.1016/j.jaci.2013.07.006.
- **Cannella F, Scagnolari C, Selvaggi C, Stentella P, Recine N, Antonelli G et al.** Interferon lambda 1 expression in cervical cells differs between low-risk and high-risk human papillomavirus-positive women. *Med Microbiol Immunol.* 2014 Jun;203(3):177-84. doi: 10.1007/s00430-014-0330-9.
- **Capobianchi MR, Uleri E, Caglioti C, Dolei A.** Type I IFN family members: similarity, differences and interaction. *Cytokine Growth Factor Rev*. 2015 Apr;26(2):103-11. doi: 10.1016/j.cytogfr.2014.10.011.
- **Carter GM, Esmaeili A, Shah H, Indyk D, Johnson M, Andreae M et al.** Probiotics in Human Immunodeficiency Virus Infection: A Systematic Review and Evidence Synthesis of Benefits and Risks. *Open Forum Infect Dis.* 2016 Jul 29;3(4):ofw164. doi: 10.1093/ofid/ofw164.
- **Ceccarelli G, Cavallari EN, Savinelli S, Bianchi L, Pierangeli A, Vullo F, et al.** Clearance of human papillomavirus related anal condylomas after oral and endorectal multistrain probiotic supplementation in an HIV positive male: A case report. *Medicine (Baltimore).* 2018 Apr;97(16):e0329. doi: 10.1097/MD.0000000000010329.

- **Chang YE, Laimins LA.** Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. *J Virol.* 2000 May; 74(9):4174-82.
- **Chaturvedi AK, Madeleine MM, Biggar RJ, Engels EA.** Risk of human papillomavirus-associated cancers among persons with AIDS. *J Natl Cancer Inst*. 2009 Aug 19;101(16):1120-30. doi: 10.1093/jnci/djp205.
- **Chelimo C, Wouldes TA, Cameron LD, Elwood JM.** Risk factors for and prevention of human papillomaviruses (HPV), genital warts and cervical cancer. *J Infect.* 2013 Mar; 66(3):207-17
- **Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC.** Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol Cell.* 2000 Mar;5(3):557-67. doi: 10.1016/s1097-2765(00)80449-9.
- **Cheng M, Chen Y, Xiao W, Sun R, Tian Z.** NK cell-based immunotherapy for malignant diseases. *Cell Mol Immunol.* 2013;10:230–52
- **Chiang B, Sheih Y, Wang L, Liao C, Gill H.** Enhancing immunity by dietary consumption of a probiotic lactic acid bacteria (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *Eur J Clin Nutr.* 2000;54:849–55.
- **Chiao EY, Giordano TP, Palefsky JM, Tyring S, El Serag H.** Screening HIV infected individuals for anal cancer precursor lesions: a systematic review. *Clin Infect Dis* 2006;43(2):223–33.
- **Cho HW, So KA, Lee JK, Hong JH.** Type-specific persistence or regression of human papillomavirus genotypes in women with cervical intraepithelial neoplasia 1: A prospective cohort study. *Obstet Gynecol Sci.* 2015 Jan;58(1):40-5. doi: 10.5468/ogs.2015.58.1.40.
- **Christensen ND.** HPV disease transmission protection and control. *Microb Cell.* 2016 Sep 5; 3(9): 476–490. doi: 10.15698/mic2016.09.530

- **Coggin JR and zur Hausen H.** Workshop on papillomaviruses and cancer. *Cancer Res.* 1979.39, 545–46.
- **Coghill AE, Shiels MS, Rycroft RK, Copeland G, Finch JL, Hakenewerth AM et al.** Rectal squamous cell carcinoma in immunosuppressed populations: is this a distinct entity from anal cancer? *AIDS.* 2016 Jan 2;30(1):105-12. doi: 10.1097/QAD.0000000000000873.
- **Collett D, Mumford L, Banner NR, Neuberger J, Watson C.** Comparison of the incidence of malignancy in recipients of different types of organ: a UK Registry audit. *Am J Transplant* 2010;10(8):1889–96.
- **Colmenares V, Noyola DE, Monsiváis-Urenda A, Salgado-Bustamante M, Estrada-Capetillo L, González-Amaro R et al.** Human papillomavirus immunization is associated with increased expression of different innate immune regulatory receptors. *Clin Vaccine Immunol.* 2012 Jul;19(7):1005-11. doi: 10.1128/CVI.00043-12.
- **Colón-López V, Shiels MS, Machin M, Ortiz AP, Strickler H, Castle PE et al.** Anal Cancer Risk Among People With HIV Infection in the United States. *J Clin Oncol.* 2018 Jan 1;36(1):68-75. doi: 10.1200/JCO.2017.74.9291.
- **Conesa-Zamora P.** Immune responses against virus and tumor in cervical carcinogenesis: Treatment strategies for avoiding the HPV-induced immune escape. *Gyn Onc.* 2013; 131: 480-488.
- **Cooper MA, Fehniger TA, Caligiuri MA.** The biology of human natural killer-cell subsets. *Trends Immunol.* 2001 Nov;22(11):633-40. doi: 10.1016/s1471-4906(01)02060-9.
- Coscia MF, Monno R, Ballini A, Mirgaldi R, Dipalma G, Pettini F et al. Human papilloma virus (HPV) genotypes prevalence in a region of South Italy (Apulia). *Ann Ist Super Sanita*, 2015;51(3):248-51. doi: 10.4415/ANN_15_03_14.
- **Crakes KR, Jiang G.** Gut Microbiome Alterations During HIV/SIV Infection: Implications for HIV Cure. *Front Microbiol*. 2019 May 22;10:1104. doi: 10.3389/fmicb.2019.01104.

- **Crome SQ, Lang PA, Lang KS, Ohashi PS.** Natural killer cells regulate diverse T cell responses. *Trends Immunol.* 2013 Jul;34(7):342-9. doi: 10.1016/j.it.2013.03.002. Epub 2013 Apr 17. PMID: 23601842.
- **D'Souza G, Wiley DJ, Li X, Chmiel JS, Margolick JB, Cranston RD et al.** Incidence and epidemiology of anal cancer in the multicenter AIDS cohort study. *J Acquir Immune Defic Syndr.* 2008 Aug 1;48(4):491-9. doi: 10.1097/QAI.0b013e31817aebfe.
- **Daling JR, Weiss NS, Hislop TG, Maden C, Coates RJ, Sherman KJ et al.** Sexual practices, sexually transmitted diseases, and the incidence of anal cancer. *N Engl J Med.* 1987 Oct 15;317(16):973-7. doi: 10.1056/NEJM198710153171601.
- **Darragh TM, Birdsong GG, Luff RD, Davey DD.** Anal-rectal cytology. In: Solomon D, Nayar R, eds. The Bethesda System for Reporting Cervical Cytology: Definitions, Criteria and Explanatory Notes (2nd ed.). New York, NY: *Springer*, 2004:169–75.
- **Darragh TMBJ, Jay N, Palefsky J.** The Anal Canal and Perianus: HPV-Related Disease. American Society for Colposcopy and Cervical Pathology (Author), E. J. Mayeaux Jr. (Editor), J. Thomas Cox MD (Editor) 2011. Modern Colposcopy Textbook and Atlas; Third Edition.
- **Darragh TM, Winkler B.** Anal cancer and cervical cancer screening: key differences. *Cancer Cytopathology*. 2011;119(1):5–19.
- **Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, et al.** The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Arch Pathol Lab Med* . 2012 Oct;136(10):1266-97. doi: 10.5858/arpa.LGT200570.
- **Darwich L, Cañadas MP, Videla S, Coll J, Molina-López RA, Sirera G et al.** Prevalence, clearance, and incidence of human papillomavirus type-specific infection at the anal and

penile site of HIV-infected men. *Sex Transm Dis.* 2013 Aug;40(8):611-8. doi: 10.1097/01.OLQ.0000430798.61475.08.

- **Davis TW, Goldstone SE, Chen G.** Tolerability of anal dysplasia screening. *J Low Genit Tract Dis.* 2013;17(4):404–408
- **Day PM, Baker CC, Lowy DR, Schiller JT.** Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci U S A*. 2004 Sep 28;101(39):14252-7. doi: 10.1073/pnas.0404229101.
- **Desaintes C., Demeret C**. Control of papillomavirus DNA replication and transcription. *Semin. Cancer Biol.* 1996;7:339–347.
- **d'Ettorre G, Paiardini M, Ceccarelli G, Silvestri G, Vullo V.** HIV-associated immune activation: from bench to bedside. *AIDS Res Hum Retroviruses.* 2011 Apr;27(4):355-64. doi: 10.1089/aid.2010.0342.
- **d'Ettorre G, Rossi G, Scagnolari C, Andreotti M, Giustini N, Serafino S et al.** Probiotic supplementation promotes a reduction in T-cell activation, an increase in Th17 frequencies, and a recovery of intestinal epithelium integrity and mitochondrial morphology in ART-treated HIV-1-positive patients. *Immun Inflamm Dis.* 2017 Sep;5(3):244-260. doi: 10.1002/iid3.160.
- **de Gruijl TD, Bontkes HJ, van den Muysenberg AJ, van Oostveen JW, Stukart MJ, Verheijen RH et al.** Differences in cytokine mRNA profiles between premalignant and malignant lesions of the uterine cervix. *Eur J Cancer.* 1999 Mar;35(3):490-7. doi: 10.1016/s0959-8049(98)00371-2.

- **De Maria A, Fogli M, Costa P, Murdaca G, Puppo F, Mavilio D, Moretta A, Moretta L.** The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a

reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol*. 2003 Sep;33(9):2410-8. doi: 10.1002/eji.200324141.

- **de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H.** Classification of papillomaviruses. *Virology.* 2004;324(1):17–27
- **Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH.** Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol.* 2000 Aug;1(2):119-26. doi: 10.1038/77793.
- **Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK et al.** An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunol.* 2014 Jul;7(4):983-94. doi: 10.1038/mi.2013.116.
- **Donà MG, Palamara G, Di Carlo A, Latini A, Vocaturo A, Benevolo M et al.** Prevalence, genotype diversity and determinants of anal HPV infection in HIV-uninfected men having sex with men. *J Clin Virol.* 2012 Jun;54(2):185-9. doi: 10.1016/j.jcv.2012.02.014.
- **Dong H, Rowland I, Yaqoob P.** Comparative effects of six probiotic strains on immune function in vitro. *Br J Nutr.* 2012 Aug;108(3):459-70. doi: 10.1017/S0007114511005824.
- **Donnelly N, Gorman AM, Gupta S, Samali A.** The eIF2α kinases: their structures and functions. *Cell Mol Life Sci*. 2013 Oct; 70(19):3493-511.
- **Donnelly RP, Kotenko SV.** Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res.* 2010 Aug;30(8):555-64. doi: 10.1089/jir.2010.0078.
- **Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR et al.** The biology and life-cycle of human papillomaviruses. *Vaccine.* 2012 Nov 20;30 Suppl 5:F55-70. doi: 10.1016/j.vaccine.2012.06.083.
- **Dornan D, Eckert M, Wallace M, Shimizu H, Ramsay E, Hupp TR et al.** Interferon regulatory factor 1 binding to p300 stimulates DNA-dependent acetylation of p53. *Mol Cell Biol.* 2004 Nov;24(22):10083-98. doi: 10.1128/MCB.24.22.10083-10098.2004.

- **Durbin RK, Kotenko SV, Durbin JE.** Interferon induction and function at the mucosal surface. *Immunol Rev.* 2013 Sep;255(1):25-39. doi: 10.1111/imr.12101.
- **Dyson N, Howley PM, Munger K, Harlow E.** The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science.* 1989;243:934–937.
- **Duensing S, Münger K.** Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *Int J Cancer.* 2004 Mar 20; 109(2):157-62.
- **Egawa N, Egawa K, Griffin H, Doorbar J.** Human papillomaviruses; epithelial tropisms, and the development of neoplasia. *Viruses.* 2015; 7:3863–90. doi: 10.3390/v7072802
- **Egli A, Santer DM, O'Shea D, Tyrrell DL, Houghton M.** The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. *Emerg Microbes Infect.* 2014 Jul;3(7):e51. doi: 10.1038/emi.2014.51.
- **Einstein MH, Schiller JT, Viscidi RP, Strickler HD, Coursaget P, Tan T et al.** Clinician's guide to human papillomavirus immunology: knowns and unknowns. *Lancet Infect Dis.* 2009 Jun;9(6):347-56. doi: 10.1016/S1473-3099(09)70108-2.
- **Elinav E, Strowig T, Henao-Mejia J, Flavell RA.** Regulation of the antimicrobial response by NLR proteins. *Immunity.* 2011 May 27;34(5):665-79. doi: 10.1016/j.immuni.2011.05.007.
- **Favre D, Mold J, Hunt PW, Kanwar B, Loke P, Seu L, et al.** Tryptophan catabolism by indoleamine 2, 3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med* 2010 May 19;2(32):32ra36. doi: 10.1126/scitranslmed.3000632.
- **Fehrmann F, Klumpp DJ, and Laimins LA.** Human Papillomavirus Type 31 E5 Protein Supports Cell Cycle Progression and Activates Late Viral Functions upon Epithelial Differentiation. *J Virol.* 2003 Mar; 77(5): 2819–2831. doi: 10.1128/JVI.77.5.2819-2831.2003
- **Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD.** The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr Biol.* 2007 Jul 3;17(13):1140-5. doi: 10.1016/j.cub.2007.05.074.

- **Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM.** Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127(12):2893–917
- **Finnen RL, Erickson KD, Chen XS, Garcea RL.** Interactions between papillomavirus L1 and L2 capsid proteins. *J Virol.* 2003 Apr;77(8):4818-26. doi: 10.1128/jvi.77.8.4818-4826.2003.
- **Flores ER, Allen-Hoffmann BL, Lee D, Lambert PF.** The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. *J Virol.* 2000 Jul;74(14):6622-31. doi: 10.1128/jvi.74.14.6622-6631.2000.
- **Flórez-Álvarez L, Hernandez JC, Zapata W.** NK Cells in HIV-1 Infection: From Basic Science to Vaccine Strategies. *Front Immunol.* 2018 Oct 17;9:2290. doi: 10.3389/fimmu.2018.02290.
- **Frazer IH.** Interaction of human papillomaviruses with the host immune system: a well evolved relationship. *Virology.* 2009 Feb 20;384(2):410-4. doi: 10.1016/j.virol.2008.10.004.
- **Frazer IH, Cox JT, Mayeaux EJ Jr, Franco EL, Moscicki AB, Palefsky JM et al.** Advances in prevention of cervical cancer and other human papillomavirus-related diseases. *Pediatr Infect Dis J.* 2006 Feb;25(2 Suppl):S65-81, quiz S82. doi: 10.1097/01.inf.0000196485.86376.46.
- **Gad HH, Dellgren C, Hamming OJ, Vends S, Paludan SR, Hartmann R.** Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family. *J Biol Chem.* 2009 Jul 31;284(31):20869-75. doi: 10.1074/jbc.M109.002923.
- **Gadducci A, Guerrieri ME, Greco C.** Tissue biomarkers as prognostic variables of cervical cancer. *Crit Rev Oncol Hematol.* 2013 May;86(2):104-29. doi: 10.1016/j.critrevonc.2012.09.003.
- **Gameiro SF, Zhang A, Ghasemi F, Barrett JW, Nichols AC, and Mymryk JS.** Analysis of Class I Major Histocompatibility Complex Gene Transcription in Human Tumors Caused by Human Papillomavirus Infection. *Viruses.* 2017 Sep; 9(9): 252. doi: 10.3390/v9090252

- **Gammoh N, Grm HS, Massimi P, Banks L.** Regulation of human papillomavirus type 16 E7 activity through direct protein interaction with the E2 transcriptional activator. *J Virol*. 2006;80(4):1787-1797. doi:10.1128/JVI.80.4.1787-1797.2006
- **Gao L, Zhou F, Li X, Yang Y, Ruan Y, Jin Q.** Anal HPV infection in HIV-positive men who have sex with men from China. *PLoS One.* 2010; 5(12):e15256. pmid:21151900;
- **Garbuglia AR, Gentile M, Del Nonno F, Lorenzini P, Lapa D, Lupi F et al.** An anal cancer screening program for MSM in Italy: Prevalence of multiple HPV types and vaccine-targeted infections. *J Clin Virol.* 2015 Nov;72:49-54. doi: 10.1016/j.jcv.2015.09.001.
- **Garcia-Iglesias T, Del Toro-Arreola A, Albarran-Somoza B, Del Toro-Arreola S, Sanchez-Hernandez PE, Ramirez-Dueñas MG et al.** Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions. *BMC Cancer*. 2009 Jun 16;9:186. doi: 10.1186/1471-2407-9-186.
- **Garcia- Piñeres AJ, Hildesheim A, Trivett M, Williams M, Wu L, Kewalramani VN et al.** Role of DC-SIGN in the activation of dendritic cells by HPV-16 Ll virus-like particle vaccine. *Eur J Immunol* 2006 Feb;36(2):437-45.doi: 10.1002/eji.200535068.
- **Garrett WS.** Cancer and the microbiota. *Science* 2015; 348:80–86;
- **Gasteiger G, Rudensky AY.** Interactions between innate and adaptive lymphocytes. *Nat Rev Immunol.* 2014 Sep;14(9):631-9. doi: 10.1038/nri3726.
- **Geskus RB, González C, Torres M, Del Romero J, Viciana P, Masiá M et al.** Incidence and clearance of anal high-risk human papillomavirus in HIV-positive men who have sex with men: estimates and risk factors. *AIDS.* 2016 Jan 2;30(1):37-44. doi: 10.1097/QAD.0000000000000874.
- **Gey A, Kumari P, Sambandam A, Lecuru F, Cassard L, Badoual C et al.** Identification and characterisation of a group of cervical carcinoma patients with profound downregulation of intratumoral Type 1 (IFNgamma) and Type 2 (IL-4) cytokine mRNA expression. Eur J Cancer. 2003 Mar;39(5):595-603. doi: 10.1016/s0959-8049(02)00839-0.

- **Gheit T.** Mucosal and Cutaneous Human Papillomavirus Infections and Cancer Biology. *Front Oncol*. 2019;9:355. doi:10.3389/fonc.2019.00355
- **Gill H, Rutherford K, Cross M, Gopal P.** Enhancement of immunity in the elderly by dietary supplementation with the probiotic Bifidobacterium lactis HN019. *Am J Clin Nutr.* 2001;74:833–9;
- **Goldschmidt MH, Kennedy JS, Kennedy DR, Yuan H, Holt DE, Casal ML et al.** Severe papillomavirus infection progressing to metastatic squamous cell carcinoma in bone marrow-transplanted X-linked SCID dogs. *J Virol.* 2006 Jul;80(13):6621-8. doi: 10.1128/JVI.02571-05.
- **Goldstone SE.** High-resolution anoscopy is a crucial component of anal dysplasia screening. *Dis Colon Rectum* 2010;53:364-5. DOI: 10.1007/DCR.0b013e3181c3b54b
- **Goldstone S, Palefsky JM, Giuliano AR, Moreira ED Jr, Aranda C, Jessen H, et al.** Prevalence of and risk factors for human papillomavirus (HPV) infection among HIV-seronegative men who have sex with men. *J Infect Dis*. 2011; 203(1):66–74. pmid:21148498
- **Gonzalez VD, Landay AL, Sandberg JK.** Innate immunity and chronic immune activation in HCV/HIV-1 co-infection. *Clin Immunol.* 2010 Apr;135(1):12-25. doi: 10.1016/j.clim.2009.12.005.
- **Goodier MR, Imami N, Moyle G, Gazzard B, Gotch F.** Loss of the CD56hiCD16- NK cell subset and NK cell interferon-gamma production during antiretroviral therapy for HIV-1: partial recovery by human growth hormone. *Clin Exp Immunol.* 2003 Dec;134(3):470-6. doi: 10.1111/j.1365-2249.2003.02329.x.
- **Grabowska AK, Riemer AB.** The invisible enemy - how human papillomaviruses avoid recognition and clearance by the host immune system. *Open Virol J*. 2012;6:249-56. doi: 10.2174/1874357901206010249.

- **Gras Navarro A, Björklund AT, Chekenya M.** Therapeutic potential and challenges of natural killer cells in treatment of solid tumors. *Front Immunol.* 2015 Apr 29;6:202. doi: 10.3389/fimmu.2015.00202.
- **Gui Q, Wang A, Zhao X, Huang S, Tan Z, Xiao C et al.** Effects of probiotic supplementation on natural killer cell function in healthy elderly individuals: a meta-analysis of randomized controlled trials. *Eur J Clin Nutr*. 2020 Dec;74(12):1630-1637. doi: 10.1038/s41430-020-0670-z.
- **Hamid NA, Brown C, Gaston K.** The regulation of cell proliferation by the papillomavirus early proteins. *Cell Mol Life Sci.* 2009 May;66(10):1700-17. doi: 10.1007/s00018-009-8631-7.
- **Harper KN.** HIV-altered gut microbiome may be driving disease progression. *AIDS.* 2017 Jan 14;31(2):N1. doi: 10.1097/QAD.0000000000001295
- **Harro CD, Pang YY, Roden RB, Hildesheim A, Wang Z, Reynolds MJ et al.** Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J Natl Cancer Inst.* 2001 Feb 21; 93(4):284-92. doi: 10.1093/jnci/93.4.284.
- **Hasan UA, Bates E, Takeshita F, Biliato A, Accardi R, Bouvard V et al.** TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J Immunol.* 2007 Mar 1;178(5):3186-97. doi: 10.4049/jimmunol.178.5.3186.
- **Hasan UA, Zannetti C, Parroche P, Goutagny N, Malfroy M, Roblot G et al.** The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the Toll-like receptor 9 promoter. *J Exp Med*. 2013 Jul 1;210(7):1369-87. doi: 10.1084/jem.20122394.
- **Hasanzadeh M, Rejali M, Mehramiz M, Akbari M, Mousavi Seresht L, Yazdandoost Y et al.** The interaction of high and low-risk human papillomavirus genotypes increases the risk of developing genital warts: A population-based cohort study. *J Cell Biochem*. 2019 Aug;120(8):12870-12874. doi: 10.1002/jcb.28557.

- **Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT.** HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* 1989 Dec 1;8(12):3905-10.
- **Heard I, Schmitz V, Costagliola D, Orth G, Kazatchkine MD.** Early regression of cervical lesions in HIV-seropositive women receiving highly active antiretroviral therapy. *AIDS.* 1998 Aug 20;12(12):1459-64. doi: 10.1097/00002030-199812000-00007.
- **Heard I, Tassie JM, Kazatchkine MD, Orth G**. Highly active antiretroviral therapy enhances regression of cervical intraepithelial neoplasia in HIV-seropositive women. *AIDS.* 2002 Sep 6;16(13):1799-802. doi: 10.1097/00002030-200209060-00013.
- **Hearps AC, Maisa A, Cheng WJ, Angelovich TA, Lichtfuss GF, Palmer CS et al.** HIV infection induces age-related changes to monocytes and innate immune activation in young men that persist despite combination antiretroviral therapy. *AIDS.* 2012 Apr 24;26(7):843-53. doi: 10.1097/QAD.0b013e328351f756.
- **Hebner CM, Laimins LA**. Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. *Rev Med Virol.* 2006 Mar-Apr;16(2):83-97. doi: 10.1002/rmv.488.
- **Hebner C, Beglin M, Laimins LA.** Human papillomavirus E6 proteins mediate resistance to interferon-induced growth arrest through inhibition of p53 acetylation. *J Virol.* 2007 Dec;81(23):12740-7. doi: 10.1128/JVI.00987-07
- **Herberman RB, Holden HT.** Natural cell-mediated immunity. *Adv Cancer Res.* 1978;27:305-77. doi: 10.1016/s0065-230x(08)60936-7.
- **Hernandez AL, Efird JT, Holly EA, Berry JM, Jay N, Palefsky JM.** Incidence of and risk factors for type-specific anal human papillomavirus infection among HIV-positive MSM. *AIDS.* 2014 Jun 1;28(9):1341-9. doi: 10.1097/QAD.0000000000000254.
- **Hernández-Ramírez RU, Shiels MS, Dubrow R, Engels EA.** Cancer risk in HIV-infected people in the USA from 1996 to 2012: a population-based, registry-linkage study. *Lancet HIV.* 2017 Nov;4(11):e495-e504. doi: 10.1016/S2352-3018(17)30125-X.

- **Hertzog P, Forster S, Samarajiwa S.** Systems biology of interferon responses. *J Interferon Cytokine Res.* 2011 Jan;31(1):5-11. doi: 10.1089/jir.2010.0126.
- **Holder KA, Lajoie J, Grant MD.** Natural Killer Cells Adapt to Cytomegalovirus Along a Functionally Static Phenotypic Spectrum in Human Immunodeficiency Virus Infection. *Front Immunol.* 2018 Nov 12;9:2494. doi: 10.3389/fimmu.2018.02494.
- **Hong HS, Eberhard JM, Keudel P, Bollmann BA, Ballmaier M, Bhatnagar N et al.** HIV infection is associated with a preferential decline in less-differentiated CD56dim CD16+ NK cells. *J Virol.* 2010 Jan;84(2):1183-8. doi: 10.1128/JVI.01675-09.
- **Hong S, Mehta KP, Laimins LA.** Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. *J Virol.* 2011 Sep;85(18):9486-94. doi: 10.1128/JVI.05007-11.
- **Houlihan CF, Larke NL, Watson-Jones D, Smith-McCune KK, Shiboski S, Gravitt PE et al.** HPV infection and increased risk of HIV acquisition. A systematic review and meta-analysis. *AIDS.* 2012 November 13; 26(17):doi:10.1097/QAD.0b013e328358d908
- **Hubert WG, Laimins LA.** Human papillomavirus type 31 replication modes during the early phases of the viral life cycle depend on transcriptional and posttranscriptional regulation of E1 and E2 expression. *J Virol.* 2002 Mar;76(5):2263-73. doi: 10.1128/jvi.76.5.2263-2273.2002.
- **Hubert P, Caberg JH, Gilles C, Bousarghin L, Franzen-Detrooz E, Boniver J et al.** E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J Pathol.* 2005 Jul;206(3):346-55. doi: 10.1002/path.1771.
- **Hughes FJ, Romanos MA.** E1 protein of human papillomavirus is a DNA helicase/ATPase. *Nucleic Acids Res.* 1993 Dec 25;21(25):5817-23. doi: 10.1093/nar/21.25.5817.

- **IARC** Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 90, IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon (FR): International Agency for Research on Cancer; 2007
- **IARC** Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100B, 2011
- **Ishov AM, Maul GG.** The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J Cell Biol.* 1996 Aug;134(4):815-26. doi: 10.1083/jcb.134.4.815.
- **Ismail A, Yusuf N.** Type I interferons: key players in normal skin and select cutaneous malignancies. *Dermatol Res Pract*. 2014;2014:847545. doi: 10.1155/2014/847545.
- **Isolauri E, Salminen S, Ouwehand AC.** Microbial-gut interactions in health and disease. Probiotics. *Best Pract Res Clin Gastroenterol.* 2004 Apr;18(2):299-313. doi: 10.1016/j.bpg.2003.10.006.
- **Ivashkiv LB, and Donlin LT.** Regulation of type I interferon responses. *Nat Rev Immunol.* 2014 Jan; 14(1): 36–49. doi: 10.1038/nri3581
- **Jaitin DA, Roisman LC, Jaks E, Gavutis M, Piehler J, Van der Heyden J, Uze G, Schreiber G.** Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. *Mol Cell Biol*. 2006 Mar;26(5):1888-97. doi: 10.1128/MCB.26.5.1888-1897.2006.
- **Kalliolias GD, Ivashkiv LB.** Overview of the biology of type I interferons. *Arthritis Res Ther.* 2010;12 Suppl 1(Suppl 1):S1. doi: 10.1186/ar2881.
- **Karre K.** Natural killer cell recognition of missing self. *Nat Immunol.* 2008 May;9(5):477-80. doi: 10.1038/ni0508-477.
- **Karstensen B, Poppelreuther S, Bonin M, Walter M, Iftner T, Stubenrauch F.** Gene expression profiles reveal an upregulation of E2F and downregulation of interferon targets by HPV18 but no changes between keratinocytes with integrated or episomal viral genomes. *Virology.* 2006 Sep 15;353(1):200-9. doi: 10.1016/j.virol.2006.05.030.

- **Kim JJ.** Targeted human papillomavirus vaccination of men who have sex with men in the USA: a cost-effectiveness modelling analysis. *Lancet Infect Dis*. 2010 Dec;10(12):845-52. doi: 10.1016/S1473-3099(10)70219-X.
- **Klingelhutz AJ, Roman A.** Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. *Virology.* 2012 Mar 15;424(2):77-98. doi: 10.1016/j.virol.2011.12.018.
- **Kost BP, Hofmann J, Stoellnberger S, Bergauer F, Blankenstein T, Alba-Alejandre I et al.** Prevalence of human papillomavirus infection of the anal canal in women: A prospective analysis of high-risk populations. *Oncol Lett.* 2017 Apr;13(4):2495-2501. doi: 10.3892/ol.2017.5714.
- **Kotenko SV, Durbin JE.** Contribution of type III interferons to antiviral immunity: location, location, location. *J Biol Chem*. 2017 May 5;292(18):7295-7303. doi: 10.1074/jbc.R117.777102.
- **Kroon SJ, Ravel J, Huston WM.** Cervicovaginal microbiota, women's health, and reproductive outcomes. *Fertil Steril.* 2018 Aug;110(3):327-336. doi: 10.1016/j.fertnstert.2018.06.036.
- **Lace MJ, Anson JR, Klingelhutz AJ, Harada H, Taniguchi T, Bossler AD et al.** Interferon-beta treatment increases human papillomavirus early gene transcription and viral plasmid genome replication by activating interferon regulatory factor (IRF)-1. *Carcinogenesis.* 2009 Aug;30(8):1336-44. doi: 10.1093/carcin/bgp150.
- **Laidlaw SM, Dustin LB.** Interferon lambda: opportunities, risks, and uncertainties in the fight against HCV. *Front Immunol.* 2014 Oct 31;5:545. doi: 10.3389/fimmu.2014.00545.
- **Lanier LL.** NK cell recognition. *Annu Rev Immunol*. 2005;23:225-74. doi: 10.1146/annurev.immunol.23.021704.115526.
- **Lasfar A, Abushahba W, Balan M, Cohen-Solal KA.** Interferon lambda: a new sword in cancer immunotherapy. *Clin Dev Immunol.* 2011;2011:349575. doi: 10.1155/2011/349575.

- **Leeds Il, and Fang SH.** Anal cancer and intraepithelial neoplasia screening: A review. *World J Gastrointest Surg.* 2016 Jan 27; 8(1): 41–51. doi: 10.4240/wjgs.v8.i1.41
- **Lehtinen M, Dillner J.** Clinical trials of human papillomavirus vaccines and beyond. *Nat Rev Clin Oncol.* 2013 Jul;10(7):400-10. doi: 10.1038/nrclinonc.2013.84.
- **Levy DE, Marié IJ, and Durbin JE.** Induction and Function of Type I and III Interferon in Response to Viral Infection. *Curr Opin Virol.* 2011 Dec 1; 1(6): 476–486. doi: 10.1016/j.coviro.2011.11.001
- **Li S, Labrecque S, Gauzzi MC, Cuddihy AR, Wong AH, Pellegrini S et al.** The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. *Oncogene.* 1999 Oct 14;18(42):5727-37. doi: 10.1038/sj.onc.1202960.
- **Li W, Deng XM, Wang CX, Zhang X, Zheng GX, Zhang J et al.** Down-regulation of HLA class I antigen in human papillomavirus type 16 E7 expressing HaCaT cells: correlate with TAP-1 expression. *Int J Gynecol Cancer.* 2010 Feb;20(2):227-32. doi: 10.1111/IGC.0b013e3181cceec5.
- **Lillo FB, Ferrari D, Veglia F, Origoni M, Grasso MA, Lodini S et al.** Human papillomavirus infection and associated cervical disease in human immunodeficiency virus-infected women: effect of highly active antiretroviral therapy. *J Infect Dis* 2001; 184:547–51;
- **Liu D, Chang CH, Rossi EA, Cardillo TM, Goldenberg DM.** Interferon-λ1 linked to a stabilized dimer of Fab potently enhances both antitumor and antiviral activities in targeted cells. *PLoS One.* 2013 May 16;8(5):e63940. doi: 10.1371/journal.pone.0063940.
- **Longworth MS, Laimins LA.** The binding of histone deacetylases and the integrity of zinc finger-like motifs of the E7 protein are essential for the life cycle of human papillomavirus type 31. *J Virol.* 2004 Apr;78(7):3533-41. doi: 10.1128/jvi.78.7.3533-3541.2004.

- **Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ.** Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. *Obstet Gynecol*. 1992 Mar;79(3):328-37. doi: 10.1097/00006250-199203000-00002.
- **Lucia MB, Froio N, Tacconelli E, Tumbarello M, Rutella S, Rumi C et al.** CD16+CD56+CD8+ natural killer (NK) cells are decreased during HIV infection. *Eur J Histochem.* 1997;41 Suppl 2:197-8. PMID: 9859846.
- **Machalek DA, Poynten M, Jin F, Fairley CK, Farnsworth A, Garland SM et al.** Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. *Lancet Oncol.* 2012 May; 13(5):487-500
- **Maglennon GA, McIntosh PB, Doorbar J.** Immunosuppression facilitates the reactivation of latent papillomavirus infections. *J Virol.* 2014 Jan;88(1):710-6. doi: 10.1128/JVI.02589-13.
- **Mantovani F, Banks L.** The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene*. 2001;20:7874–7887. doi: 10.1038/sj.onc.1204869
- **Marra E, Lin C, Clifford GM.** Type-Specific Anal Human Papillomavirus Prevalence Among Men, According to Sexual Preference and HIV Status: A Systematic Literature Review and Meta-Analysis. *J Infect Dis.* 2019 Jan 29;219(4):590-598. doi: 10.1093/infdis/jiy556;
- **Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E et al.** Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A.* 2005 Feb 22;102(8):2886-91. doi: 10.1073/pnas.0409872102.
- **McFarland AP, Savan R, Wagage S, Addison A, Ramakrishnan K, Karwan M** et al. Localized delivery of interferon-β by Lactobacillus exacerbates experimental colitis. *PLoS One.* 2011 Feb 18;6(2):e16967. doi: 10.1371/journal.pone.0016967.

- **Meites E.** Use of a 2-dose schedule for Human Papillomavirus Vaccination—updated recommendations of the Advisory Committee on Immunization Practices. *MMWR Morb Mortal Wkly Rep.* Dec 16;65(49):1405-1408. doi: 10.15585/mmwr.mm6549a5.
- **Mesplède T, Gagnon D, Bergeron-Labrecque F, Azar I, Sénéchal H, Coutlée F** et al. p53 Degradation Activity, Expression, and Subcellular Localization of E6 Proteins from 29 Human Papillomavirus Genotypes. *J Virol.* 2012 Jan; 86(1): 94–107. doi: 10.1128/JVI.00751-11
- **Miknis ZJ, Magracheva E, Li W, Zdanov A, Kotenko SV, Wlodawer A.** Crystal structure of human interferon-λ1 in complex with its high-affinity receptor interferon-λR1. *J Mol Biol.* 2010 Dec 10;404(4):650-64. doi: 10.1016/j.jmb.2010.09.068.
- **Milner DA.** Diagnostic Pathology: Infectious Diseases. *Elsevier Health Sciences*, 2015. p. 40. ISBN 9780323400374
- **Mirjačić Martinović KM, Vuletić AM, Lj Babović N, Džodić RR, Konjević GM, Jurišić VB.** Attenuated in vitro effects of IFN-α, IL-2 and IL-12 on functional and receptor characteristics of peripheral blood lymphocytes in metastatic melanoma patients. *Cytokine.* 2017 Aug;96:30-40. doi: 10.1016/j.cyto.2017.02.024.
- **Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y et al.** Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell* . 1988 54 (6): 903–13. doi:10.1016/S0092-8674(88)91307-4.).
- **Modis Y, Trus BL, Harrison SC.** Atomic model of the papillomavirus capsid. *EMBO J.* 2002 Sep 16;21(18):4754-62. doi: 10.1093/emboj/cdf494
- **Moody CA, Laimins LA.** Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer.* 2010 Aug;10(8):550-60. doi: 10.1038/nrc2886.
- **Mooij SH, van Santen DK, Geskus RB, van der Sande MA, Coutinho RA, Stolte IG et al.** The effect of HIV infection on anal and penile human papillomavirus incidence and

clearance: a cohort study among MSM. *AIDS.* 2016 Jan 2;30(1):121-32. doi: 10.1097/QAD.0000000000000909.

- **Mordstein M, Neugebauer E, Ditt V, Jessen B, Rieger T, Falcone V et al.** Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *J Virol.* 2010 Jun;84(11):5670-7. doi: 10.1128/JVI.00272-10.
- **Moscicki AB, Ellenberg JH, Farhat S, Xu J**. Persistence of human papillomavirus infection in HIV-infected and -uninfected adolescent girls: risk factors and differences, by phylogenetic type. *J Infect Dis.* 2004 Jul 1;190(1):37-45. doi: 10.1086/421467.
- **Moscicki AB, Ma Y, Farhat S, Jay J, Hanson E, Benningfield S et al.** Natural history of anal human papillomavirus infection in heterosexual women and risks associated with persistence. *Clin Infect Dis.* 2014 Mar;58(6):804-11. doi: 10.1093/cid/cit947.
- **Münger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M et al.** Mechanisms of human papillomavirus-induced oncogenesis. *J Virol.* 2004 Nov;78(21):11451-60. doi: 10.1128/JVI.78.21.11451-11460.2004.
- **Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV et al.** Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 2003 Feb 6;348(6):518-27. doi: 10.1056/NEJMoa021641.
- **Muñoz N, Castellsagué X, de González AB, Gissmann L.** Chapter 1: HPV in the etiology of human cancer. *Vaccine.* 2006;24 Suppl 3:S3/1-10. doi: 10.1016/j.vaccine.2006.05.115.

- **Muto V, Stellacci E, Lamberti AG, Perrotti E, Carrabba A, Matera G et al.** Human papillomavirus type 16 E5 protein induces expression of beta interferon through interferon regulatory factor 1 in human keratinocytes. *J Virol*. 2011 May;85(10):5070-80. doi: 10.1128/JVI.02114-10.

- **Nayar R, Wilbur DC**. The Pap test and Bethesda 2014. *Cancer Cytopathol.* 2015 May;123(5):271-81. doi: 10.1002/cncy.21521.
- **Nees M, Geoghegan JM, Hyman T, Frank S, Miller L, Woodworth CD.** Papillomavirus type 16 oncogenes downregulate expression of interferon responsive genes and upregulate proliferation associated and NF-kappa B-responsive genes in cervical keratinocytes. *J Viro1* 2001; 75:4283-96
- **Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ.** Skin immune sentinels in health and disease. *Nat Rev Immunol.* 2009 Oct;9(10):679-91. doi: 10.1038/nri2622.
- **Newman PA, Roberts KJ, Masongsong E, Wiley D.** Anal cancer screening: barriers and facilitators among ethnically diverse gay, bisexual, transgender, and other men who have sex with men. *J Gay Lesbian Soc Serv*. 2008 Oct 1;20(4):328-353. doi: 10.1080/10538720802310733.
- **Nicholls PK, Moore PF, Anderson DM, Moore RA, Parry NR, Gough GW** et al. Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes. *Virology*. 2001 Apr 25;283(1):31-9. doi: 10.1006/viro.2000.0789.
- **Nyitray A, Nielson CM, Harris RB, Flores R, Abrahamsen M, Dunne EF et al.** Prevalence of and risk factors for anal human papillomavirus infection in heterosexual men. *J Infect Dis.* 2008 Jun 15;197(12):1676-84. doi: 10.1086/588145.
- **Nyitray AG, Smith D, Villa L, Lazcano-Ponce E, Abrahamsen M, Papenfuss M et al.** Prevalence of and risk factors for anal human papillomavirus infection in men who have sex with women: a cross-national study. *J Infect Dis.* 2010 May 15;201(10):1498-508. doi: 10.1086/652187.
- **Olagnier D, Hiscott J.** Type I and type III interferon-induced immune response: it's a matter of kinetics and magnitude. *Hepatology.* 2014 Apr;59(4):1225-8. doi: 10.1002/hep.26959.
- **Olesen TB, Sand FL, Rasmussen CL, Albieri V, Toft BG, Norrild B et al.** Prevalence of human papillomavirus DNA and $p16^{INK4a}$ in penile cancer and penile intraepithelial neoplasia:

a systematic review and meta-analysis. *Lancet Oncol.* 2019 Jan;20(1):145-158. doi: 10.1016/S1470-2045(18)30682-X.

- **Olivares M, Diaz-Ropero M, Gomez N, Lara-Villoslada F, Sierra S, Maldonado J et al.** The consumption of two new probiotic strains, Lactobacillus gasseri CECT 5714 and Lactobacillus coryniformis CECT 5711, boosts the immune system of healthy humans. Int Microbiol. 2006;9:47–52
- **Orange JS.** Natural killer cell deficiency. *J Allergy Clin Immunol*. 2013 Sep;132(3):515-525. doi: 10.1016/j.jaci.2013.07.020.
- **Orth G, Favre M, Croissant O.** Characterization of a new type of human papillomavirus that causes skin warts. *J Virol.* 1977 Oct;24(1):108-20. doi: 10.1128/JVI.24.1.108-120.1977.
- **Ozbun MA, Meyers C.** Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. *J Virol.* 1997 Jul;71(7):5161-72. doi: 10.1128/JVI.71.7.5161-5172.1997.
- **Pahl JHW, Cerwenka A, Ni J.** Memory-Like NK Cells: Remembering a Previous Activation by Cytokines and NK Cell Receptors. *Front Immunol*. 2018;9:2796. doi:10.3389/fimmu.2018.02796
- **Palefsky JM, Holly EA, Hogeboom CJ, Berry JM, Jay N, Darragh TM.** Anal cytology as a screening tool for anal squamous intraepithelial lesions. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1997 Apr 15;14(5):415-22. doi: 10.1097/00042560-199704150-00004.
- **Palefsky JM, Holly EA, Efirdc JT, Da Costa M, Jay N, Berry JM et al.** Anal intraepithelial neoplasia in the highly active antiretroviral therapy era among HIV-positive men who have sex with men. *AIDS.* 2005 Sep 2;19(13):1407-14. doi: 10.1097/01.aids.0000181012.62385.4a.

- **Palefsky JM, Giuliano AR, Goldstone S, Moreira ED Jr, Aranda C, Jessen H et al.** HPV vaccine against anal HPV infection and anal intraepithelial neoplasia. *N Engl J Med.* 2011 Oct 27;365(17):1576-85. doi: 10.1056/NEJMoa1010971.
- **Palefsky J.** Human papillomavirus-related disease in people with HIV. *Curr Opin HIV AIDS*. 2009 Jan; 4(1): 52–56. doi: 10.1097/COH.0b013e32831a7246
- **Palefsky JM, Holly EA, Ralston ML, Jay N.** Prevalence and risk factors for human papillomavirus infection of the anal canal in human immunodeficiency virus (HIV)-positive and HIV-negative homosexual men. *J Infect Dis.* 1998 Feb;177(2):361-7. doi: 10.1086/514194.
- **Palefsky J and Jolly EA.** Molecular virology and epidemiology of human papillomavirus and cervical cancer. *Cancer Epidemiol Biomarkers & Prev* 1995; 4:415-28.
- **Paludan SR, Bowie AG.** Immune sensing of DNA. *Immunity.* 2013 May 23;38(5):870-80. doi: 10.1016/j.immuni.2013.05.004.
- **Panda SK, Colonna M.** Innate Lymphoid Cells in Mucosal Immunity. *Front Immunol.* 2019 May 7;10:861. doi: 10.3389/fimmu.2019.00861.
- **Papasavvas E, Surrey LF, Glencross DK, Azzoni L, Joseph J, Omar T et al.** High-risk oncogenic HPV genotype infection associates with increased immune activation and T cell exhaustion in ART-suppressed HIV-1-infected women. *Oncoimmunology.* 2016 Jan 19;5(5):e1128612. doi: 10.1080/2162402X.2015.1128612.

- **Park J S, Kim E J, Kwon H J, Hwang E S, Namkoong S E, Um S J.** Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. *J Biol Chem*. 2000 Mar 10;275(10):6764-9. doi: 10.1074/jbc.275.10.6764.

- **Parisi SG, Cruciani M, Scaggiante R, Boldrin C, Andreis S, Dal Bello F et al.** Anal and oral human papillomavirus (HPV) infection in HIV-infected subjects in northern Italy: a longitudinal cohort study among men who have sex with men. *BMC Infect Dis.* 2011 May 25;11:150. doi: 10.1186/1471-2334-11-150.
- **Patel P, Hanson DL, Sullivan PS, Novak RM, Moorman AC, Tong TC et al.** Incidence of types of cancer among HIV-infected persons compared with the general population in the United States, 1992-2003. *Ann Intern Med.* 2008 May 20;148(10):728-36. doi: 10.7326/0003-4819-148-10-200805200-00005.
- **Pazina T, Shemesh A, Brusilovsky M, Porgador A, Campbell KS.** Regulation of the Functions of Natural Cytotoxicity Receptors by Interactions with Diverse Ligands and Alterations in Splice Variant Expression. *Front Immunol.* 2017 Mar 30;8:369. doi: 10.3389/fimmu.2017.00369.
- **Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating** and inhibitory receptors of natural killer cells. *Immunol Cell Biol.* 2011 Feb;89(2):216-24. doi: 10.1038/icb.2010.78.
- **Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB.** Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol*. 2004;22:929-79. doi: 10.1146/annurev.immunol.22.012703.104622.
- **Pett MR, Alazawi WO, Roberts I, Dowen S, Smith DI, Stanley MA et al.** Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. *Cancer Res*. 2004 Feb 15;64(4):1359-68. doi: 10.1158/0008-5472.can-03-3214.
- **Phelps WC, Münger K, Yee CL, Barnes JA, Howley PM.** Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. *J Virol.* 1992 Apr;66(4):2418-27. doi: 10.1128/JVI.66.4.2418-2427.1992.

- **Pierangeli A, Degener AM, Ferreri ML, Riva E, Rizzo B, Turriziani O et al.** Interferon-induced gene expression in cervical mucosa during human papillomavirus infection. *Int J Immunopathol Pharmacol*. 2011 Jan-Mar;24(1):217-23. doi: 10.1177/039463201102400126.
- **Piketty C, Selinger-Leneman H, Grabar S, Duvivier C, Bonmarchand M, Abramowitz L et al.** Marked increase in the incidence of invasive anal cancer among HIV-infected patients despite treatment with combination antiretroviral therapy. *AIDS.* 2008 Jun 19;22(10):1203-11. doi: 10.1097/QAD.0b013e3283023f78.
- **Pinacchio C, Scheri GC, Statzu M, Santinelli L, Ceccarelli G, Innocenti GP et al**. Type I/II Interferon in HIV-1-Infected Patients: Expression in Gut Mucosa and in Peripheral Blood Mononuclear Cells and Its Modification upon Probiotic Supplementation. *J Immunol Res.* 2018 Aug 12;2018:1738676. doi: 10.1155/2018/1738676.
- **Pinidis P, Tsikouras P, Iatrakis G, Zervoudis S, Koukouli Z, Bothou A et al.** Human Papilloma Virus' Life Cycle and Carcinogenesis. *Maedica (Bucur).* 2016 Mar;11(1):48-54.
- **Plesa A, Iancu IV, Botezatu A, Huica I, Stoian M and Anton G.** The Involvement of Epigenetic Mechanisms in HPV-Induced Cervical Cancer. Human Papillomavirus – Research in a Global Perspective. *Rajkumar R*: 9InTech. 2016.
- **Reed AC, Reiter PL, Smith JS, Palefsky JM, Brewer NT.** Gay and bisexual men's willingness to receive anal Papanicolaou testing. *Am J Public Health.* 2010 Jun;100(6):1123-9. doi: 10.2105/AJPH.2009.176446.
- **Renoux VM, Bisig B, Langers I, Dortu E, Clémenceau B, Thiry M et al.** Human papillomavirus entry into NK cells requires CD16 expression and triggers cytotoxic activity and cytokine secretion. *Eur J Immunol.* 2011 Nov;41(11):3240-52. doi: 10.1002/eji.201141693.
- **Richel O, Prins JM, de Vries HJ.** Screening for anal cancer precursors: what is the learning curve for high-resolution anoscopy? *AIDS.* 2014 Jun 1;28(9):1376-7. doi: 10.1097/QAD.0000000000000227.

- **Ries L, Melbert D, Krapcho M, et al.** Age-adjusted SEER Incidence by site: Table I–4. *National Cancer Institute*; 2007.
- **Robbins SH, Bessou G, Cornillon A, Zucchini N, Rupp B, Ruzsics Z, et al.** Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog*. 2007; 3:e123. doi: 10.1371/journal.ppat.0030123
- **Robertson MJ.** Role of chemokines in the biology of natural killer cells. J Leukocyte Biol 2002 Feb;71(2):173-83.doi: 10.1189/jlb.71.2.173
- **Roden RBS, Stern PL.** Opportunities and challenges for human papillomavirus vaccination in cancer. *Nat Rev Cancer*. 2018 Apr;18(4):240-254. doi: 10.1038/nrc.2018.13.
- **Rodríguez AC, Schiffman M, Herrero R, Wacholder S, Hildesheim A, Castle PE et al.** Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections. *J Natl Cancer Inst.* 2008 Apr 2;100(7):513-7. doi: 10.1093/jnci/djn044.
- **Rositch AF, Koshiol J, Hudgens MG, Razzaghi H, Backes DM, Pimenta JM et al.** Patterns of persistent genital human papillomavirus infection among women worldwide: a literature review and meta-analysis. *Int J Cancer.* 2013 Sep 15;133(6):1271-85. doi: 10.1002/ijc.27828.
- **Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H et al.** Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res.* 2013 Jan;41(Database issue):D1040-6. doi: 10.1093/nar/gks1215.
- **Ruttkay-Nedecky B, Jimenez AMJ, Nejdl L, Chudobova D, Gumulec J, Masarik M et al.** Relevance of infection with human papillomavirus: the role of the p53 tumor suppressor protein and E6/E7 zinc finger proteins. *Int J Oncol.* 2013 Dec;43(6):1754-62. doi: 10.3892/ijo.2013.2105.
- **Sainz B Jr, LaMarca HL, Garry RF, Morris CA.** Synergistic inhibition of human cytomegalovirus replication by interferon-alpha/beta and interferon-gamma. *Virol J.* 2005 Feb 23;2:14. doi: 10.1186/1743-422X-2-14.

- **Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, et al.** Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* 2003 Aug 15;102(4):1389-96. doi: 10.1182/blood-2003-01-0019.
- **Samuel CE.** Antiviral Actions of Interferons. *Clin Microbiol Rev.* 2001 Oct; 14(4): 778–809. doi: 10.1128/CMR.14.4.778-809.2001
- **Sanchez-Martínez D, Krzywinska E, Rathore MG, Saumet A, Cornillon A, Lopez-Royuela N et al.** All-trans retinoic acid (ATRA) induces miR-23a expression, decreases CTSC expression and granzyme B activity leading to impaired NK cell cytotoxicity. *Int J Biochem Cell Biol.* 2014 Apr;49:42-52. doi: 10.1016/j.biocel.2014.01.003.
- **Sancho D, Reis e Sousa C.** Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol.* 2012;30:491-529. doi: 10.1146/annurev-immunol-031210-101352.
- **Scheinfeld N, Lehman DS.** An evidence-based review of medical and surgical treatments of genital warts. *Dermatol Online J.* 2006;12((3)):5.
- **Shiels MS, Engels EA.** Evolving epidemiology of HIV-associated malignancies. Curr Opin HIV *AIDS.* 2017 Jan;12(1):6-11. doi: 10.1097/COH.0000000000000327.
- **Shiels MS, Cole SR, Kirk GD, Poole C.** A meta-analysis of the incidence of non-AIDS cancers in HIV-infected individuals. *J Acquir Immune Defic Syndr*. 2009 Dec;52(5):611-22. doi: 10.1097/QAI.0b013e3181b327ca.
- **Schiller JT, Day PM, Kines RC.** Current understanding of the mechanism of HPV infection *Gynecol Oncol.* 2010 Jun; 118(1 Suppl): S12–S17. doi: 10.1016/j.ygyno.2010.04.004
- **Schmeisser H, Bekisz J, Zoon KC.** New function of type I IFN: induction of autophagy. *J Interferon Cytokine Res.* 2014 Feb;34(2):71-8. doi: 10.1089/jir.2013.0128.
- **Schneider WM, Chevillotte MD, Rice CM.** Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annu Rev Immunol*. 2014 32:513–45. doi: 10.1146/annurev-immunol-032713-120231.

- **Schoggins JW.** Interferon-Stimulated Genes: What Do They All Do? *Annu Rev Virol.* 2019 Sep 29;6(1):567-584. doi: 10.1146/annurev-virology-092818-015756.
- **Schuman P, Ohmit SE, Klein RS, Duerr A, Cu-Uvin S, Jamieson DJ et al.** Longitudinal study of cervical squamous intraepithelial lesions in human immunodeficiency virus (HIV)-seropositive and at-risk HIV-seronegative women. *J Infect Dis.* 2003 Jul 1;188(1):128-36. doi: 10.1086/375783.
- **Scott M, Nakagawa M, Moscicki AB.** Cell-mediated immune response to human papillomavirus infection. *Clin Diagn Lab Immunol*. 2001;8(2):209-220. doi:10.1128/CDLI.8.2.209-220.2001
- **Seaberg EC, Wiley D, Martínez-Maza O, Chmiel JS, Kingsley L, Tang Y et al.** Cancer incidence in the multicenter AIDS Cohort Study before and during the HAART era: 1984 to 2007. *Cancer.* 2010 Dec 1;116(23):5507-16. doi: 10.1002/cncr.25530.
- **Sen P, Ganguly P, Ganguly N.** Modulation of DNA methylation by human papillomavirus E6 and E7 oncoproteins in cervical cancer. *Oncol Lett.* 2018 Jan;15(1):11-22. doi: 10.3892/ol.2017.7292.
- **Serrano-Villar S, Vásquez-Domínguez E, Pérez-Molina JA, Sainz T, de Benito A, Latorre A et al.** HIV, HPV, and microbiota: partners in crime? *AIDS.* 2017 Feb 20;31(4):591-594. doi: 10.1097/QAD.0000000000001352.
- **Serrano-Villar S, Rojo D, Martínez-Martínez M, Deusch S, Vázquez-Castellanos JF, Bargiela R, Sainz T et al.** Gut Bacteria Metabolism Impacts Immune Recovery in HIV-infected Individuals. *EBioMedicine.* 2016 Jun;8:203-216. doi: 10.1016/j.ebiom.2016.04.033.
- **Simard EP, Pfeiffer RM, Engels EA.** Spectrum of cancer risk late after AIDS onset in the United States. *Arch Intern Med.* 2010 Aug 9;170(15):1337-45. doi: 10.1001/archinternmed.2010.253.

- **Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM et al.** Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science.* 2015 Nov 27;350(6264):1084-9. doi: 10.1126/science.aac4255.
- **Smotkin D, Wettstein FO.** Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA.* 1986;83:4680–4684. doi: 10.1073/pnas.83.13.4680
- **Sojka DK, Yang L, Yokoyama WM.** Uterine Natural Killer Cells. *Front Immunol.* 2019 May 1;10:960. doi: 10.3389/fimmu.2019.00960.
- **Sommereyns C, Paul S, Staeheli P, Michiels T.** IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog*. 2008 Mar 14;4(3):e1000017. doi: 10.1371/journal.ppat.1000017.
- **Sowah LA, Buchwald UK, Riedel DJ, Gilliam BL, Khambaty M, Fantry L et al.** Anal Cancer Screening in an Urban HIV Clinic: Provider Perceptions and Practice. *J Int Assoc Provid AIDS Care*. 2015 Nov-Dec;14(6):497-504. doi: 10.1177/2325957415601504.
- **Stanley MA.** Epithelial Cell Responses to Infection with Human Papillomavirus. *Clin Microbiol Rev.* 2012 Apr; 25(2): 215–222. doi: 10.1128/CMR.05028-11
- **Stark GR, Darnell JE Jr.** The JAK-STAT pathway at twenty. *Immunity.* 2012 Apr 20;36(4):503-14. doi: 10.1016/j.immuni.2012.03.013.
- **Steenbergen RD, de Wilde J, Wilting SM, Brink AA, Snijders PJ, Meijer CJ.** HPV-mediated transformation of the anogenital tract. *J Clin Virol*. 2005 Mar;32 Suppl 1:S25-33. doi: 10.1016/j.jcv.2004.11.019.
- **Stojanovic A, Cerwenka A.** Natural killer cells and solid tumors. *J Innate Immun.* 2011;3(4):355-64. doi: 10.1159/000325465.
- **Strickler HD, Burk RD, Fazzari M, Anastos K, Minkoff H, Massad LS et al.** Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus-positive women. *J Natl Cancer Inst.* 2005 Apr 20;97(8):577-86. doi: 10.1093/jnci/dji073.

- **Stubenrauch F, Colbert AM, Laimins LA.** Transactivation by the E2 protein of oncogenic human papillomavirus type 31 is not essential for early and late viral functions. *J Virol.* 1998 Oct;72(10):8115-23. doi: 10.1128/JVI.72.10.8115-8123.1998.
- **Stünkel W, Bernard HU.** The Chromatin Structure of the Long Control Region of Human Papillomavirus Type 16 Represses *Viral Oncoprotein Expression*. J Virol. 1999; 73(3):1918-30. doi: 10.1128/JVI.73.3.1918-1930.1999
- **Sudenga SL, Nyitray AG, Torres BN, Silva R, Villa L, Lazcano-Ponce E et al.** Comparison of anal HPV natural history among men by country of residence: Brazil, Mexico, and the United States. *J Infect.* 2017 Jul;75(1):35-47. doi: 10.1016/j.jinf.2017.03.010.
- **Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A et al.** Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021 Feb 4. doi: 10.3322/caac.21660.
- **Sutlu T, Alici E.** Natural killer cell-based immunotherapy in cancer: current insights and future prospects. *J Intern Med.* 2009 Aug;266(2):154-81. doi: 10.1111/j.1365-2796.2009.02121.x.
- **Takeuchi O, Akira S.** Pattern recognition receptors and inflammation. *Cell.* 2010 Mar 19;140(6):805-20. doi: 10.1016/j.cell.2010.01.022.
- **Takiishi T, Fenero CIM, Câmara NOS.** Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. *Tissue Barriers*. 2017 Oct 2;5(4):e1373208. doi: 10.1080/21688370.2017.1373208.
- **Teeraananchai S, Kerr SJ, Amin J, Ruxrungtham K, Law MG.** Life expectancy of HIV-positive people after starting combination antiretroviral therapy: a meta-analysis. *HIV Med.* 2017 Apr;18(4):256-266. doi: 10.1111/hiv.12421.
- **Tay SS, Roediger B, Tong PL, Tikoo S, Weninger W.** The Skin-Resident Immune Network. *Curr Dermatol Rep*. 2013 Nov 28;3(1):13-22. doi: 10.1007/s13671-013-0063-9.

- **Thomas JT, Hubert WG, Ruesch MN, Laimins LA.** Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc Natl Acad Sci U S A.* 1999 Jul 20;96(15):8449-54. doi: 10.1073/pnas.96.15.8449.
- **Terra Junior ON, Maldonado Gde C, Alfradique GR, Lisboa Vda C, Arnóbio A, de Lima DB et al.** Study of Natural Cytotoxicity Receptors in Patients with HIV/AIDS and Cancer: A Cross-Sectional Study. *ScientificWorldJournal*. 2016;2016:2085871. doi: 10.1155/2016/2085871.
- **Tjalsma H, Boleij A, Marchesi JR, Dutilh BE.** A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. Nat Rev Microbiol. 2012 Jun 25;10(8):575-82. doi: 10.1038/nrmicro2819.
- **Tomaić V.** Functional Roles of E6 and E7 Oncoproteins in HPV-Induced Malignancies at Diverse Anatomical Sites. *Cancers (Basel).* 2016 Oct; 8(10): 95. doi: 10.3390/cancers8100095
- **Tommasino M, Accardi R, Caldeira S, Dong W, Malanchi I, Smet A et al.** The role of TP53 in Cervical carcinogenesis. *Hum Mutat.* 2003 Mar;21(3):307-12. doi: 10.1002/humu.10178.
- **Tulay P, Serakinci N.** The role of human papillomaviruses in cancer progression. *J Cancer Metastasis Treat* 2016; 2:201-213. 10.20517/2394-4722.2015.67
- **Tummers B, Goedemans R, Pelascini LP, Jordanova ES, van Esch EM, Meyers C, et al.** The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NF activation. *Nat Commun*, 2015; 6:6537. doi:10.1038/ncomms7537
- **Turner MD, Nedjai B, Hurst T, Pennington DJ**. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta.* 2014 Nov;1843(11):2563-2582. doi: 10.1016/j.bbamcr.2014.05.014.

- **Vázquez-Castellanos JF, Serrano-Villar S, Latorre A, Artacho A, Ferrús ML et al.** Altered metabolism of gut microbiota contributes to chronic immune activation in HIV-infected individuals. *Mucosal Immunol.* 2015 Jul;8(4):760-72. doi: 10.1038/mi.2014.107.
- **Venuti A, Paolini F, Nasir L, Corteggio A, Roperto S, Campo MS et al.** Papillomavirus E5: the smallest oncoprotein with many functions. *Mol Cancer.* 2011; 10: 140. doi: 10.1186/1476-4598-10-140
- **Verhoeven V, Renard N, Makar A, Van Royen P, Bogers JP, Lardon F et al.** Probiotics enhance the clearance of human papillomavirus-related cervical lesions: a prospective controlled pilot study. *Eur J Cancer Prev.* 2013 Jan;22(1):46-51. doi: 10.1097/CEJ.0b013e328355ed23.
- **Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ et al.** Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci Transl Med.* 2013 Jul 10;5(193):193ra91. doi: 10.1126/scitranslmed.3006438.
- **Wakabayashi R, Nakahama Y, Nguyen V, Espinoza JL.** The Host-Microbe Interplay in Human Papillomavirus-Induced Carcinogenesis. *Microorganisms.* 2019 Jul 13;7(7):199. doi: 10.3390/microorganisms7070199.
- **Wang H, Hu H and Zhang K.** Overview of interferon: characteristics, signaling and anticancer effect. *Arch Biotechnol Biomed.* 2017; 1: 001-016. doi: 10.29328/journal.hjb.1001001
- **Wang B, Amerio P, Sauder DN.** Role of cytokines in epidermal Langerhans cell migration. *J Leukoc Biol.* 1999 Jul;66(1):33-9. doi: 10.1002/jlb.66.1.33.
- **Watson FS, Spendlove I, Madjd Z, McGilvray R, Green AR, Ellis IO, et al**. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. *Int J Cancer*. 2006; 118:1445–52. doi: 10.1002/ijc.21510
- **Weber F.** Antiviral Innate Immunity: Introduction Reference Module in Life Sciences. 2020; B978-0-12-809633-8.21290-9. doi: 10.1016/B978-0-12-809633-8.21290-9

- **Wells JS, Holstad MM, Thomas T, Bruner DW**. An integrative review of guidelines for anal cancer screening in HIV-infected persons. *AIDS Patient Care STDS*. 2014 Jul;28(7):350-7. doi: 10.1089/apc.2013.0358.
- **Werness BA, Levine AJ, Howley PM.** Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science.* 1990 Apr 6;248(4951):76-9. doi: 10.1126/science.2157286.
- **Wechsler EI, Tugizov S, Herrera R, Da Costa M, and Palefsky JM.** E5 can be expressed in anal cancer and leads to epidermal growth factor receptor-induced invasion in a human papillomavirus 16-transformed anal epithelial cell line. *J Gen Virol*. 2018 May; 99(5): 631–644. doi: 10.1099/jgv.0.001061
- **Wilkin TJ, Palmer S, Brudney KF, Chiasson MA, Wright TC.** Anal intraepithelial neoplasia in heterosexual and homosexual HIV-positive men with access to antiretroviral therapy. *J Infect Dis.* 2004 Nov 1;190(9):1685-91. doi: 10.1086/424599.
- **Wilson VG, West M, Woytek K, Rangasamy D.** Papillomavirus E1 proteins: form, function, and features. *Virus Genes.* 2002 Jun;24(3):275-90. doi: 10.1023/a:1015336817836.
- **Wong IKJ, Poynten IM, Cornall A, Templeton DJ, Molano M, Garland SM et al.** Sexual behaviours associated with incident high-risk anal human papillomavirus among gay and bisexual men. *Sex Transm Infect.* 2021 Mar 16:sextrans-2020-054851. doi: 10.1136/sextrans-2020-054851.
- **Wu J, Chen ZJ.** Innate immune sensing and signaling of cytosolic nucleic acids. *Annu Rev Immunol.* 2014;32:461-88. doi: 10.1146/annurev-immunol-032713-120156.
- **Xiao TS, Fitzgerald KA.** The cGAS-STING pathway for DNA sensing. *Mol Cell.* 2013 Jul 25;51(2):135-9. doi: 10.1016/j.molcel.2013.07.004.

- **Yim EK, Park JS.** The Role of HPV E6 and E7 Oncoproteins in HPV-associated Cervical Carcinogenesis. *Cancer Res Treat*. 2005 Dec; 37(6): 319–324. doi: 10.4143/crt.2005.37.6.319
- **Yokota J, Sugimura T.** Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. *FASEB J.* 1993 Jul;7(10):920-5. doi: 10.1096/fasebj.7.10.8344488.
- **Yuan T, Fitzpatrick T, Ko NY, Cai Y, Chen Y, Zhao J et al.** Circumcision to prevent HIV and other sexually transmitted infections in men who have sex with men: a systematic review and meta-analysis of global data. *Lancet Glob Health.* 2019 Apr;7(4):e436-e447. doi: 10.1016/S2214-109X(18)30567-9.
- **Zevin AS, McKinnon L, Burgener A, Klatt NR.** Microbial translocation and microbiome dysbiosis in HIV-associated immune activation. *Curr Opin HIV AIDS*. 2016 Mar;11(2):182-90. doi: 10.1097/COH.0000000000000234.
- **Zhou J, Sun XY, Louis K, Frazer IH.** Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. *J Virol.* 1994 Feb;68(2):619-25. doi: 10.1128/JVI.68.2.619-625.1994.

- **Zhou F, Chen J, Zhao KN.** Human papillomavirus 16-encoded E7 protein inhibits IFN-γ-mediated MHC class I antigen presentation and CTL-induced lysis by blocking IRF-1 expression in mouse keratinocytes. *J Gen Virol.* 2013; 94(Pt 11):2504-2514. doi: 10.1099/vir.0.054486-0.
- **Zoon KC, ZurNedden, DL, Enterline, JC, Manischewitz, JF, Dyer et al.** Chemical and biological characterization of natural human lymphoblastoid interferon alphas. In: Cantell K, Schellekens H, eds. The Biology of the Interferon System. Dordecht:Martinus Nijhoff Pub. pp 567–569, 1986.

- **zur Hausen H, Gissmann L, Steiner W, Dippold W, Dreger I.** Human papilloma viruses and cancer. *Bibl Haematol.* 1975 Oct;(43):569-71. doi: 10.1159/000399220.
- **zur Hausen H.** In Viruses and Cancer eds Rigby, P. W. J. & Wilkie, N. M. 83-90 Cambridge Univ. Press, Cambridge, 1986.
- **zur Hausen H.** Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst.* 2000 May 3;92(9):690-8. doi: 10.1093/jnci/92.9.690.
- **zur Hausen H.** Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer.* 2002 May;2(5):342-50. doi: 10.1038/nrc798.