UNIVERSITÀ
DEGLI STUDI
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DIPARTIMENTO DI MEDICINA MOLECOLARE

SCUOLA DI DOTTORATO DI RICERCA IN BIOMEDICINA

CICLO: XXV

Evaluation of HPV type-specific antibody response induced by the prophylactic quadrivalent vaccine

Direttore della Scuola : Ch.mo Prof. Giorgio Palù

Supervisore : Ch.ma Prof.ssa Luisa Barzon

Dottoranda : Dr.ssa Laura Squarzon

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ABSTRACT

Human papillomavirus (HPV) is one of the most common sexually transmitted infections worldwide and affects approximately 300 million new individuals each year. HPV is considered the primary etiological agent involved in the development of cervical cancer and causes half a million cases per year worldwide. Prevention of genital HPV infection through immunization has led investigators to employ a number of strategies to develop candidate HPV vaccines. Until today, two different vaccines are available on the market: a quadrivalent vaccine that protects against HPV types 16, 18, 6, and 11 (Gardasil®, Merck Sharp and Dohme), and a bivalent vaccine that protects against HPV types 16 and 18 (Cervarix™, Glaxo SmithKline). Current data regarding the efficacy of these vaccines derive mainly from studies performed by the manufacturers and standardized assays are not commercially available to measure HPV immunity.

In this context, the aim of this PhD research project is to set up and standardize HPV pseudovirion-based neutralization (PBNA) and enzyme-linked immunosorbent (ELISA) assays and to use these tests to evaluate and compare the immunogenicity and cross-reactivity levels of the two prophylactic HPV vaccines, which are offered free in Italy to 12-year old girls and are recommended to women aged 12-45 years, according to World Health Organization (WHO) guidelines.

First, pseudovirions of HPV types 6, 11, 16, 18, 31, 45, 52, 58 were obtained with a titer of 10^9 transducing units/ml and neutralization and ELISA assays standardized. Subsequently, a cross sectional study to evaluate the humoral immune response against HPV-volunteers, adolescents, and healthy vaccinated adults with Gardasil® or Cervarix™ was approved by ethics committee of University Hospital of Padua. Comprehensive results were obtained from a group of 100 subjects from Veneto Region, where the quadrivalent Gardasil® vaccine was offered. The study group included 81 subjects investigated within 1-6 months after the completion of the three doses of vaccine and 7, 7, and 5 subjects investigated at 2, 3, and 4 years after vaccination, respectively.

At 1-6 months after the completion of the vaccination cycle, 100% vaccinees had neutralizing antibodies (NAbs) against HPV16, 98,8% had NAbs against HPV18, while 91% had NAbs against HPV6 and 50% had NAbs against HPV11. The NAbs titer ranged widely from 1:40 to over 1:10,240 and was lower for NAbs against HPV6 and HPV11 as compared with NAbs titers against HPV16 and HPV18. A progressive reduction of NAbs titer was observed over time and, at 4 years from vaccination, 80% of subjects had NAbs against HPV16, HPV18 and HPV6, and 60% against HPV11. Low level cross-NAbs titer against HPV31 (1:40) was detected in

50% (3/6) of subjects at 1-6 months after vaccination, while no cross-NAbs were detected against HPV45, HPV52 and HPV58.

We also evaluated the presence of HPV type-specific NAbs in a group of 6 young girls vaccinated with Cervarix™ at 1-6 months after the completion of the vaccination cycle. All subjects presented specific NAbs against HPV16 and HPV18. Titers were higher as compared with titers observed in Gardasil® vaccinated subjects. 100% of subjects presented also cross-NAbs against HPV31, whereas 16,6% presented cross-NAbs against HPV45 and HPV58. None presented cross-NAbs against HPV52.

Thanks to these results we can conclude that high-level NAbs were induced with both Gardasil® and Cervarix™ vaccines. For the first vaccine, we observed the decline of NAbs titers and the limited cross-neutralization against HPV31. For the second one, cross-neutralizing NAbs were observed against HPV31 in all subjects, together with the presence of NAbs against HPV45 and HPV58 in some subjects.

RIASSUNTO

L'infezione da papilloma virus umano (HPV) è una delle più comuni infezioni trasmesse per via sessuale in tutto il mondo e colpisce circa 300 milioni di nuovi individui ogni anno. L'infezione persistente da tipi di HPV definiti ad alto rischio è la causa necessaria per lo sviluppo del cancro del collo dell'utero. Annualmente, vengono registrati circa 500.000 casi di carcinomi del collo dell'utero in tutto il mondo. La necessità di prevenire questo tipo di infezione ha portato nel corso degli ultimi anni allo sviluppo di diverse strategie vaccinali. Ad oggi, sono disponibili due diversi vaccini profilattici: un vaccino quadrivalente che protegge contro HPV16, 18, 6, e 11 (Gardasil®, Merck Sharp & Dohme), e un vaccino bivalente che protegge contro HPV 16 e 18 (Cervarix™, Glaxo SmithKline). I dati riguardanti l'efficacia e l'immunogenicità di questi due vaccini derivano principalmente da studi effettuati dalle ditte produttrici. Non sono disponibili inoltre test standardizzati commerciali in grado di valutare l'immunità nei confronti dei diversi tipi di HPV.

Obiettivo di questo progetto di ricerca di dottorato è quello di sviluppare e standardizzare un test specifico per la ricerca di anticorpi anti-HPV basato sulla neutralizzazione di diversi tipi di HPV mediante pseudovirioni (PBNA) e un test immunoenzimatico (ELISA), e di utilizzare questi test per valutare e confrontare i livelli di immunogenicità e cross-reattività dei due vaccini profilattici anti-HPV che sono offerti gratuitamente in Italia alle ragazze nel loro dodicesimo anno di età e che vengono raccomandati per le donne di età compresa tra i 12 e i 45 anni, secondo le linee guida dell'Organizzazione Mondiale della Sanità (OMS).

A tal fine, sono stati prodotti diversi lotti di pseudovirioni corrispondenti ai tipi HPV6, 11, 16, 18, 31, 45, 52, 58 con un titolo pari a 10^9 unità trasducenti/ml e sono stati standardizzati i saggi di neutralizzazione tipo-specifica e il saggio ELISA. E' stato disegnato uno studio *cross-sectional* per valutare la risposta immunitaria umorale contro i diversi tipi di HPV in soggetti sani, adolescenti e adulti, vaccinati con Gardasil® o Cervarix™.

I risultati sono stati ottenuti analizzando un gruppo di 100 soggetti della Regione Veneto, dove era offerta la vaccinazione con Gardasil®. In particolare, sono stati esaminati 81 soggetti a distanza di 1-6 mesi dal completamento del ciclo vaccinale, 7 soggetti valutati a 2 anni dalla vaccinazione, 7 soggetti a 3 anni dalla vaccinazione, e 5 a 4 anni dalla vaccinazione.

A distanza di 1-6 mesi dal completamento della vaccinazione con Gardasil®, il 100% dei soggetti presentava anticorpi neutralizzanti contro HPV16, il 98,8% contro HPV18, il 91% contro HPV6 e il 50% contro HPV11. Sono stati ottenuti titoli di anticorpi neutralizzanti compresi tra 1:40 e 1:10,240. I titoli osservati nei confronti di HPV6 e HPV11 sono risultati inferiori rispetto a quelli osservati nei confronti di

HPV16 e HPV18. E' stata, inoltre, osservata una riduzione progressiva nel titolo in base al tempo intercorso dall'ultima dose vaccinale. A 4 anni dalla vaccinazione, l'80% dei soggetti presentava anticorpi neutralizzanti contro HPV16, HPV18 e HPV6, mentre il 60% nei confronti di HPV11. Per quanto riguarda la presenza di anticorpi cross-neutralizzanti, è stato osservato un titolo pari a 1:40 nei confronti di HPV31 nel 50% (3/6) dei soggetti entro i primi 6 mesi dalla vaccinazione, mentre non sono stati rilevati anticorpi cross-neutralizzanti nei confronti di HPV45, HPV52 e HPV58.

E' stata valutata, inoltre, la presenza di anticorpi neutralizzanti nei confronti dei diversi tipi di HPV in un gruppo di 6 ragazze vaccinate con Cervarix™ a distanza di 1-6 mesi dal completamento della vaccinazione. Tutti i soggetti presentavano anticorpi neutralizzanti nei confronti di HPV16 e HPV18, a titoli più elevati rispetto ai titoli osservati nei soggetti vaccinati con Gardasil®. Il 100% dei soggetti presentava, inoltre, anticorpi cross-neutralizzanti contro HPV31, mentre il 16,6% aveva anticorpi cross-neutralizzanti contro HPV45 e HPV58. Nessun soggetto ha presentato anticorpi cross-neutralizzanti contro HPV52.

In conclusione, entrambi i vaccini sono in grado di indurre elevati livelli di specifici anticorpi neutralizzanti i tipi di HPV vaccinali. Per quanto riguarda il vaccino Gardasil® è stata osservata una diminuzione dei titoli anticorpali nel tempo e una limitata cross-neutralizzazione nei confronti di HPV31. Per quanto riguarda il vaccino Cervarix™, invece, è stata osservata la presenza di anticorpi cross-neutralizzanti contro HPV31 in tutti i soggetti, unitamente alla presenza degli anticorpi neutralizzanti contro HPV45 e HPV58 in alcuni soggetti.

1. INTRODUCTION

1.1. Human papillomaviruses

Human papillomaviruses (HPVs) are small, non-enveloped viruses that belong to the *Papillomaviridae* family. HPVs comprise five evolutionary groups with different epithelial tropisms and disease associations. Alpha papillomaviruses include low-risk mucosal types that cause genital warts, and high-risk mucosal types that can cause cervical pre-cancer and cancer (Figure 1). Although cutaneous HPV types (Alpha, Beta and Gamma) are not generally associated with cancer, certain Beta types have been implicated in the development of non-melanoma skin cancers (NMSC) in immunosuppressed individuals and in epidermodysplasia verruciformis (EV) patients.

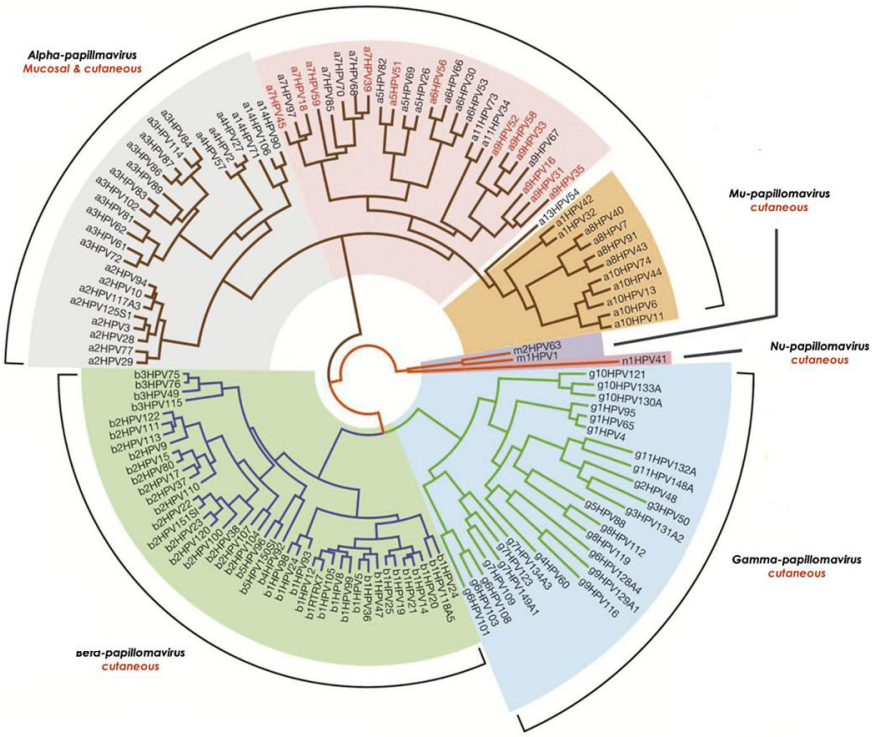


Figure 1. Evolutionary relationship between HPVs (adapted from Doorbar *et al.*, 2012).

The genome of HPVs is composed by a circular double-stranded DNA molecule of 8 kilobases (kb). Viral capsid consists of two main structural proteins, L1 (called major) and L2 (called minor), both with late expression. At molecular level, L1 sequence is the most conserved among different genotypes of HPVs such as N-terminal domain of L2 sequence (Gambhira *et al.*, 2007; Pereira *et al.*, 2009). More than 150 HPV types have been completely sequenced and classified on the basis of major capsid protein L1 differences in the amino acid sequence (de Villiers *et al.*,

2004; Bernard *et al.*, 2010; see Papillomavirus Episteme (PaVE); http://pave.niaid.nih.gov/#home). L1 proteins are also able to self assemble forming *virus-like particles* (VLPs) (IARC, 2007), while L2 proteins are internally located with roles in genome encapsidation, L1 interaction and capsid stabilization, endosomal escape of virions and nuclear transport of HPV genome. Assembled particles contain 360 molecules of L1 arranged into 72 pentameric capsomeres linked via disulphide bonds, with a much smaller and variable number of L2 molecules, which can occupy capsomeres at the 5-fold axis of symmetry. Viral genome is composed by other six early expression proteins: E5, E6, E7 with cellular proliferation, survivor and carcinogenesis functions and E1, E2, E4 with transcriptional control and viral replication involvement (Figure 2). In particular, E7 protein is responsible of Rb destruction and progression in S-phase of cellular cycle, while E6 protein interferes with pro-apoptotic factors p53 and Bak and activates telomerase enzyme.

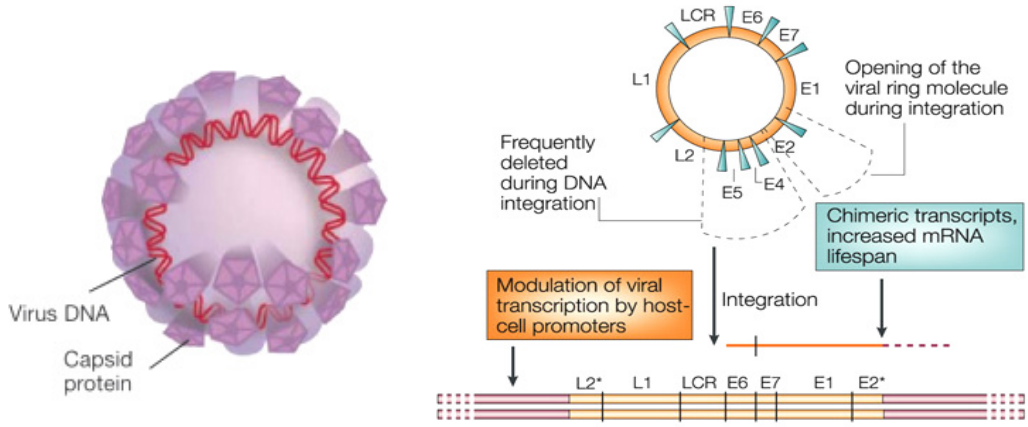


Figure 2. HPV structure and genome (adapted from zur Hausen, 2002).

At pathogenetic level, HPV infection is one of the most common sexually transmitted infections worldwide and affects around 300 million new individuals each year. Twelve HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are defined by the WHO as being high-risk cancer causing types (category 1, according to IARC classification), with additional types (68, 73) being recognized as possibly cancer-causing (category 2, according to IARC classification). Together, these viruses cause approximately half a million cases of cervical cancer per year worldwide, with approximately half of these being fatal (WHO/ICO Information Centre on Human Papilloma Virus and Cervical Cancer; http://www.who.int/hpvcentre/en/). In particular, HPV types 16 and 18 cause approximately 70% of all cervical cancers (Day *et al.*, 2007; Villa, 2011). Other oncogenic HPV types, including HPV types 31, 33, 35, 45, 52 and 58, account for additional 18% of all cervical cancers (Figure 3) (Stanley *et al.*, 2006; Lu *et al.*, 2011; Crow, 2012).

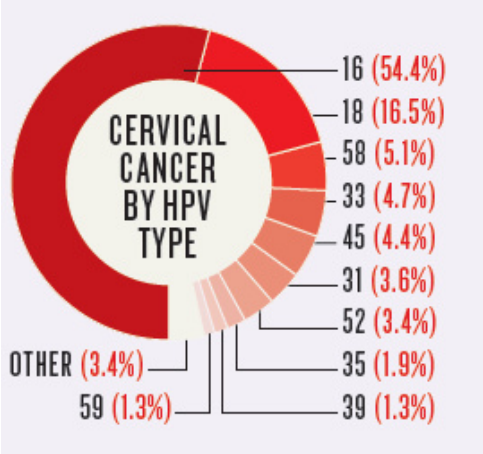


Figure 3. HPV and cervical cancer (adapted from WHO/ico information.
Center on HPV and cervical cancer. http://www.nature.com/nature/journal/v488/).

Prospective studies have shown that women persistently infected with high-risk HPV types are at a significantly greater risk of developing cervical intraepithelial neoplasia (CIN) compared with women who are only transiently infected (Schlecht *et al.*, 2003). HPV 16 is found in about half of cervical tumors examined and is strongly associated with CIN, carcinoma *in situ* (CIS), squamous intraepithelial lesions (SIL) and cervical cancer. Importantly, all fourteen HPV types mentioned before, are also associated with cancers at other sites, including the penis in men, the vagina and vulva in women and, in both genders, the anal transformation zone, the tonsils, oropharynx and base of tongue (D'Souza *et al.*, 2007; Giuliano, Tortolero-Luna *et al.*, 2008). Deregulation of viral gene expression may occur to different extents at different sites of high-risk HPV infection, and that squamo-columnar junctions, such as the cervical transformation zone, are particularly prone to neoplastic disease. Nevertheless, high-risk HPVs do not cause cancer in the vast majority of the individuals that they infect (Doorbar, 2006; zur Hausen, 2009). The natural history of HPV infection in men has also been evaluated. A recent study has shown that genital HPV infection is common and multifocal in young men, and its incidence seems to be higher than previously reported (Giuliano, Lazcano-Ponce *et al.*, 2008). The low-risk types HPV6 and HPV11 are responsible for anogenital warts, which are common in the general population (Brown *et al.,* 1999), and for a rare but severe and potentially lethal disease, i.e., recurrent respiratory papillomatosis (RRP) (Wiatrak, 2003).

1.2. Mechanism of HPV infection

Papillomaviruses are species-specific viruses with a strong tropism for epithelial tissues such as skin and mucosa. Their process of infection is interesting and, in

some ways, unique. Penetration of the virus occurs in the basal layer of the epithelium, following cuts or abrasions. Experimental models suggest that infection requires access of virus particles to the basal lamina, and the interaction with heparan sulphate proteoglycans (HSPGs) (Combita *et al.*, 2001; Giroglou *et al.*, 2001; Johnson *et al.*, 2009) and possibly also laminin (Culp *et al.*, 2006). Structural changes in the virion capsid, which includes furin cleavage of L2, facilitate transfer to a secondary receptor on the basal keratinocyte, which is necessary for virus internalization and subsequent transfer of the viral genome to the nucleus (Richards *et al.*, 2006; Kines *et al.*, 2009; Schiller *et al.*, 2010; Bienkowska-Haba *et al.*, 2009). Although the Alpha 6 Integrin and growth factor receptors have been implicated in this process (Evander *et al.*, 1997; McMillan *et al.*, 1999; Shafti-Keramat *et al.*, 2003; Licitra *et al.*, 2006; Scheurer *et al.*, 2007; Surviladze *et al.*, 2012), the precise nature of the entry receptor remains somewhat controversial (Patterson *et al.*, 2005; Sapp *et al.*, 2009; Schiller *et al.*, 2010; Horvath *et al.*, 2010; Surviladze *et al.*, 2012) (Figure 4).

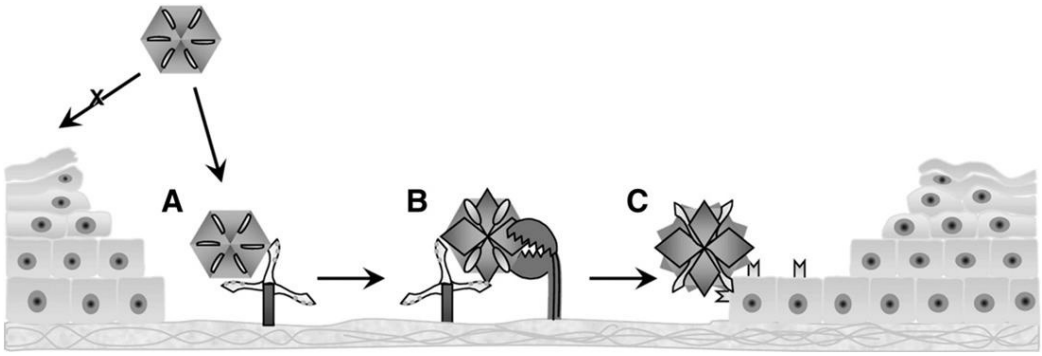


Figure 4. HPV initial infection steps (adapted from Schiller *et al.*, 2010). The virion first binds to HSPGs on the base membrane exposed after disruption (A). This induces a conformational change exposing a site on L2 susceptible to furin cleavage (B). After L2 cleavage, an L2 neutralizing epitope is exposed and a previously unexposed region of L1 binds to an unidentified secondary receptor on the invading edge of the epithelial cells (C).

Once internalized, virions undergo endosomal transport, uncoating, and cellular sorting. The L2 protein-DNA complex ensures the correct nuclear entry of the viral genomes, while the L1 protein is retained in the endosome and ultimately subjected to lysosomal degradation (Bergant Marušič *et al.*, 2012; Schelhaas *et al.*, 2012) (Figure 5).

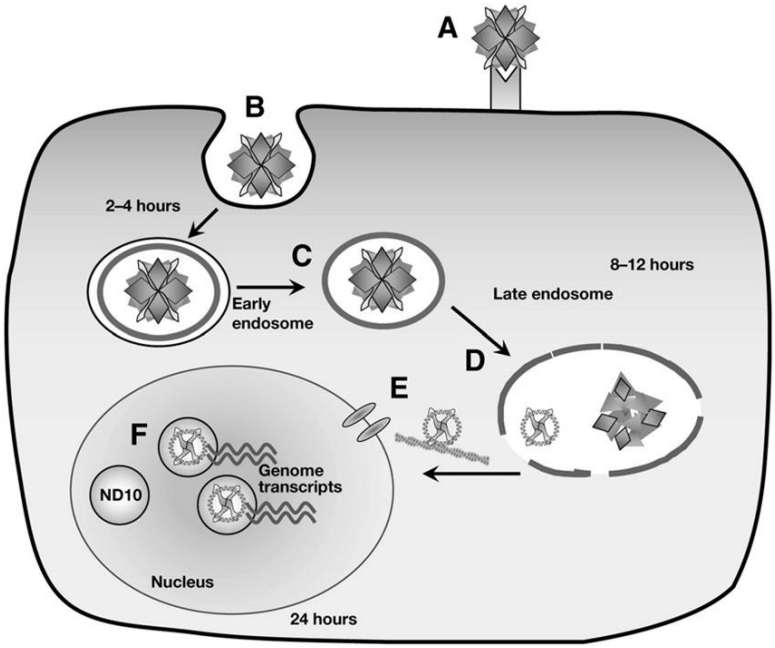


Figure 5. HPV virion processing after cell entry (adapted from Schiller *et al.*, 2010). After initial binding to HSPGs and furin cleavage, the virus is transferred to an unidentified receptor on the cell surface (A). The virus then enters the cell via an endocytic pathway (B) and within 4 hours localizes in the early endosome (C). By 12 hours, the virus uncoats within the late endosome, and the viral genome complexed with L2 is released (D). The L2–genome complex traffics through the cytoplasm, perhaps via microtubules, and enters the nucleus by 24 hours (E). After nuclear entry, the complex co-localizes with ND10 and RNA transcription begins (F).

After the entry into the nucleus, the genome of HPV can remain in an episomal state at low copy number (about 100 copies per cell). Viral genome is able to replicate and segregate into daughter cells persisting in the episomal state until basal cells (keratinocytes) migrate to higher levels, such as differentiated layers (spinous and granular layers of squamous epithelium). Thanks to E6 and E7 oncoproteins, HPV stimulates the proliferation of differentiated cells, thus ensuring its survival and replication. Finally, with the production of L1 and L2 proteins, new mature virions can form (Figure 6). Although not precisely defined, the abundant E4 protein is thought to contribute to virion release and infectivity in the upper epithelial layers, as it assembles into amyloid fibres that disrupt keratin structure and compromise the normal assembly of the cornified envelope (Wang *et al.*, 2004; Brown *et al.*, 2006; McIntosh *et al.*, 2008). Time between infection and release of mature virions is approximately 3 weeks. This gap is necessary for the differentiation of keratinocytes and desquamation. Subsequently, in a period ranging between weeks and months, benign lesions such as warts or papillomas arise. These formations often regress spontaneously. However, sometimes, the lesions may persist and evolve, giving rise

to tumor formation, such as warts, laryngeal papillomas and cervical cancers (Kanodia *et al.*, 2007).

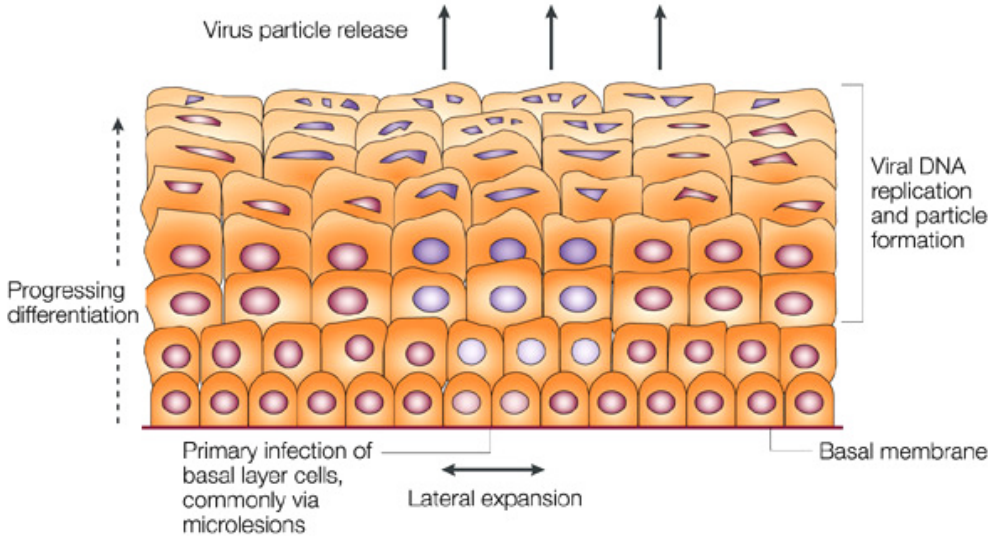


Figure 6. Mechanism of HPV infection (adapted from zur Hausen, 2002).

1.3. HPV and the immune system

Most cervical HPV infections are transient, resolving within 1 to 2 years in 70-90% of cases (Steben and Duarte-Franco, 2007), implying that the host immune system must play an important role in preventing, controlling and eliminating HPV infection at the cervix. In some cases, the immune system is unable to control the infection, thus persistent infection is established and viral DNA can be found in the cervix for at least 6-12 months. This situation may lead to the development of high-grade cervical intraepithelial lesions (CIN2/3) that may progress to achieve invasive properties. In general, during infection, innate immunity, cell-mediated immunity (CMI) and humoral immunity play a key role in the response at entry and replication phases, although it is not yet known the exact sequence in which one follows after the other. Neutralizing antibodies are believed to be the main effectors of protection against infection, preventing the initial entry of virus and internalization into basal epithelial cells. On the other hand, clearance of infection is thought to be mediated predominantly by cellular immunity. At humoral level, antibodies are directed against conformational epitope(s) in the variable regions of the major viral coat protein L1 displayed on the outer surface of the intact virus particle and are the only ones that can neutralize the viral particles circulating at extracellular level. After natural infection, humoral response is very slow and weak, with low levels of detectable type-specific antibodies to L1 in about 50-70% of infected individuals (Viscidi *et al.*,1997; Carter *et al.*, 2000). Seroconversion appears to occur 6-18 months following

infection, defined as detection of HPV DNA. The presence of HPV L1-specific antibodies can be considered as marker for past or current infection, because they are frequently detected in subjects with persistent infections and precancerous lesions (Mariani and Venuti, 2010). Most serologic studies have focused only on immunoglobulin G (IgG) antibody concentrations. Isotype-specific serum antibody responses against HPV16 have been evaluated in a few cross-sectional, prospective, and population-based seroepidemiological studies. IgG1 and immunoglobulin A (IgA) are the most abundant isotypes in natural infection. IgA is induced earlier, and does not appear to be as persistent as the IgG response after HPV DNA clearance (van Doornum *et al.*, 1998). However, not all individuals develop serum antibodies after HPV infection, and protection degree and duration in those subjects who seroconverted are still unknown. In addition, some of these individuals remain susceptible to re-infection with the same viral type. In this context, memory B cells are essential for protection against subsequent re-infections by the same papillomavirus type (Schiller *et al.*, 2008) and CMI responses against HPV proteins may participate in early defense against HPV infection. In particular, T helper responses play a key role in antiviral cytokines release (i.e. IFN gamma; TNF alfa) which contribute to inhibition and control of infection and in the generation and maintenance of protective B cell responses. Cytotoxic T lymphocytes (CTL) responses against L1 may also be important for maximization of the prophylactic efficacy by eliminating or limiting the number of HPV-infected cells that have escaped antibody neutralization. In contrast, HPV has implemented a series of strategies to evade immune responses and many of them are dependent from its replication cycle. In fact, HPV has only intraepithelial and not cytolytic replication. Therefore, there is no viremia or systemic infection development and the expression of viral proteins does not reach detectable levels. In addition, pro-inflammatory cytokines are released to activate dendritic cells, but no inflammatory reactions arise. In fact, interferon response is suppressed by viral oncoproteins E6 and E7 of high-risk HPV types, such as HPV16 and down-regulation of the toll-like receptor 9 expression (TLR9) by the E7 protein has been recently reported (Schwartz, 2008). In this scenario, the innate immune response is compromised and the adaptive immune response delayed.

1.4. Prophylactic HPV L1 VLP vaccines

A number of strategies to develop candidate HPV vaccines to prevent or treat genital HPV infection through immunization have been investigated (Schiller *et al.*, 2001). Back in the nineties, the first proof-of-principle clinical trials were conducted with extremely positive results: administration of papillomaviruses L1 self-assembled

VLPs protected against infections and diseases caused by common papillomavirus, in a type specific manner. High titers of neutralizing antibodies are induced by VLP vaccines as measured by type-specific antibody responses to each HPV VLP in different immunogenicity studies (Villa, 2007). From the first studies to licensing of HPV prophylactic vaccines only about 15 years passed by. Two vaccines have been developed and are available for clinical use, a quadrivalent vaccine that protects primarily against HPV16, 18, 6, and 11 (Gardasil®, Merck Sharp and Dohme), and a bivalent vaccine that protects primarily against HPV16 and 18 (Cervarix™, Glaxo SmithKline). Both vaccines are composed of HPV L1 proteins assembled into VLPs; they are produced in different cells (via recombinant *Saccharomyces pombe* vector and via recombinant baculovirus vector, respectively) and contain different adjuvants (proprietary alum adjuvant for Gardasil® and ASO4 adjuvant comprising 500 g of aluminium hydroxide and 50 g of 3-deacylated monophosphoryl lipid A for Cervarix™). Both are administered by intramuscular injection, in three doses (0, 1 or 2 and 6 months), and are generally well tolerated (Harper *et al.*, 2004; Villa *et al.*, 2005). Systemic immunization with L1 VLPs generates antibody concentrations 1-4 logs higher than those measured in natural infections (Harro *et al.*, 2001); this is most likely due to the route of immunization and antigen concentration. Some clinical trials have also reported the vaccine efficacy after fewer than three doses of the bivalent vaccine (Kreimer *et al.*, 2011). Most common vaccine-related adverse events were local transient mild to moderate pain and erythema at the site of injection. The proportion of women experiencing serious adverse events was much the same in vaccinated and placebo recipients. Although women were encouraged to use reliable methods of birth control, pregnancies did occur in numerous women enrolled in the trials. Overall, there was no difference in pregnancy outcomes, including congenital abnormalities, between VLP vaccines and placebo for either vaccines. In fact, none of the serious adverse events referred either in clinical trials or post-licensure have been related to the vaccines (Slade *et al.*, 2009). Both vaccines are able to induce the expected memory B cell response, which is a characteristic of vaccines with durable immune responses (Olsson *et al.*, 2007). In one study, memory B cell responses appeared to correlate with neutralization titers, but not with antibody avidity (Dauner *et al.*, 2010). Long-term follow-up studies have shown that efficacy is maintained for at least 7 years, and modeling studies suggest protection may last much longer (Villa *et al.*, 2006a; Lehtinen *et al.*, 2006). Both vaccines were shown to be highly immunogenic in clinical trials, resulting in essentially 100% seroconversion in the different populations studied. Peak geometric mean antibody titers (GMTs) were approximately 10- to 100-fold higher that the GMTs generated after natural infection (Villa *et al.*, 2006b; Schwarz *et al.*, 2008). Several clinical trials of two prophylactic HPV vaccines have been conducted in different countries including approximately

50.000 individuals. Phase III trials were blinded, randomized, and placebo-controlled and included young (mean age 20 years old) and adult women (up to 55 years old) and more recently also men (16-23 years old) from different countries worldwide. Prophylactic efficacy was measured considering HPV infection and disease endpoints, particularly CIN2 or worse (CIN2+) for the bivalent and quadrivalent vaccines, as well as Vulvar Intraepithelial Neoplasia (VIN) or Vaginal Intraepithelial Neoplasia (VaIN) and genital warts, for the quadrivalent vaccine only. In these trials, HPV DNA assays and antibody measurements were applied both to defining naïve, HPV-susceptible per-protocol populations and to determine the effects of vaccination, although only 50% of individuals mount an immune response after natural exposure to HPV. The per-protocol populations included women who were naïve at baseline to HPV16 and 18, or to HPV6, 11, 16, and 18, as determined by serology testing for the presence of HPV type-specific antibodies or polymerase chain reaction (PCR) testing of genital samples for the presence of HPV DNA. For both the bivalent and quadrivalent vaccines, very high prophylactic efficacies (approximately 100%) were recorded (Harper *et al.*, 2004; Villa *et al.*, 2005; Harper *et al.*, 2006; Future II Study Group, 2007; Ault *et al.*, 2007; Joura *et al.*, 2007; Paavonen *et al.*, 2007; Einstein *et al.*, 2011a). Recently, the bivalent vaccine was demonstrated to induce significantly higher anti-HPV16 and 18 neutralizing antibody titers in women aged 27-45 years at all time points through to month 24, as compared to the quadrivalent vaccine. Moreover, higher anti-HPV16 and 18 neutralizing antibody titers have been observed in younger age groups (18-26 years old and 27-35 years old) compared with the older age group (36-45 years old) at all time points from month 7 to 24 (Einstein *et al.*, 2011a). These results confirmed previous observations, by Reisenger and Pedersen regarding higher immune response observed in children and adolescent than in adults (Reisinger *et al.*, 2007; Pedersen *et al.*, 2007). As expected from observations in other studies for both vaccines, levels of vaccine-induced anti-HPV16 and 18 antibodies peaked at month 7 and subsequently declined toward *plateau* by month 18 (Einstein *et al.*, 2011a). Anyway, prior or prevalent infection by one HPV type did not appear to influence the effectiveness of the vaccine against other types (Hildesheim *et al.*, 2007). Cross-protection against incident infection with HPV type 31 has been observed for both the bivalent and the quadrivalent vaccines (Ault, 2007). For additional HPV types different levels of protection were recorded (Bonanni *et al.*, 2009). In particular, Einstein and collaborators (Einstein *et al.*, 2011b) demonstrated that both vaccines are able to induce cross-reactive serologic responses against HPV31 and HPV45, but at lower titer than those for vaccine types HPV16/18. These finding are in line with cross-protection efficacy against infections and lesions associated with HPV 31, 33, and 45 that has been reported for the bivalent vaccine (Malagon *et al.*, 2012). At month 24,

there were no significant differences between the bivalent vaccine and the quadrivalent vaccine in terms of anti-HPV31/45 circulating antibodies (Einstein *et al.*, 2011b). However, it should be noted that circulating neutralizing antibodies were at levels close to or below the limit of detection of each assay (Einstein *et al.*, 2011b).

1.5. Immune correlates of protection

Data from published clinical trials on HPV L1 VLP vaccines demonstrate clearly that both the bivalent and quadrivalent vaccines protect the vaccinees from persistent cervical HPV16/18 infection and cervical HPV16/18-induced disease. The quadrivalent vaccine also protects women against HPV6/11-induced mucosal and cutaneous genital disease. The mechanisms of protection are not fully understood. VLPs are highly immunogenic and VLP-immunized individuals have anti-VLP antibody responses substantially greater (at least 1-4 logs) than in natural infections. In natural infections, L1 antibody levels are low and there is probably little or no viraemia and no evolutionary pressure on the virus to escape the antibody response. In contrast to natural infection, L1 VLP vaccines are delivered by intramuscular injection, which allows antigens to gain access to the draining lymphatics and small vessels at the injection site, thereby effectively mimicking a viraemia. One can speculate that this explains, in part, the intensity of the antibody responses induced by these HPV L1 VLP vaccines (Stanley *et al.*, 2006; Stanley *et al.*, 2012). Although a direct correlation between antibody levels and protection may seem intuitively obvious, it is still unclear whether differing antibody titers indicate better disease protection or longer duration of immune protection (Schwarz and Leo, 2008). Given that virtually all vaccinated women are seroconverted and the vaccines are highly efficacious, to date there is no immune correlate of protection against infection or disease; the minimum level of antibody needed for such protection and the role of B cell memory if antibody wanes have yet to be established (Stanley *et al.*, 2012). As already stated in the last WHO position paper, the question still remains unanswered (WHO position paper No 15, 2009). Only long-term follow up of vaccinated cohorts in human populations could answer such questions unequivocally.

1.6. Next generation HPV vaccines

Neutralizing antibodies evocated by vaccines based on HPV L1 are type-restricted and there is limited cross-reactivity. On the other hand, minor capsid protein L2 does not evoke a neutralizing antibody response in natural infections. However, deliberate immunization with L2 protein has been demonstrated to generate neutralizing antibodies that are protective against viral challenge in cows and rabbits. Importantly,

these antibodies have been implicated in cross-neutralization of a broad range of mucosal and cutaneous HPV types, suggesting that an L2 vaccine could act as a pan-HPV vaccine (Karanam *et al.*, 2009). However, protein vaccines such as L2 are considered as poorly immunogenic, as compared to the L1 VLPs. In fact, L1-based VLPs are optimal immunogens both in terms of particle size, which allow easy transfer across endothelium into lymphatics, and in terms of geometry of the highly organized pentamer repeat structure across the capsid, which induces robust antibody and memory responses in all B cell subsets. Recent studies have focused on strategies to enhance L2 immunogenicity by multimerisation, epitope display and adjuvantation. In a recent report, mice immunised with a concatenated multitype L2 fusion protein with a variety of different adjuvants, including alum, generated robust antibody concentrations that were only slightly lower than those elicited by commercially available VLP vaccines (Jagu *et al.*, 2009). The L2 vaccine is due to enter phase I clinical trials in early 2013 (Nieto *et al.*, 2012). Many efforts have been done to develop therapeutic vaccines. These vaccines generally targets E6 and E7 oncoproteins. A prototype vaccine that contains whole E7 proteins from the five most common cancer-causing HPV types, as well as poly(I:C) - a double-stranded RNA molecule that signals the presence of a virus has been generated. Together E7 and poly(I:C) stimulate a swift build-up of CD8. In addition, the whole-protein formulation of this vaccine, called Pentarix, makes it more stable than the minimal peptide approaches, without the need for refrigeration, thus rendering it useful for developing-world settings (Wick and Webb, 2011).

1.7. Immunoassays

Measurement of specific serum antibodies anti-L1 VLP by immunoassays in vaccinated and unvaccinated individuals is the main parameter used in the current vaccine trials to monitor vaccine-induced immune responses. The methodologies that have been used in the quadrivalent Gardasil® vaccine trials differ from those employed in the evaluation of the bivalent Cervarix™ vaccine and direct comparisons of antibody responses to the different vaccines are therefore not feasible yet. For the quadrivalent vaccine, serum antibodies against HPV6, 11, 16 and 18 were measured using a competitive radioimmunoassay (cRIA) or a competitive Luminex immunoassay (cLIA). In brief, in the cRIA, polystyrene beads are coated with a limiting amount of VLP antigen and incubated with the corresponding HPV type-specific, neutralizing, mouse monoclonal antibody (MAb), and the serum sample. The amount of MAbs bound to the VLP antigen is measured after incubation with ^{125}I-labelled goat anti-IgG. In the cLIA, HPV L1 VLPs are conjugated to Luminex microspheres and incubated with phycoerythrin labelled type-

specific neutralizing MAbs and a serum sample. The amount of bound dye is quantified with a Luminex100 Biopex instrument. These competitive assays measure only those serum antibodies that bind to, or compete with, the single neutralizing epitope that binds the MAbs on the specific VLPs and are not a measurement of total serum anti-L1 VLP IgG. A disadvantage of such assays is that, since affinities of binding and other parameters of antibody–antigen interaction will differ between the different MAbs used, direct comparisons of antibody titers generated by the different VLPs (HPV 6, 11, 16, 18 VLPs) in the vaccine cannot be made. The advantages of the competitive assays are that neutralizing antibody is assayed specifically, the antibody response to each VLP is measured in the same test and, in general in such assays, backgrounds are low, sensitivity is enhanced and spurious cross-reactions are reduced. For the bivalent HPV16/18 vaccine, measurement of serum antibody to HPV16 and 18 VLPs was performed using a conventional ELISA. In this assay, microwell plates coated with purified recombinant HPV16 or 18 VLPs are incubated with serial dilutions of sera, the bound antibody reacted with horseradish peroxidase (HRP) conjugated goat anti-human IgG and the colour intensity is quantified by optical density measurements. The advantages of such assay are that total serum anti-VLP IgG is measured and direct comparisons can be made between responses to individual VLPs. A disadvantage is that the antibody response to individual VLPs is assayed in separate tests, the fraction of serum IgG attributable to neutralizing antibody specifically cannot be quantified, but the available data suggest that neutralizing and ELISA antibodies titers are usually highly correlated. At the present, the *gold standard* method to evaluate specific antibody responses is a neutralization assay based on pseudovirion (PBNA). This test involves the use of pseudovirion made by L1 and L2 proteins of different HPV types (HPV type 6, 11 16 18, etc.) containing a reporter gene, such as alkaline phosphatase. To test neutralizing titers in serum samples, target cells (293TT) are seeded firstly, and then serum and pseudovirion are added. After 72 hours of incubation, supernatants are collected to measure alkaline phosphatase activity using a luminometer. Neutralizing titers are expressed as the dilution of serum in which there is 50% reduction in alkaline phosphatase activity. Other methods, just as important for assessing the response of memory B cells and CD4+ T cells, are based on the use of ELISPOT assays. B-cell ELISPOT assay is able to quantify HPV-specific memory B cells after differentiation into plasma cells secreting antibodies *in vitro*, as well as to determine the production of IFN-γ and IL-10. Mononuclear cells or B lymphocytes are purified from peripheral blood samples and 96-well plates are pre-adsorbed with the specific HPV VLPs. Mononuclear cells or B cells are added to the plates and left at 37 °C for several hours. During the incubation secreted antibodies bind to the VLPs. Cells are then removed and a secondary biotinylated antibody directed against human

immunoglobulins is added to the plates. The subsequent addition of streptavidin and chromogenic substrate allows the detection of immune complexes and the quantification of the spots. Reading is performed by an ELISPOT reader. T-cell ELISPOT assay allows to evaluate cellular immunity induced by the vaccination, analyzing T cells that produce IFN-γ. Specific monoclonal antibodies are immobilized on the bottom of 96-well plates. To stimulate the production of cytokines, cells under examination are incubated with the CD4 or CD8 epitopes of the L1 protein of HPV. Secreted cytokines are then immobilized by the antibodies on the bottom of the wells and their detection occurs after a washing step, adding a secondary biotinylated antibody or conjugated with an enzyme. The subsequent addition of the substrate allows the registration of spots corresponding to active T cells by the ELISPOT reader.

2. AIM OF THE STUDY

Available data regarding HPV vaccine efficacy and immunogenicity derive mainly from studies performed by the manufacturer companies. In addition, standardized assays are not commercially available to measure HPV immunity. In this context, the development of standardized tests for the evaluation of antibody immune response after vaccination and the design of independent studies comparing the immunogenicity of the two vaccines could provide new useful diagnostic tools and scientific data to support national and regional public health authorities in the evaluation of vaccination programs.

The main purpose of this PhD research project is to set up and standardize HPV pseudovirion-based neutralization assays (PBNA) and ELISA assays, and to use these assays to compare the immunogenicity of the two prophylactic HPV vaccines which are currently offered in Italy, in terms of neutralizing and cross-neutralizing antibody titers. No comparative data on the immunogenicity of two vaccines in the target population of adolescent girls are yet available in the literature. Moreover, no data from independent studies comparing efficacy and immunogenicity of prophylactic HPV vaccines have been published until now. Therefore, insights emerging from this 3-year project will provide information on the humoral immune response after the completion of three vaccine doses and at long-term follow-up which could be useful to decide if further booster doses are required. The assessment on the possible different levels of cross-protection against non-vaccinal high-risk HPV types induced by two vaccines is another important evaluation from this study.

3. MATERIALS AND METHODS

MATERIALS

3.1. Cell line

Cell line used for transfection and transduction experiments in this project consists of an adenovirus-transformed human embryonic kidney cell line with a stably integrated Simian vacuolating virus 40 (SV40) genome, called 293TT, kindly provided by the groups of Professor Chris Buck and Professor John Schiller at the National Cancer Institute - Center for Cancer Research (NCI-CCR) in Bethesda, USA. These engineered cells constitutively express the SV40 Large T antigen. This line supports high level replication of plasmids containing the SV40 origin of replication (ori). Co-transfection of 293TT cells with plasmids containing strong eukaryotic promoters driving codon modified L1 and L2, together with the pseudogenome plasmid containing the SV40 ori and a marker gene, results in assembly of reporter pseudovirions in the nucleus of the transfected cell. The growth medium suitable for the maintenance of these cells is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) inactivated at 56 °C, 1% non-essential amino acids and 1% Glutamax (Invitrogen™). The cells were also subjected to selection with the antibiotic hygromycin B 250 μg/ml (Invitrogen™), in order to promote the maintenance of T antigen. Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

3.2. Plasmids

Plasmids used in this study were kindly provided by the groups of Professor Chris Buck and Professor John Schiller at NCI-CCR in Bethesda, USA. Plasmids contain the coding sequences of viral structural proteins of different HPV types necessary for the virions formation that are not replication-competent (p6shell, p11shell.L1, p11shell.L2, p16shell, p18shell, p31shell, p45shell, p52shell and p58shell). Plasmid containing sequences of two different reporter genes were also used (pfwb containing the coding sequence of enhanced green fluorescence protein (EGFP), and pYSEAP containing the coding sequence of human placental alkaline phosphatase protein (SEAP)). All plasmids were amplified in high-efficiency commercial prokaryotic cells (MAX Efficiency®Stbl2™ Competent Cells, Invitrogen™). Bacteria were grown in TB medium (Terrific Broth: Bacto tryptone, yeast extract, glycerol and phosphate buffer) shaking at 30 °C.

3.3. Study subjects

Serum samples were obtained from 100 female subjects aged between 12 and 46 years old (mean age: 15,66 years old) who completed the vaccination with Gardasil® at different time points (1-6 months, 2 years, 3 years and 4 years). Blood samples were collected at Microbiology and Virology Unit of Padova University Hospital. To all the participants, or to a parent in the case of a minor, was required to subscribe an informed consent. Other 11 non-vaccinated subjects (8 males and 3 females, aged between 24 and 47 years old) tested positive for molecular research HPV were included in the study, as well as a vaccinated female and a vaccinated male as first control experiments. Thanks to a kind collaboration with Microbiology and Virology Unit of Sant'Orsola Malpighi University Hospital of Bologna, we included also 6 female serum samples (age range: 11-20 years old; mean age: 13,99 years old), collected 1-6 months after the completion of vaccination cycle with Cervarix™ . All blood samples were centrifuged at 3000 rpm for 10 minutes and sera were then aliquoted and stored at -20 °C until use.

METHODS

3.4. Bacterial transformation

HPV DNA plasmids were incubated with 100 μl of competent bacteria on ice for 30 minutes. Subsequently, bacteria were subjected to thermal shock by exposure to 42 °C for 25 seconds, cooled on ice and incubated at 30 °C for 90 minutes in 900 μl of SOC medium (Invitrogen™), allowing the expression and the synthesis of the protein that confers resistance to the antibiotic selection. Finally, 100 μl of 1:40 diluted suspension were seeded in Petri dishes containing LB-agar supplemented with ampicillin 100 mg/ml or alternatively with blasticidin 100 g/ml or with kanamycin 25 mg/ml, depending on the selection, and incubated 16 hours at 30 °C in order to select transformed bacteria.

3.5. Plasmid DNA preparation

To obtain plasmid preparations of high purity and large-scale (maxi prep) suitable for transfection of eukaryotic cells, the plasmid DNA was extracted using alternatively the QIAGEN Plasmid Maxi Kit or QIAfilter Plasmid Maxi kit based both on the use of anion exchange columns by QIAGEN®. Through this process plasmid DNA binds to the resin column using buffers with low salt concentration and is purified from RNA, proteins and high molecular weight impurities. Plasmid DNA is then

eluted from the column by buffers at high salt concentration. Subsequently, the plasmid is concentrated by precipitation with isopropanol and then washed with ethanol 70% (v/v). DNA was quantified by spectrophotometric reading with NanoDropND 1000 Spectrophotometer (ThermoScientific) at the wavelength of 260 nm, corresponding to the maximum absorption peak of the nitrogenous bases. Contaminations have been detected by the reading of the preparation at a wavelength of 280 nm, corresponding to the maximum absorption peak of the peptide bond.

3.6. Enzyme restriction

Different DNA restriction reactions were performed using the appropriate enzymes in their reaction buffer (Biolabs). p16shell, p58shell, p18shell, p45shell plasmids were assessed by digestion with the restriction enzyme PstI, p31shell with EcoRI, p6shell with ClaI, p52shell with EcoRI, p11shell.L1 and p11shell.L2 with BamHI, pYSEAP with SacI, pfwb with NcoI. The reactions were conducted at the optimum temperature of the enzyme (37 °C in all cases) for about 2 hours. An aliquot of the digestion products was controlled by electrophoretic migration in agarose gel 1% (w/v) using 20X TBE buffer (11 mM Tris base, boric acid, 5.5 mM EDTA, 0.5 M pH 8) to determine the correct restriction.

3.7. Transfection of 293TT cells

In this experimental work, lipofection method was used to transfect 293TT cells. Cells were seeded at a density of $7x10^4$ in 6-well plates in 4 ml of DMEM medium without antibiotics, but enriched in serum. This operation was performed 24 hours before the lipofection, in order to achieve a perfect confluence of 30-50% of the cells. Next day, cells were transfected with 4 μg of each plasmid in conjunction with 4 μg of pfwb plasmid or pYSEAP plasmid using 12 μl of Lipofectamine™ 2000 (1 mg/ml, Invitrogen™) according to the manufacturer's instructions.

3.8. Production of HPV VLPs containing EGFP and SEAP reporter genes

Different HPV VLPs, containing L1 and L2 coding sequences of different HPV types and EGFP or SEAP reporter genes, were produced by lipofection of 293TT cells with p6shell, p11shell.L1, p11shell.L2, p16shell, p18shell, p31shell, p45shell, p52shell, p58shell and pfwb or pYSEAP plasmids (see paragraphs 3.2. and 3.7.). VLPs that are released from 293TT cells lysis are immediately infectious, but require further maturation overnight at 37 °C to assume the correct conformation. For this purpose, 48 hours after lipofection 293TT cells were tripsinized, counted and

resuspended in DPBS-Mg. The lysate was then incubated for 24 hours at 37 °C with 1/20th of a volume of 10% Triton X-100. All manipulations of transfected cells and their lysates were carried out under a laminar flow hood type BL2. Following maturation, the lysate was partially frozen at -80 °C and partially purified by polyacrilamide desalting column (see paragraph 3.9.).

3.9. Extraction and purification of the salts by Optiprep or polyacrilamide desalting column

Before Optiprep (Sigma®) or polyacrylamide desalting column (Thermo Scientific Pierce) purification, cell lysates containing VLPs were subjected to salt extraction. Lysates were incubated on ice, 5 M NaCl was added and cells were centrifuged at 10.000 g for 10 minutes. Supernatant was centrifuged, and stratified on the Optiprep gradient or on a pre-balanced desalting column. Optiprep gradient is made of solution containing 60% iodixanol appropriately diluted with DPBS/NaCl in order to obtain 27%, 33% and 39% iodixanol solutions and to be placed in 5 ml ultracentrifuge tubes. Lysates were subjected to ultracentrifugation stratification at 50.000 rpm at 16 °C for 3,5 hours. After that, different fractions were collected. Purification with polyacrilamide desalting column was made by subsequent elutions with buffer containing DPBS and 0.5 M NaCl.

3.10. Screening of the fractions

Fractions obtained after ultracentrifugation and layered with Optiprep were analyzed using Quant-iT PicoGreen dsDNA assay. This assay allows to assess the presence of DNA encapsidation, thanks to the permeability of these capsids to small solutes, such as fluorescent markers of DNA. In addition, fractions containing L1 protein, which are the most represented in the central fractions of the gradient (4-8), were analyzed by SDS-PAGE in non-reducing conditions. L1 content was also quantified by spectrophotometric reading with NanoDropND 1000 Spectrophotometer (ThermoScientific) at the wavelength of 280 nm, corresponding to the maximum absorption peak of the peptide bond.

3.11. Quantification of cell extracts

Protein content in cell lysate was determined by a colorimetric assay (Pierce-ThermoScientific), followed by the quantification with the spectrophotometer. The assay exploits the property of bicinchoninic acid (BCA), provided in the form of sodium salt, to react with high sensitivity with copper ions (Cu1+) to form a water-

soluble reaction product of purple color, characterized by an absorption maximum at wavelength of 492 nm. Copper ions (Cu1+) are made available in an alkaline environment owing to the interaction between proteins and Cu2+ ions (Biuret reaction). Since the reaction product is directly proportional to the total amount of protein present in the reagents, it is possible to quantify proteins by spectrophotometric analysis. For each sample, 10 µl of cell extracts were taken and seeded in 96-well plates (Falcon ®) in order to be incubated with 200 µl of the mixture of kit reagents. After an incubation of 30 minutes at 37 °C, the reaction product was quantified at the spectrophotometer and the total amount of proteins was extrapolated by comparing results with a calibration curve obtained with known amounts of bovine serum albumin.

3.12. Western blotting

In order to assess the presence and the expression of HPV16 L1 and L2 proteins in cells transfected with p16shell plasmid or transduced with HPV16 VLPs, Western blotting analysis using specific antibodies anti-HPV16 L1 (CamVir-1, Santa Cruz Biotechnology) and anti-HPV16 L2 (2JGmab#4, Santa Cruz Biotechnology, Inc.) were performed. Resolving and stacking gels were prepared at 10% of SDS in both cases. Protein extracts/elutions were loaded into gel after denaturation with β-mercaptoethanol. After gel running at a constant speed of 100 V, gels were transferred on a nitrocellulose membrane at 350 mA for 1 hour. Subsequently, saturation was performed overnight with a solution containing TBS 1X, 0.1% Tween and 1% or 5% of milk, respectively. Next day, membranes were washed with TBS 1X and 0.1% Tween solution. Blotting with the primary antibody anti-HPV16 L1 (CamVir-1, Santa Cruz Biotechnology) or anti-HPV16 L2 (2JGmab#4, Santa Cruz Biotechnology, Inc.) was performed for 4 or 1 hour respectively, followed, only in the case of p16shell and HPV16 VLPs, by the addition of goat secondary antibody anti-mouse IgG-HRP (Santa Cruz Biotechnology). Finally, the membrane was observed after revelation with ECL chemiluminescent substrate at VersaDoc instrument (Biorad).

3.13. Transduction of 293TT cells with VLPs

293TT cells were transduced with HPV VLPs (see paragraph 3.8.). One day before the transduction, $1.5x10^5$ cells were seeded in 24-wells plates in 0.5 ml of DMEM medium supplemented with serum and antibiotics. Then, cells were transduced with 1 µl of matured and purified supernatant (see paragraphs 3.8. and 3.9.). 48 hours after

transduction, cells were detached with trypsin and assessed by flow cytometry and immunofluorescence (see paragraphs 3.14. and 3.15.)

3.14. Flow cytometry

293TT transfected cells with p16shell and pfwb plasmids and 293TT transduced cells with VLPs were collected, resuspended in 300 µl of PBS and analyzed by FACS instrument (Becton-Dickinson, Rutherford, NJ). The analysis was conducted in a manner that would prevent at least 99,8% of non-transfected and non-transduced cells as control cells. Viral titer evaluation was made by selecting dilutions of VLPs with a positivity rate given by the fluorescent marker (EGFP) varying between 1% and 25%. The formula that was used is the following:
[Fraction of positive cells] x [N. inoculated cells] x [1000 µl/ml] x [dilution of the stock].

3.15. Immunofluorescence

293TT transfected cells with p16shell and pfwb plasmids and 293TT transduced cells with VLPs were evaluated by fluorescence microscopy with and without the primary antibody anti-HPV16 L1 (CamVir-1, Santa Cruz Biotechnology). After fixation with a solution containing dimethyl ketone at 95% and three washes with PBS and water, cells were stained with the specific antibody. After washing, cells were stained with fluorescein isothiocyanate (FITC) and observed under a fluorescence microscope by using various objectives.

3.16. Chemiluminescence assay for secreted alkaline phosphatase detection

Expression of alkaline phosphatase activity resulting from transfection of 293TT cells with pYSEAP plasmid or from transduction of 293TT cells with different HPV types VLPs encapsidating SEAP reporter plasmid was assessed by two chemiluminescence assays (SEAP Chemiluminescence Kit 2.0 Great Escape, Clontech; SEAP Reporter Gene Assay, Roche). Both assays provide an analysis of cell supernatant in which alkaline phosphatase is secreted after 18 hours of transfection or 72 hours of transduction. After been tested, we decided to used the second one, by Roche, because of its feasibility and reproducibility. Supernatants were diluted with a suitable buffer ready for use and then incubated in a thermal cycler at 65 °C for 30 minutes. After a centrifugation of 30 seconds at 13.000 rpm, supernatants were cooled on ice and mixed with an inactivation buffer onto 96-well micro white plate. After an incubation at room temperature for 5 minutes, substrate

was added and supernatants incubated at room temperature for 10 minutes. Finally, the reading was performed with a luminometer, set at Glow-Endpoint 0.20 sec/well.

3.17. Pseudovirion-based neutralization assay (PBNA)

VLPs encapsidating SEAP reporter plasmid were used to develop *in vitro* pseudovirion-based neutralization assay presented below. VLPs transduction of 293TT cells is monitored by SEAP activity in cell culture supernatant using a highly sensitive chemiluminescent reporter system (see paragraph 3.16.). Antibody-mediated VLPs neutralization is detected by a reduction in SEAP activity. This neutralization assay is adapted to a high-throughput 96 well plate format. Firstly, 293TT cells were counted and dilute to obtain 300.000 cells/ml in neutralization buffer (DMEM without phenol red, 10% of FBS, 1% of Glutamax™, 1% of non essential amino-acids). 100 µl of cells were dispensed in each well of a 96-well plate and incubated for 2-5 hours at 37 °C. Meanwhile, each type of HPV VLPs were diluted in neutralization buffer, as well as sera (1:40, 1:160, 1:640, 1:2,560, 1:10,240, 1:20,480). Control antibodies were also prepared (i.e. J4 for HPV 18 1:20.000, 1:100.000; D9 1:10.000, 1:20.000, 1:50.000, 1:100.000; V5 for HPV 16 1: 20.000, 1:100.000). HPV16 positive serum was used as a positive neutralization control at dilution of 1:80. After 3 hours of incubation, 80 µl of VLPs mixed with different dilution of 20 µl of serum or antibody or heparin as positive neutralization control were cooled on ice for 1 hour. Then, the mixture was added to the cells. After 72 hours, supernatant was finally used in chemiluminescence assay for secred alkaline phosphatase detection (see paragraph 3.16.).

3.18. Enzyme-linked immunosorbent assay (ELISA)

In a specific ELISA assay, intact HPV16 VLPs (0,5 µg, 1 µg) were coated onto microtiter plates in PBS at pH 7.2. Coating was performed overnight at 4 °C. After washing with PBS-Tween, plate was blocked at room temperature for 1 hour with PBS-BSA. After washing with PBS-Tween, serum samples diluted in PBS-BSA (1:10, 1: 31,6, 1:100) were added for 2 hours at room temperature. After five washing steps with PBS-Tween, specific anti-human IgHRP (γchain) antibody (dilution 1:1.000) was added to the plate for 1 hour at room temperature. After five washing steps with PBS-Tween, substrate was added and incubated for 30 minutes at room temperature. After blocking agent addiction, plate was read at 450/620 nm.

4. RESULTS

4.1. Production of HPV pseudovirions: summary of results

For this study, pseudovirion-based neutralization assay (PBNA), which is considered the *gold standard* method for the evaluation of the immune response against HPV was developed. To this purpose, constructs containing the structural proteins of different types of HPV (kindly provided by Professor Chris Buck and Professor John Schiller groups at the NCI-CCR in Bethesda, USA) were used to produce different types of HPV VLPs consisting of L1 and L2 proteins. VLPs containing EGFP and SEAP reporter plasmids were produced on small scale in 293TT hygromycin-selected cells. VLPs were then purified by using Optiprep gradient and evaluated for their transfecting and transducing capability. Structural informations of VLPs were also obtained by non-denaturing SDS-page followed by Western blotting analysis. Viral titer was evaluated by flow citometry (10^9 transducing units/ml).

4.1.1. Production of plasmids containing different HPV types sequences and EGFP and SEAP reporter genes

All plasmids containing sequences of the structural genes L1 and L2 of different HPV types and plasmids containing the sequences of reporter genes, i.e. EGFP and SEAP, were produced in quantities between 1 and 4 μg/μl by transformation into high efficiency prokaryotic cells (see paragraphs 3.2. and 3.4.) and subsequent extraction with QIAGEN Plasmid Maxi kit or QIAfilter Plasmid Maxi kit (see paragraph 3.5.). The quantification was carried out at the NanoDrop 2000 (ThermoScientific). The correctness of plasmids sequence was assessed by restriction enzyme (see paragraph 3.6.). Examples of p16shell, p18shell, p11shell.L1, p11shell.L2 and pYSEAP enzymatic restrictions are reported in figure 7.

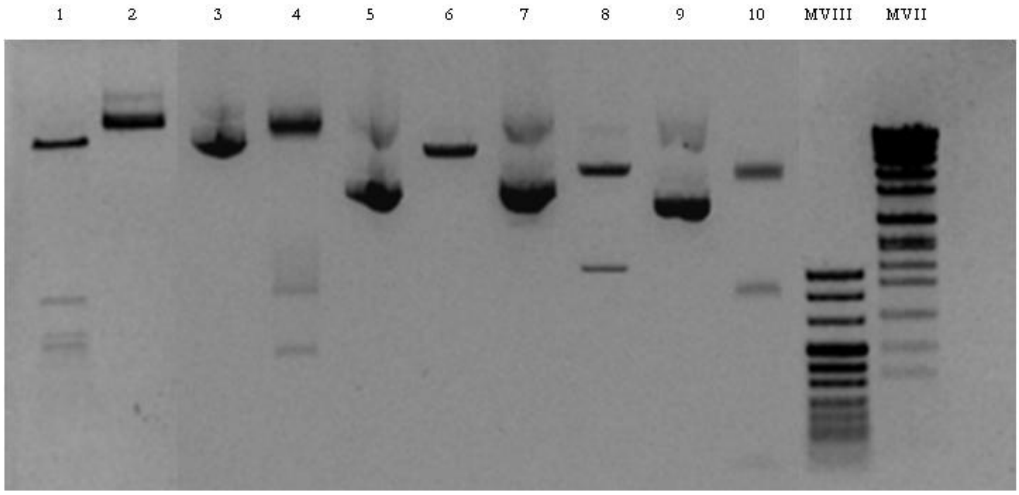


Figure 7. Examples of plasmid restrictions: p16shell plasmid after enzymatic restriction with PstI (1); uncut p16shell plasmid (2); uncut p18shell plasmid (3); p18shell plasmid after enzymatic restriction with PstI (4); uncut p11shell.L2 plasmid (5); p11shell.L2 plasmid after enzymatic restriction with BamHI (6); uncut p11shell.L1 plasmid (7); p11shell.L1 plasmid after enzymatic restriction with BamHI (8); uncut pYSEAP plasmid (9); pYSEAP plasmid after enzymatic restriction with SacI (10); molecular weight markers (11-12).

4.1.2. Evaluation of alkaline phosphatase expression by using a chemiluminescence assay in 293TT cells

Unselected and hygromycin-selected 293TT cells for 15 days were transfected with the positive control provided by the Great Escape kit (see paragraph 3.16.) in order to evaluate the transfection efficiency by the expression of alkaline phosphatase. Supernatants were collected 24, 48 and 72 hours after transfection and then processed and analyzed according to the manifacture's protocol. Chemiluminescence reading was performed by using different luminometers, one provided by Diagene and one by Promega. The results, in terms of raw luminescence (RLU: *relative light unit*), led to the conclusion that hygromycin-selected 293TT cells had the best transfection efficiency. Subsequently, unselected and hygromycin-selected 293TT cells for 15 and 30 days were transfected with pYSEAP plasmid (see paragraphs 3.2. and 3.7.). After luminometer reading we were able to conclude that transfection was carried out correctly and reading time was critical, as we observed a decrease in the chemiluminescence signal over time. 293TT cells selected with hygromycin for 15 days were the best for our experiments, according to the protocols of NCI-CCR, and were used for all the experiments here reported.

4.1.3. Evaluation of EGFP reporter gene expression in transfected cells

293TT cells selected with hygromycin for 15 days and unselected were transfected with pfwb plasmid in order to assess EGFP expression by the reporter. Direct observation of cells at fluorescence microscope (Leica 6500CTR HS) or after fixation in dimethyl ketone at 95% showed that 50-60% of cells were EGFP-positive (figure 8).

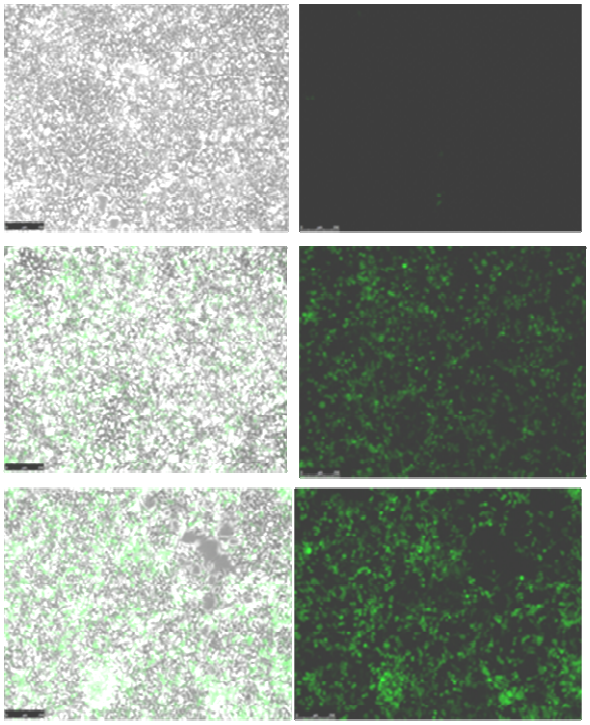


Figure 8. Direct observation of 293TT cells transfected with pfwb plasmid at fluorescence microscope: negative control (top), unselected (middle), hygromycin selected cells for 15 days (bottom).

4.1.4. Evaluation of HPV16 L1 protein expression in transfected cells

In order to observe the expression of HPV16 L1 protein in hygromycin-selected 293TT cells, cells were transfected with p16shell plasmid in single and coupled with pfwb plasmid. Subsequently, cells were harvested, fixed in dimethyl ketone at 95% and labeled with anti-HPV16 L1 specific antibody followed by the FITC dye (see paragraph 3.15.). Expression of L1 protein was observed in cells transfected with p16shell plasmid only, while the expression of EGFP was observed in cells co-transfected with p16shell and pfwb plasmids (figure 9).

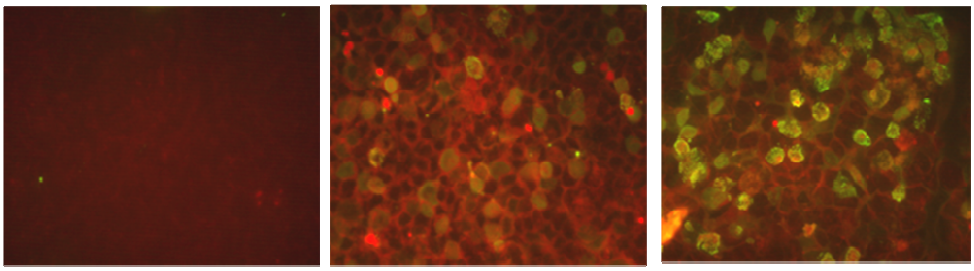


Figure 9. Immunofluorescence on 293TT transfected cells labeled with specific anti-HPV16 L1 antibody: negative control (left), cells transfected with p16shell plasmid only (middle), expression of EGFP in cells transfected with p16shell and pfwb plasmids (right).

4.1.5. Evaluation of HPV16 L2 protein expression by immunofluorescence

In order to evaluate the expression of HPV16 L2 protein in hygromycin-selected 293TT cells, cells were transfected with p16shell plasmid. After 48 hours, cells were collected, fixed in dimethyl ketone at 95% and labeled with anti-HPV16 L2 specific antibody (Santa Cruz Biotechnology), followed by the FITC dye. There was no difference between negative control and transfected cells (figure 10). This result was not surprising because, as mentioned in NCI-CCR protocols, the expression level of L2 protein was demonstrated to be lower as compared to the expression of L1 protein.

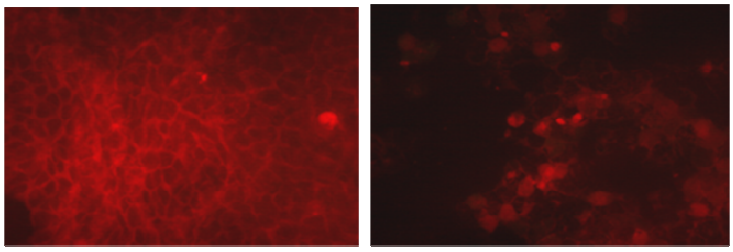


Figure 10. Immunofluorescence on 293TT transfected cells labeled with specific anti-HPV16 L2 antibody: negative control (left) and cells transfected with p16shell plasmid (right).

4.1.6. Evaluation of HPV16 L1 protein expression by Western blotting

The expression of HPV16 L1 protein was also evaluated by Western blotting. 293TT cells were seeded in 6-well plates at two different concentrations (50.000 and 150.000 per well) and subsequently transfected with p16shell plasmid. After 48 hours, cells were collected and protein concentration was quantified (see paragraphs 3.11. and 3.12.) Both cells transfected with p16shell plasmid showed the expression of 55 kDa HPV16 L1 protein (figure 11).

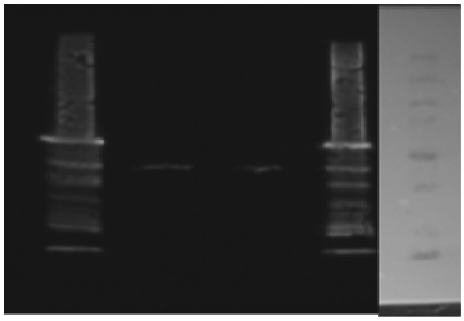


Figure 11. Western blotting analysis performed on 293TT cells transfected with p16shell plasmid and labeled with specific anti-HPV16 L1 antibody. 50.000 cells (left column), negative controls (two columns in the middle) and 150.000 cells (right column). Most intense bands correspond to HPV16 L1 protein of 55 kDa.

4.1.7. Evaluation of VLPs transducion ability by flow cytometry

First, 293TT cells were seeded in 6-well plates (200.000 cells per well) and then transduced in triplicate with different concentrations of non purified cell lysate (concentrated, 1:10, 1:100) containing VLPs. VLPs were obtained after the co-transfection of p16shell and pfwb plasmids, a maturation step at 37 °C overnight and were not purified by Optiprep gradient. After 48 hours, transduced cells were harvested and resuspended in phosphate buffer in order to be analysed by flow cytometry. Results led to the conclusion that cells transduced with the concentrated lysate and with the lysate diluted 1:100 exhibited a comparable level of EGFP expression (approximately 30%), while cells transduced with the lysate diluted 1:10 showed a percentage of EGFP expression equal to 65%. Images obtained by flow cytometry analysis are shown in figure 12.

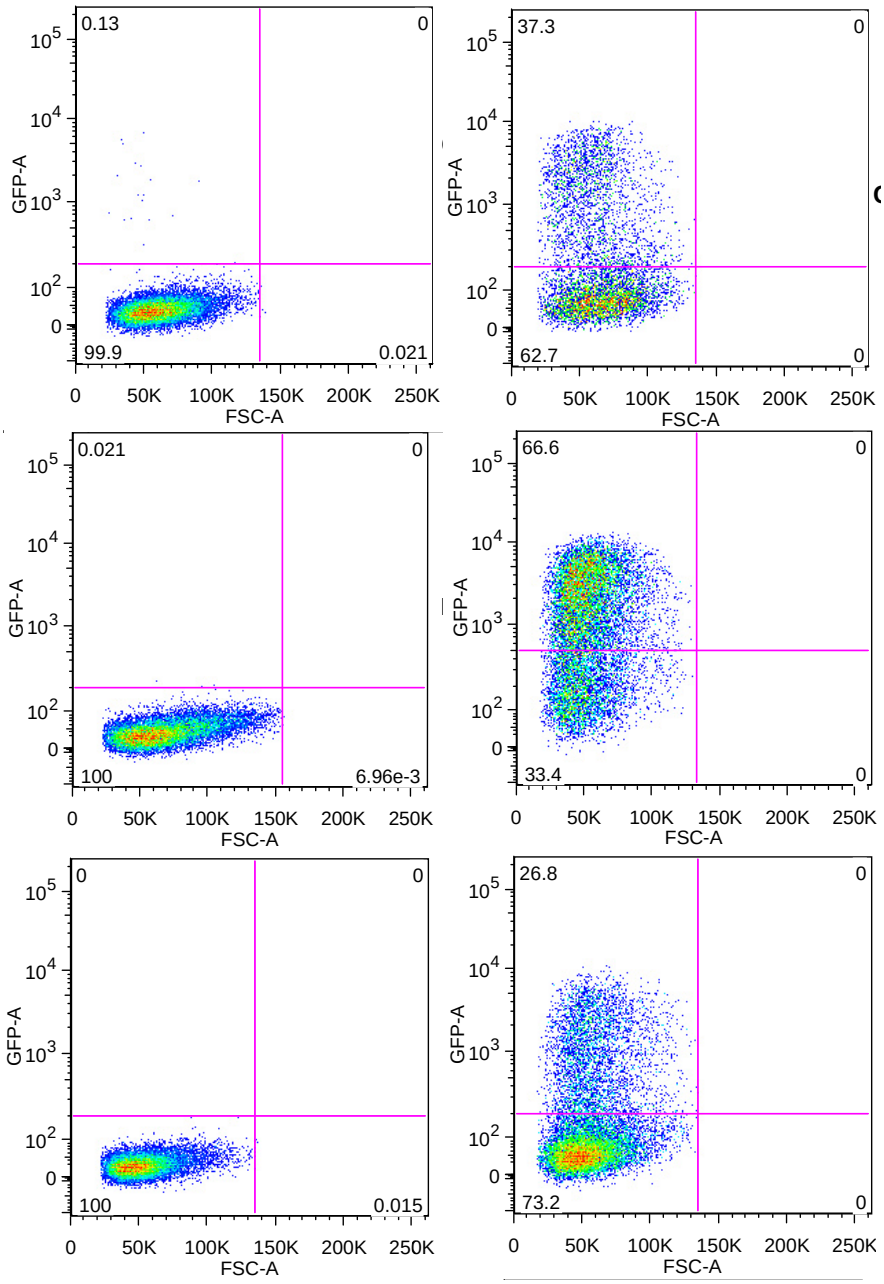


Figure 12. Images obtained by flow cytometry on crude lysate containing the VLPs: negative controls (left column from the top to the bottom: concentrated, diluted 1:10, diluted 1:100) and samples (right column from the top to the bottom: concentrated , diluted 1:10, diluted 1:100).

4.1.8. Comparison of VLPs transduction ability on 293TT cells and on keratinocytes

The experiment described in the previous paragraph was repeated on 293TT cells and keratinocytes, diluting the non-purified VLPs obtained by co-transfection of p16shell and pfwb plasmids at different concentrations (1:10, 1:20, 1:40, 1:80 and 1:160). No EGFP expression was observed in keratinocytes, whereas a gradual reduction of EGFP expression was observed in 293TT cells. With VLPs dilutions 1:80 and 1:160, highest expression levels of EGFP were achieved with percentages between 1-25%, as required by the NCI-CCR protocol. These dilutions allowed to estimate the VLPs titer in terms of 10^9 transducing units/ml.

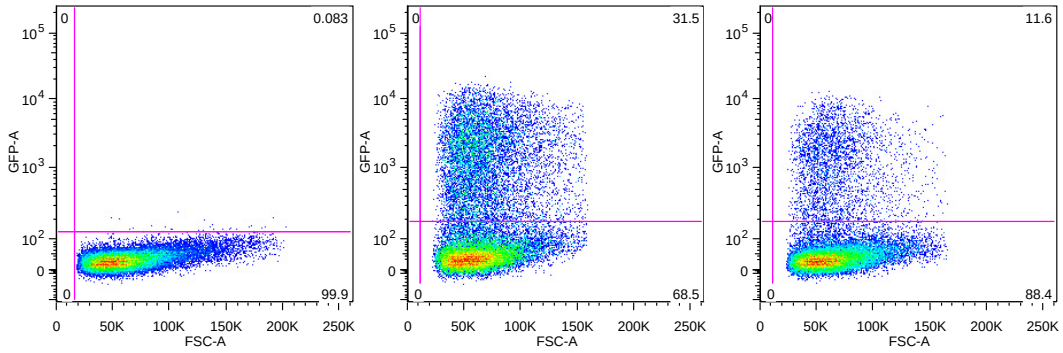


Figure 13. Images obtained by flow cytometry on the raw lysate containing the VLPs: negative control (left), raw lysate diluted 1:80 (middle) and 1:160 (right).

4.1.9. Evaluation of purified VLPs transduction ability in 293TT cells

VLPs obtained after transfection with p16shell and pfwb plasmids on 293TT cells were purified by Optiprep gradient (see paragraph 3.9.). DNA content of different fractions obtained after stratification on the gradient was estimated by fluorimeter (see paragraph 3.10.), while EGFP expression level was assessed by flow cytometry (see paragraph 3.14.). Subsequently, 293TT cells were transduced with different dilutions of single fractions of VLPs obtained by the gradient. Analysis of single fractions by fluorescence microscopy and flow cytometry, showed that fractions with highest transducing capability were those between the number 4 and the number 8, as defined by the NCI-CCR protocol. These experiments demonstrated that the purification step by Optiprep gradient was useful to identify the correct VLP fractions. Some images of transduced cells obtained by fluorescence microscope and by flow cytometry analysis are reported in figures 14 and 15.

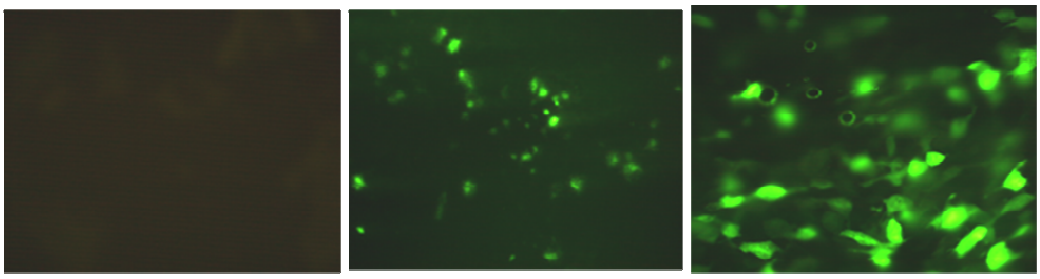


Figure 14. Fluorescence microscope images of 293TT cells transduced with fraction 7 containing purified VLPs: negative control (left), 1:10 dilution (middle) and concentrated (right)

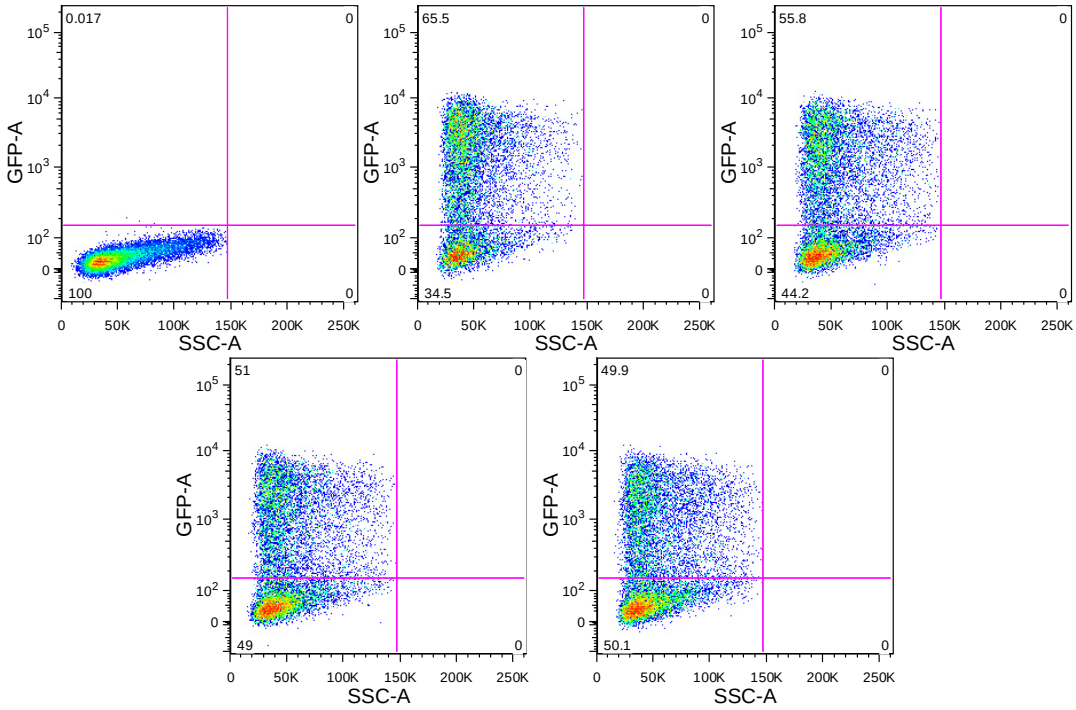


Figure 15. Flow cytometry analysis of different fractions containing purified VLPs: negative control (top left), fraction 4 (top center), fraction 5 (top right), fraction 6 (bottom left), fraction 7 (bottom right).

4.1.10. Evaluation of purified VLPs conformation by SDS-PAGE and Western blotting

Optiprep purified fractions of VLPs containing HPV16 L1 and L2 structural proteins and expressing EGFP or alternatively SEAP were analyzed by SDS-PAGE under non-reducing conditions and by Western blotting using the specific anti-HPV16 L1 antibody in order to assess the conformation of the virions. In figures 16 and 17, the dimeric and the trimeric forms of L1 protein can be appreciated. Moreover, in figure

17, the monomeric form of L1 protein can be observed after the treatment with β-mercaptoethanol.

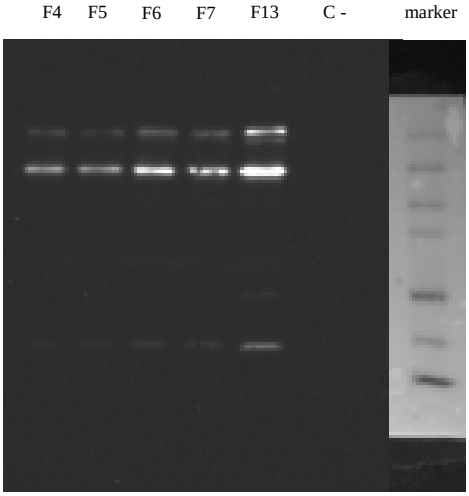


Figure 16. Western blotting performed on fractions purified by Optiprep gradient containing the VLPs (consisting of HPV16 L1 and L2 structural proteins and EGFP) after SDS-PAGE run in non-reducing conditions. The labeling was performed with specific anti-HPV16 L1 antibody. Most intense bands correspond to different forms of L1 protein: trimeric (higher bands) and dimeric (lower bands).

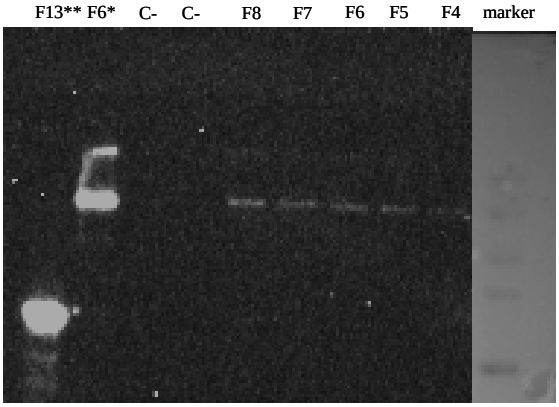


Figure 17. Western blotting performed on fractions purified by Optiprep gradient containing the VLPs (consisting of HPV16 L1 and L2 structural proteins and SEAP) after SDS-PAGE run in non-reducing conditions. The labeling was performed with specific anti-HPV16 L1. Bands correspond to different forms of L1 protein: trimeric (higher bands), dimeric (intermediate bands) and monomer (lower band). F13** corresponds to F13 reported in figure 17, treated with β-mercaptoethanol; F6* corresponds to F6 reported in figure 16. Both these fractions were used as positive controls.

4.2. Set up and standardization of HPV PBNA: summary of results

This part of the PhD research activity was focused on better characterization and production of HPV16 VLPs, in order to extend the method for other HPV VLP types. Co-expression of L2 protein in association with L1 expression was demonstrated by SDS-PAGE and Western blotting. Transducing ability and viral titer of HPV16 VLPs purified with the less time-consuming method, based on polyacrilamide desalting column, was assessed in comparison with Optiprep-purification. Neutralization experiments were set up by using HPV16 VLPs containing EGFP and SEAP reporter plasmids. Percentage of EGFP expression was evaluated by flow citometry and by immunofluorescence. Level of SEAP activity was evaluated by using SEAP Reporter Gene Assay. In particular, neutralizing effect on VLPs was observed by using the positive reference serum at different dilutions provided by WHO and by using heparin at concentration recommended by Human Papillomavirus Laboratory Manual (WHO). A comparison between two different SEAP detection kits was made on different luminometers provided by Biotek, TECAN and BMG, in order to evaluate the most sensitive and precise assay (Roche vs Clontech), as well as the most sensitive instrument. After evidences based on first set up experiments, neutralization assays were performed by using SEAP Reporter Gene Assay (Roche) on different sera, in particular, one collected from an adult female, one year after HPV vaccination with GARDASIL®. Neutralizing titer against HPV16 was higher than 1:10,240. For the set up of HPV16 neutralization assay, specific neutralizing monoclonal antibodies were tested (i.e. D9 and V5). V5 showed an excellent neutralizing activity at recommended dilutions (1:20.000 and 1:100.000) as expected from reports in the literature (Roden *et al.,* 1997; Day *et al.*, 2007). Neutralization assays were performed using VLPs containing structural proteins (L1 and L2) of the other HPV types 18, 6, and 11. Serum from the vaccinee showed neutralizing titer equal to 1:2,560 against HPV18 and HPV6, while the neutralizing titer against HPV11 was 1:1,280. VLPs containing non vaccinal high risk HPV types sequences (i.e. HPV31, 45, 52, 58) were produced, followed by the setting up of specific PBNA in order to evaluate the presence of cross-neutralizing antibodies induced by the vaccination. Pilot experiments were made by using sera from vaccinee that showed neutralizing effect against HPV31. ELISA assay was also set up in order to have data of the whole amount of anti-HPV IgG elicited by vaccination. ELISA experiments confirmed and correlated with PBNA results and indicated that the vaccine induced a specific immune response against the four vaccinal HPV types.

4.2.1. Evaluation of L2 expression in Optiprep-purified VLPs by SDS-PAGE and Western blotting

After the assessment of L1 protein, Optiprep-purified fractions of HPV16 VLPs were analyzed by SDS-PAGE and Western blotting (see paragraph 3.12.) using a specific antibody anti-HPV16 L2 (2JGmab#4, SantaCruz Biotechnology Inc.) in order to evaluate the co-expression of L2 protein in association with L1 expression. Fractions containing VLPs composed by L2 protein are shown in figure 18.

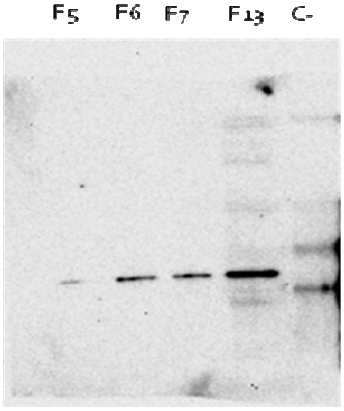


Figure 18. Western blotting performed on Optiprep-purified fractions containing HPV16 VLPs (consisting at structural level of L1 and L2 proteins and containing EGFP reporter plasmid) after SDS-PAGE. Blotting was performed with specific anti-HPV16 L2 antibody (2JGmab#4).

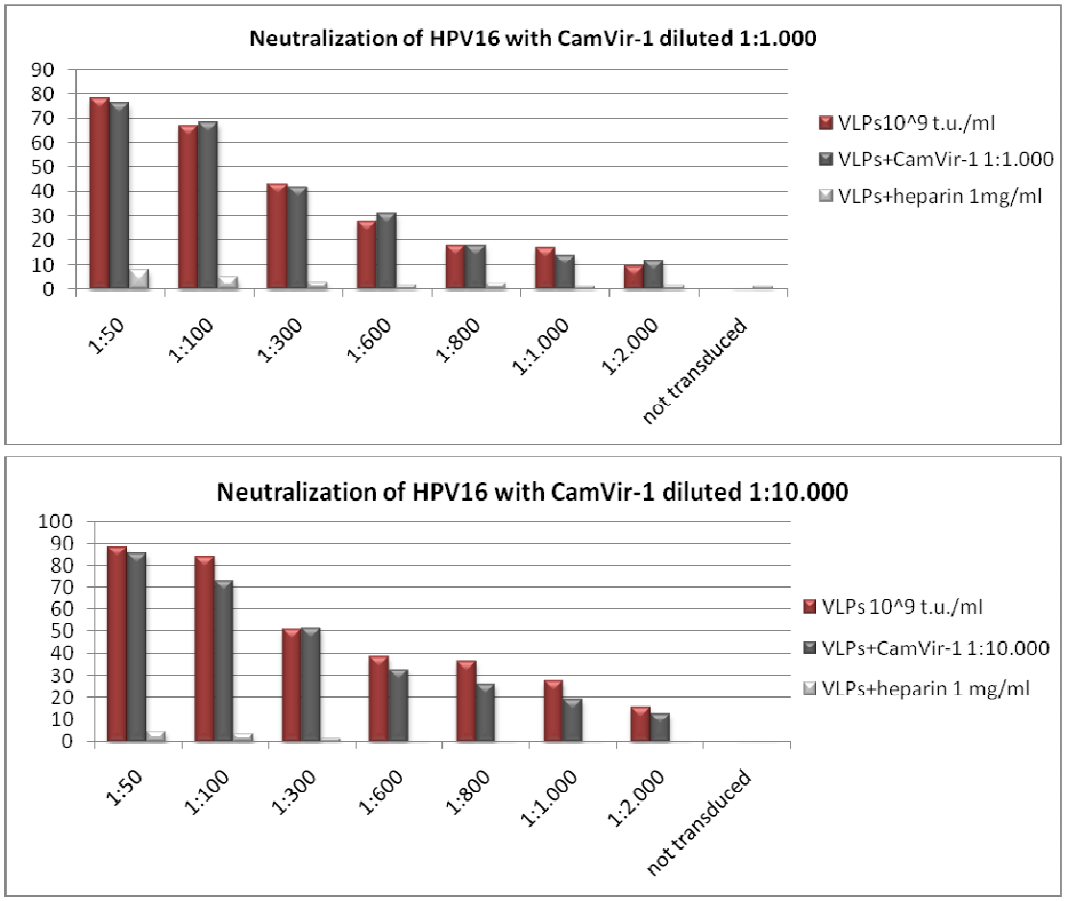
4.2.2. Assessment of transduction ability of VLPs purified with polyacrylamide desalting column

Purification of VLPs obtained by transfection of 293TT cells with p16shell and pfwb plasmids was performed also by polyacrylamide desalting column (Thermo Scientific Pierce) (see paragraph 3.9.) in order to compare this method with time-consuming Optiprep-purification. DNA content of each fraction was estimated by Quant-iT PicoGreen dsDNA assay (see paragraph 3.10.), while EGFP expression level was assessed by flow cytometry after transduction of 293TT cells with different fractions (see paragraph 3.14.). Analysis of transduced cells by fluorescence microscopy and flow cytometry showed that the fractions with higher transducing capability were those between numbers 17 and 20. Comparing two purification methods, no differences were noticed in viral titer obtained (both were 10^9 transducing units/ml). However, polyacrylamide desalting column resulted the faster purification method in

comparison with Optiprep and was applied for the following VLPs purification (i.e. HPV6, 11, 18, 31, 45, 52, 58 VLPs containing SEAP reporter plasmid).

4.2.3. PBNA set up

HPV16 VLPs containing EGFP and SEAP reporter plasmids were used to set up neutralization experiments by using a specific anti-HPV16 L1 antibody (CamVir-1, Santa Cruz Biotechnologies Inc.; dilutions 1:1.000 and 1:10.000) and heparin (1 mg/ml) as positive neutralization control. HPV16 VLPs with EGFP were diluted (1:50, 1:100, 1:300, 1:600, 1:800, 1:1.000, 1:2.000) and incubated onto 293TT cells with or without antibody or heparin. Percentage of EGFP expression was evaluated by flow citometry and also by immunofluorescence (see paragraphs 3.14. and 3.15.). Results are reported in figures 19 and 20. The antibody CamVir-1 was not able to neutralize VLPs, while heparin, used as control, blocked VLPs infection. This is due to the intrinsic characteristics of the antibody. In fact, this antibody is not able to recognize neutralizing epitopes on the VLPs surface.



Figures 19 and 20. Neutralization assay performed with HPV16 VLPs containing EGFP reporter plasmid in association with specific anti-HPV16 L1 antibody diluted 1:1.000 or 1:10.000 and heparin. Dilutions of VLPs are reported in the abscissa, while EGFP expression percentage is reported in the ordered.

HPV16 VLPs with SEAP were diluted (1:150, 1:500, 1:1.000, 1:10.000) and incubated onto 293TT cells with or without heparin at different concentrations (0,1 mg/ml and 1 mg/ml), as well as with positive reference serum (anti-human papillomavirus type 16 serum (1st International Standard) WHO International Standard or Reference Reagent NIBSC code: 05/134) at different dilutions (1:5, 1:10, 1:20, 1:40). Level of SEAP activity was evaluated by using SEAP Reporter Gene Assay (see paragraph 3.16.). Results are reported in figures 21 and 22. Heparin had greater neutralizing effect at the greatest concentration (1 mg/ml) (figure 21). This concentration is recommended by Human papillomavirus laboratory manual (WHO/IVB/10.12; First edition, 2009). In the second figure (figure 22), neutralizing effect by HPV16 positive reference serum against HPV16 VLPs is highlighted.

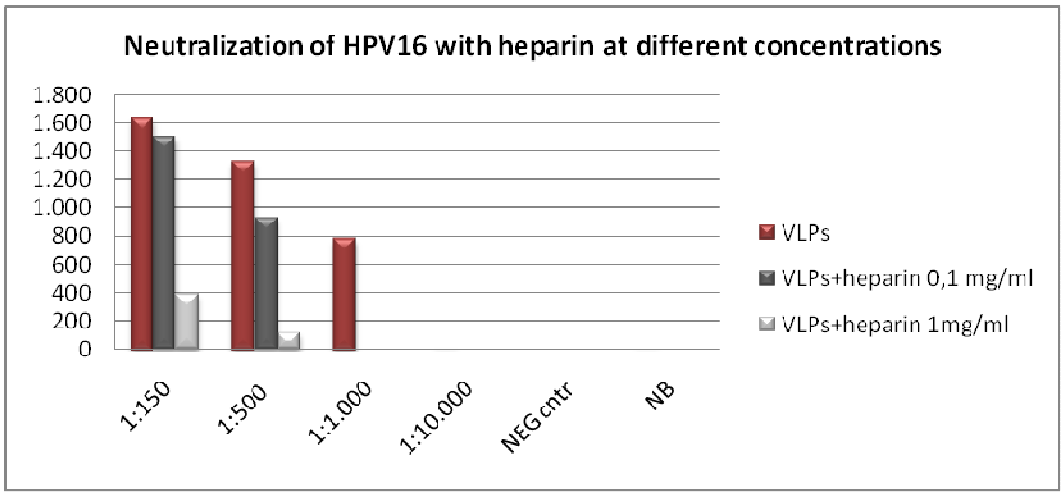


Figure 21. Neutralization assays performed with HPV16 VLPs with SEAP reporter plasmid in association with HPV16 positive heparin. Dilutions of VLPs are reported in the abscissa, while *relative light units* (RLU) of alkaline phosphatase are reported in the ordered.

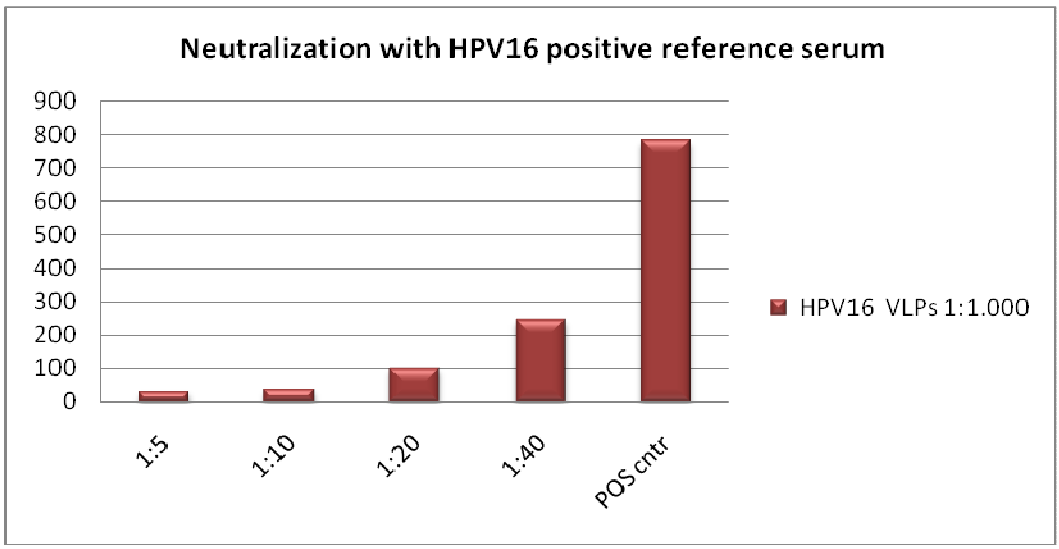


Figure 22. Neutralization assays performed with HPV16 VLPs with SEAP reporter plasmid in association with HPV16 positive reference serum. The neutralizing effect by HPV16 positive reference serum at different dilutions (between 1:5 and 1:40) against HPV16 VLPs is shown.

4.2.4. PBNA using different SEAP detection kits and luminometers

A comparison between two different commercial SEAP detection kits (see paragraph 3.16.) was made on different luminometers provided by Biotek, TECAN, and BMG, in order to identify the most sensitive and precise assay (kit by Roche vs kit by

Clontech) and the most sensitive instrument. HPV16 VLPs with SEAP reporter plasmid were diluted (1:150, 1:500, 1:1.000, 1:10.000) in neutralization buffer and three identical neutralization assays were performed. Supernatants were than evaluated in parallel with two different SEAP detection kits. SEAP Reporter Gene Assay (Roche) gave better results with all three instruments than SEAP Chemiluminescence Kit 2.0 Great Escape (Clontech). Taking in consideration both detection kits, TECAN luminometer demonstrated higher sensitivity. Results reported in RLU are shown in table 1.

	BIOTEK		TECAN		BMG	
VLPs	Roche	Clontech	Roche	Clontech	Roche	Clontech
1:150	5,59E+05	1,17E+05	6,50E+06	1,04E+06	1628	591
1:500	2,32E+05	8,29E+04	3,60E+06	6,08E+05	1323	330
1:1000	2,55E+05	4,04E+04	2,03E+06	3,44E+05	780	185
1:10,000	2,26E+03	5,04E+02	2,86E+04	3,70E+03	8	3
NEG cntr	3,60E+01	2,20E+01	7,06E+02	5,81E+02	0	2
NB	6,96E+02	1,76E+02	3,64E+03	9,96E+02	3	3

Table 1. Comparison between different SEAP detection assays (Roche vs Clontech) and luminometers (Biotek, TECAN and BMG).

4.2.5. PBNA with vaccinated female serum

After evidences based on first set up experiments, neutralization assays were performed by using different sera, in particular, one collected from an adult female, one year after HPV vaccination with GARDASIL®. Positive controls of transduction onto 293TT cells were VLPs alone. HPV16 positive and negative reference sera were provided by NIBSC. The first international standard was an anti-HPV16 serum (WHO code: 05/134), while the second one was a negative reference serum. In PBNA performed with HPV16 VLPs, the vaccinated subject showed a neutralizing titer higher than 1:80, as well as the HPV16 positive reference serum. The titer is defined as the reciprocal of the highest dilution of serum that reduces the SEAP activity by at least 50% in comparison to the reactivity in the wells that received VLPs but no antibody. Negative reference serum showed no neutralizing effect, as expected. Results are reported in figures 23, 24 and 25.

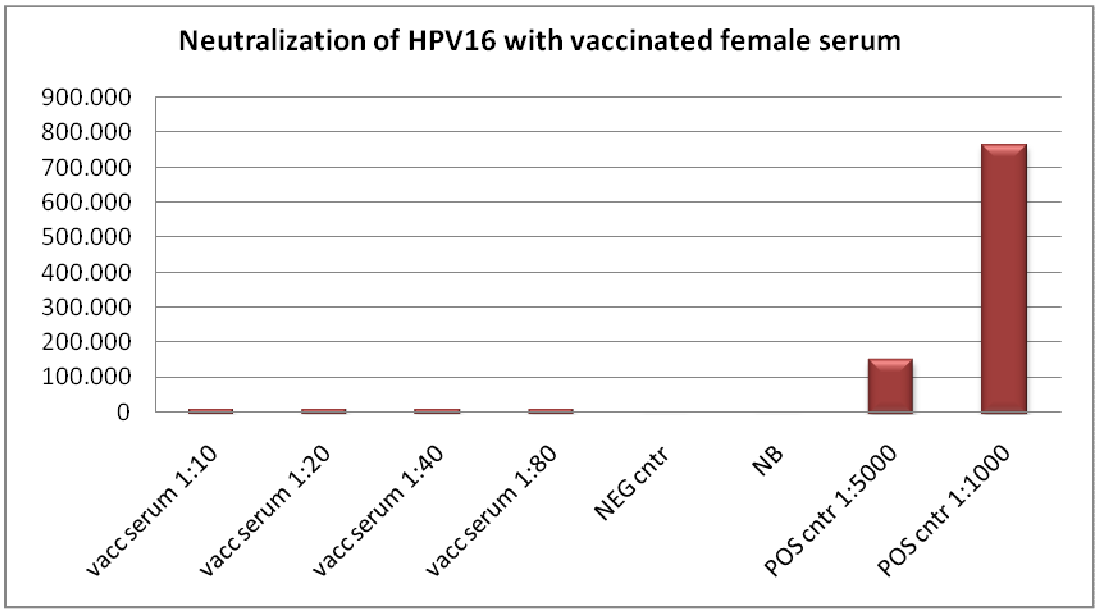


Figure 23. Neutralization of HPV16 VLPs by a vaccinated female serum, collected one year after the completion of the vaccination cycle. Neutralizing titer is higher than 1:80, thus indicating that HPV vaccination induced a good and specific humoral response.

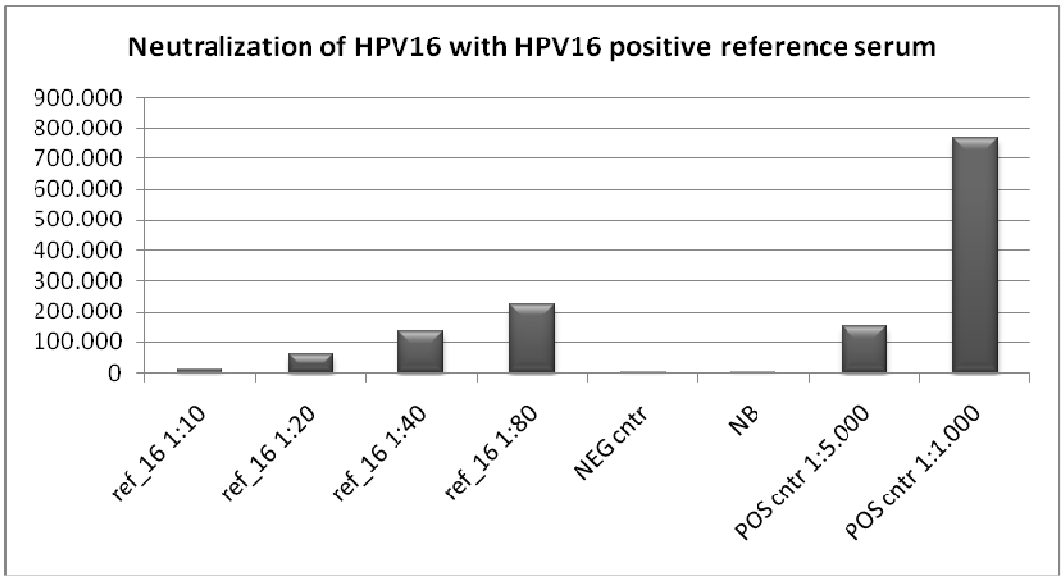


Figure 24. Neutralization of HPV16 VLPs by the HPV16 positive reference serum (WHO code: 05/134). Neutralizing titer is higher than 1:80, thus indicating that reference serum had specific neutralizing antibodies against HPV16.

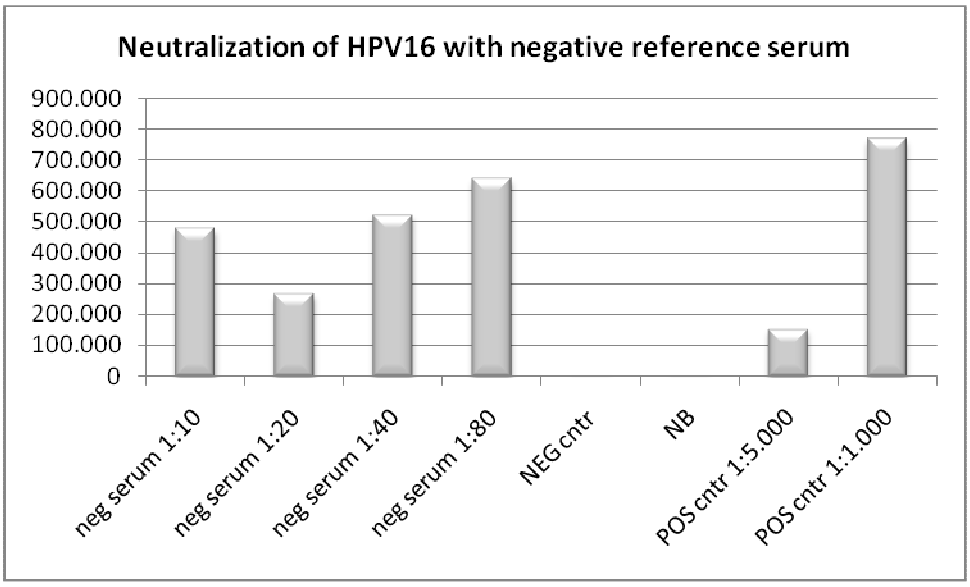


Figure 25. No neutralizing effect on HPV16 VLPs elicited by the negative reference serum.

To evaluate the neutralizing titer against HPV16 of vaccinated female with more accuracy, serial dilutions of her serum starting from 1:40 were made. Neutralizing titer was higher than 1:10,240, as reported in figure 26.

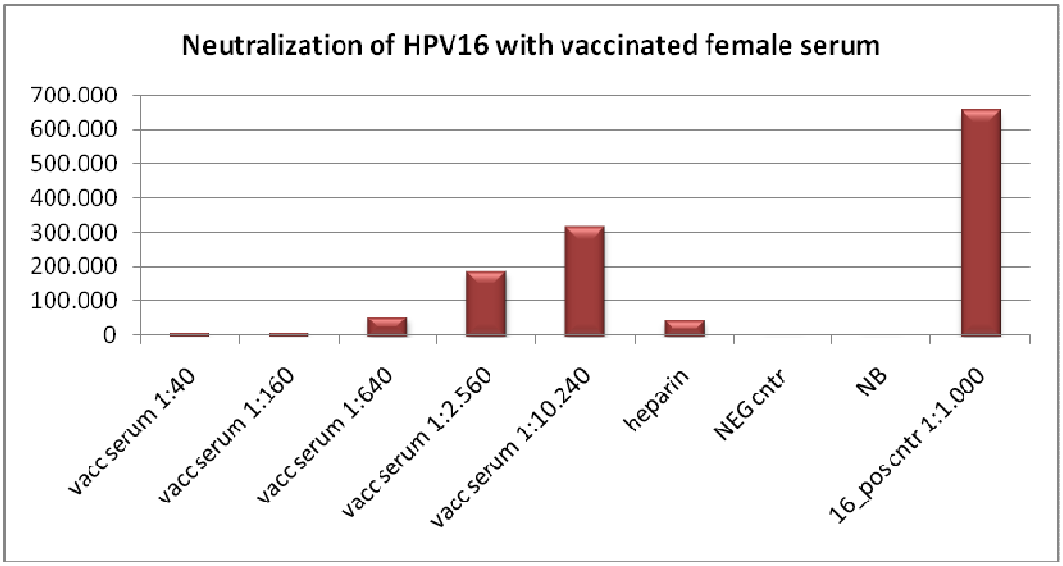


Figure 26. Neutralization of HPV16 VLPs by vaccinated female serum, collected one year after the completion of the vaccination cycle. Neutralizing titer is higher than 1:10,240, thus indicating that HPV vaccination induced a good and specific humoral response.

4.2.6. PBNA with specific neutralizing monoclonal antibodies

For HPV16 neutralization assay set up, specific neutralizing monoclonal antibodies were tested. Professor Christiansen kindly send us three of them: J4 (anti-HPV18), D9 (anti-HPV16) and V5 (anti-HPV16). D9 showed no satisfactory neutralizing effect, despite the cross-reactivity between different HPV types declared. V5 showed an excellent neutralizing activity at recommended dilutions (1:20.000 and 1:100.000) as expected from articles reported in the literature (figure 27).

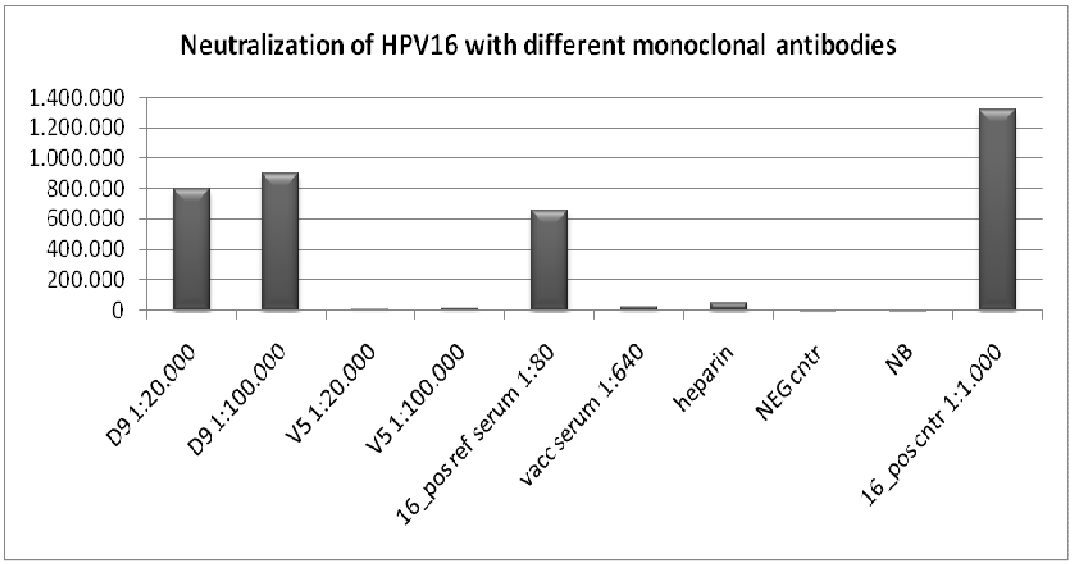


Figure 27. Neutralization of HPV16 VLPs with two different monoclonal antibodies (i.e. D9 and V5). D9 shows no satisfactory neutralizing effect, instead of V5.

4.2.7. PBNA using other HPV types (i.e. HPV types 18, 6, 11)

Neutralization assays were performed using VLPs containing L1 and L2 of other HPV types, i.e., HPV18, 6, 11. Heparin was used as a positive control for neutralization. In PBNA with HPV18 VLPs, serum from a vaccinated female showed neutralizing titer equal to 1:2,560, while negative reference serum showed no neutralizing effect (figures 28 and 29). Mild neutralizing effect was observed using specific anti-HPV18 monoclonal antibody, J4, at different dilutions (figure 30).

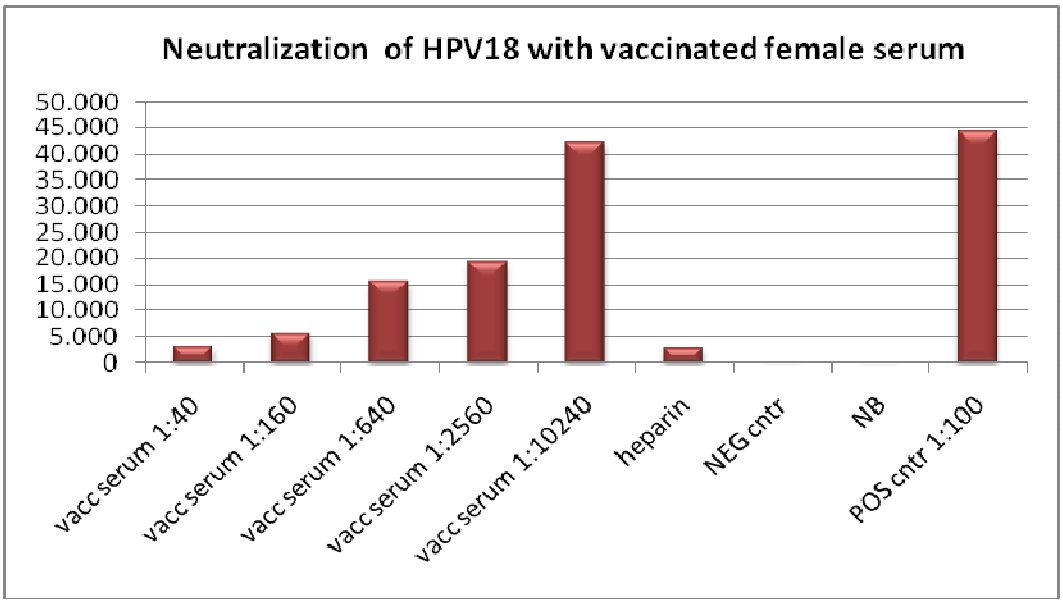


Figure 28. Neutralization of HPV18 VLPs with vaccinated female serum, collected one year after the completion of the vaccination cycle. Neutralizing titer is equal to 1:2,560.

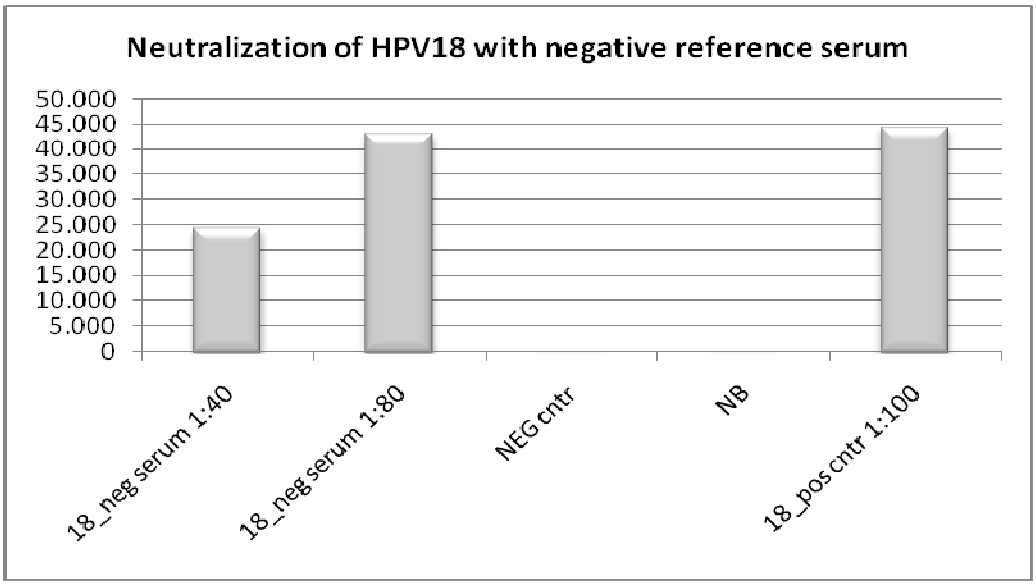


Figure 29. No neutralizing effect on HPV18 VLPs by the negative reference serum.

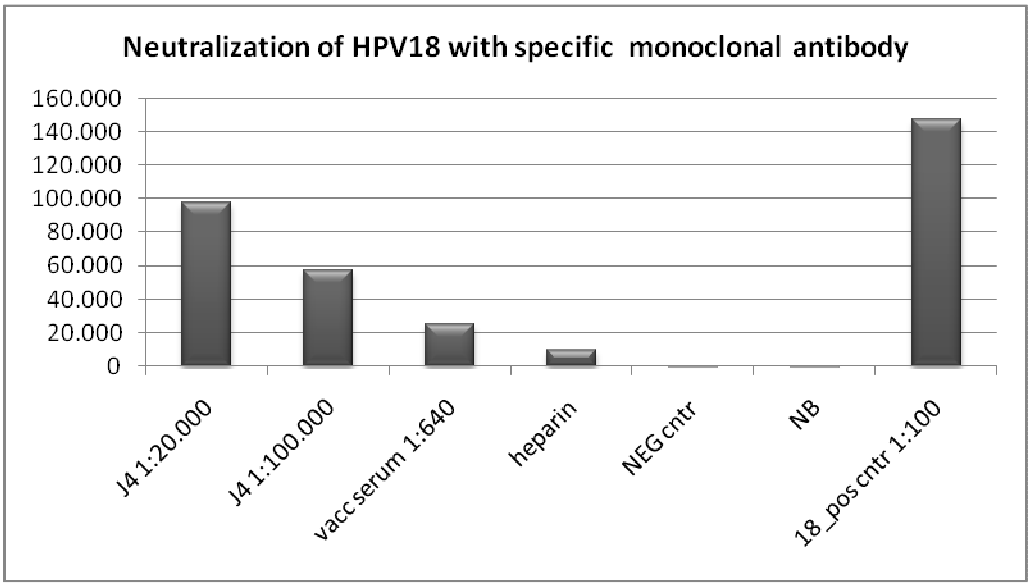


Figure 30. Neutralization of HPV18 VLPs with specific monoclonal antibody, J4, at different dilutions. Mild neutralizing effect was observed.

In PBNA with HPV6 VLPs, vaccinated female serum showed a neutralizing titer equal to 1:2,560, as reported in figure 31. Negative reference serum showed no neutralizing effect, as expected (figure 32).

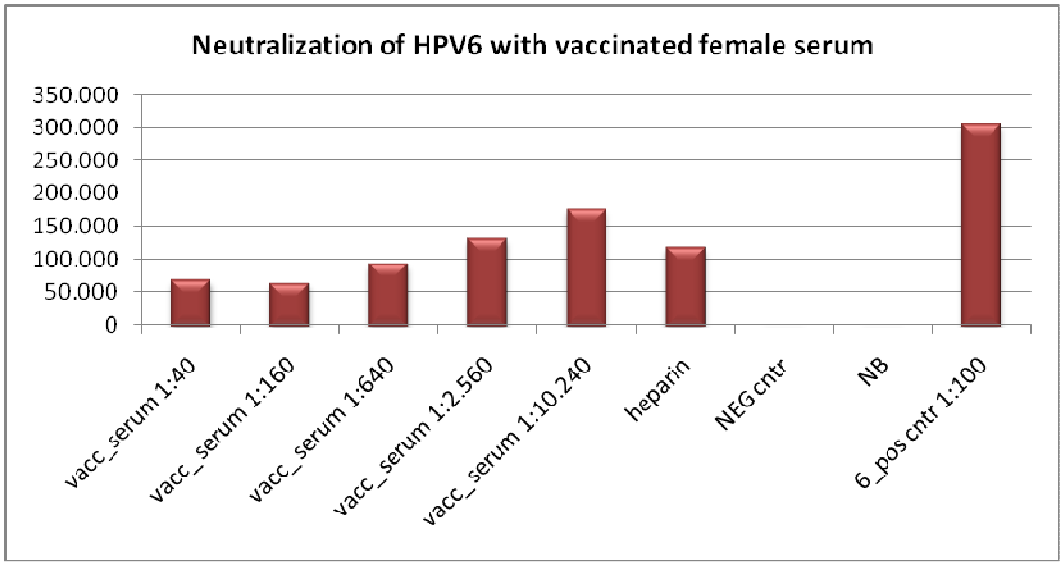


Figure 31. Neutralization of HPV6 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. Titer is equal to 1:2,560.

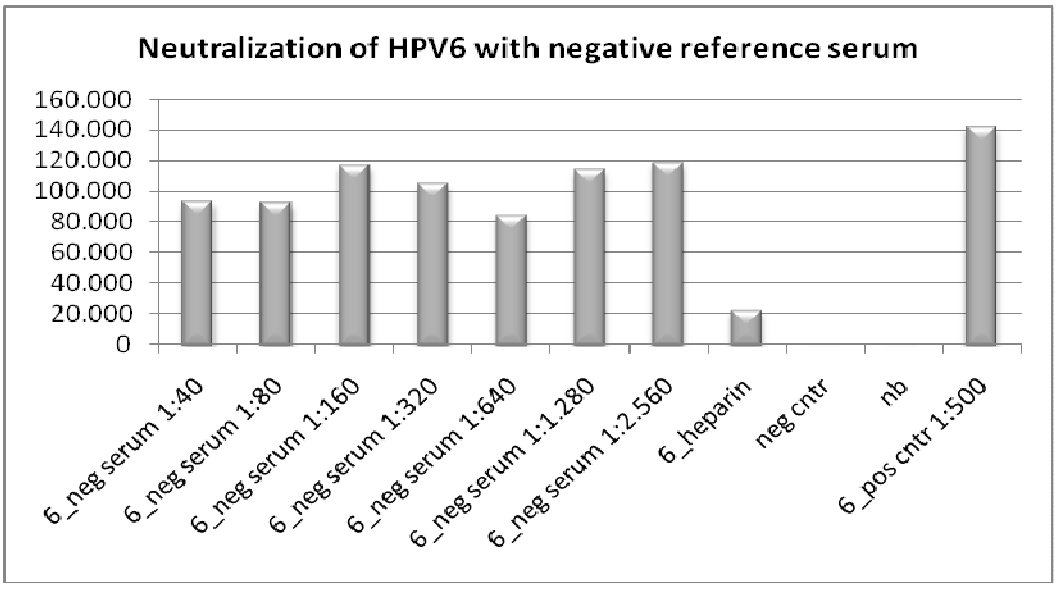


Figure 32. No neutralizing effect on HPV6 VLPs by negative reference serum.

Finally, neutralization was also performed against HPV11 VLPs. Vaccinated female serum showed a neutralizing titer equal to 1:1,280, as shown in figure 33.

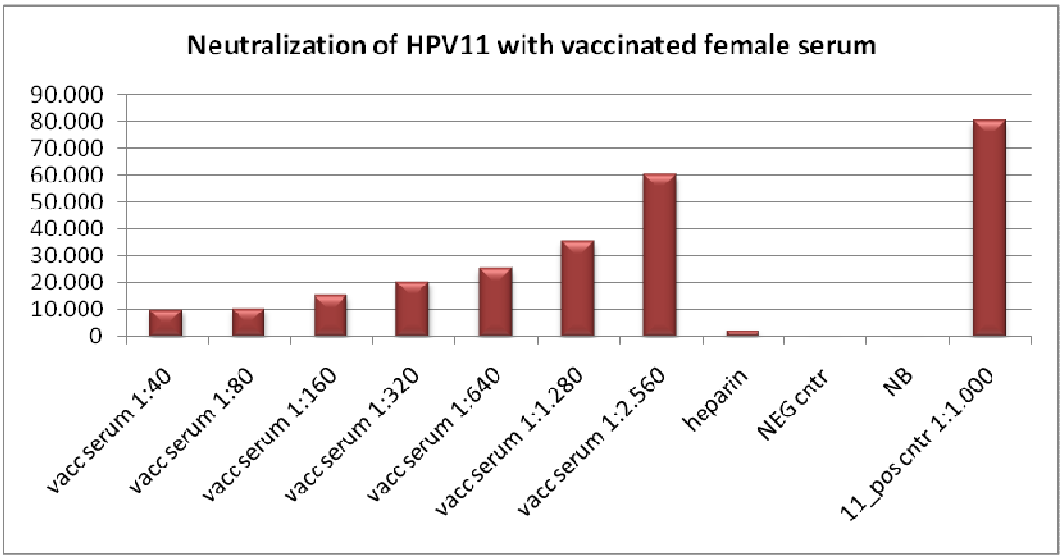


Figure 33. Neutralization of HPV11 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. Titer is equal to 1:1,280.

4.2.8. ELISA set up

ELISA for different HPVs L1 were also set up (see paragraph 3.18.) in order to have data of the whole amount of anti-HPV IgG elicited from vaccination. Coating was performed overnight at 4 °C with different concentrations of HPV16 VLPs (0.5 μg

and 1 μg) in PBS solution. Vaccinated female serum was used at dilution recommended by WHO Human Papillomavirus Laboratory Manual (i.e. 1:10, 1:31,6, 1:100). A specific anti-human IgHRP (γchain) antibody was used to detect specific human anti-HPV IgG. Best and more specific results were obtained by using the concentration of 0.5 μg of VLPs, while good results were also obtained with the concentration of 1 μg of VLPs (figures 34 and 35). The absorbance value of negative control was lower than 0.2, while the absorbance value of positive serum, which can be also considered as a positive control cut-off, was higher than 1. Correlation index was higher than 0,95. Therefore, ELISA experiments confirmed and correlated with PBNA results and indicated that the vaccine elicited a specific immune response against four vaccinal HPV types.

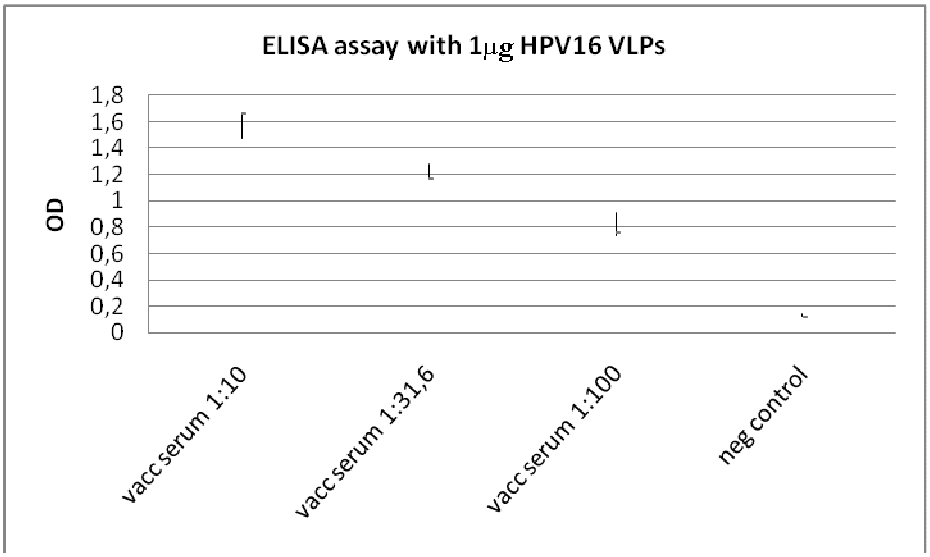


Figure 34. Results from ELISA performed with an overnight coating of 1 μg of HPV16 VLPs.

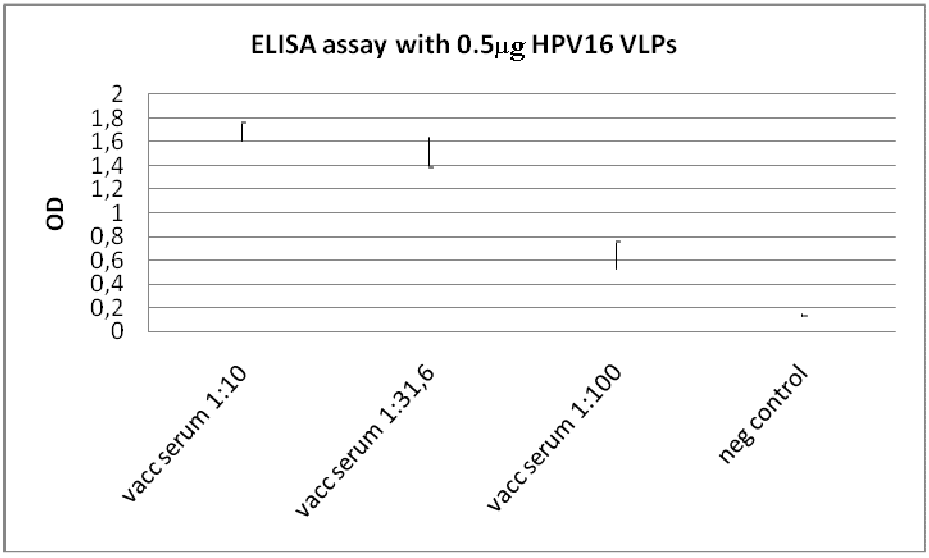


Figure 35. Results from ELISA performed with an overnight coating of 0,5 μg of HPV16 VLPs.

4.2.9. Production of VLPs containing non vaccinal high risk HPV types sequences

Different batches of VLPs containing coding sequences of structural viral proteins of non vaccinal high risk HPV types, i.e. HPV31, 45, 52 and 58, were obtained by transfecting 293TT cells with different HPV plasmids and pYSEAP plasmid containing the coding sequence of human placental alkaline phosphatase protein as reporter gene (see paragraphs 3.2., 3.7., 3.8.). All these VLPs were purified by using polyacrilamide desalting column (see paragraph 3.9.) and fractions were screened by L1 content by using NanoDropND 1000 Spectrophotometer at the wavelength of 280 nm, corresponding to the maximum absorption peak of the peptide bond (see paragraph 3.10.). Preparations were stored at -80 °C until use.

4.2.10. PBNA using non vaccinal high risk HPV types VLPs (i.e. HPV types 31, 45, 52, 58)

Neutralization assays using VLPs containing structural proteins (L1 and L2) of non vaccinal high risk HPV types, i.e. HPV31, 45, 52, 58, were set up and used to evaluate a vaccinated female serum sample. For all these assays, heparin was used as a positive control for neutralization. In PBNA with HPV31 VLPs, serum from the vaccinated female showed a neutralizing titer equal to 1:160 (figure 36). For other HPV types, no neutralizing effects were observed (figures 37, 38, 39).

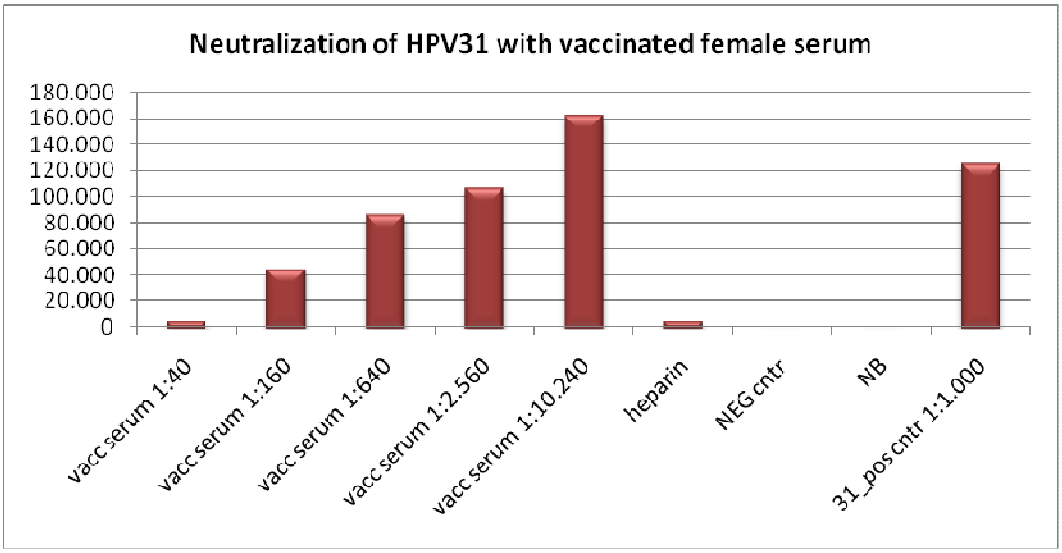


Figure 36. Neutralization of HPV31 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. Neutralizing titer is equal to 1:160.

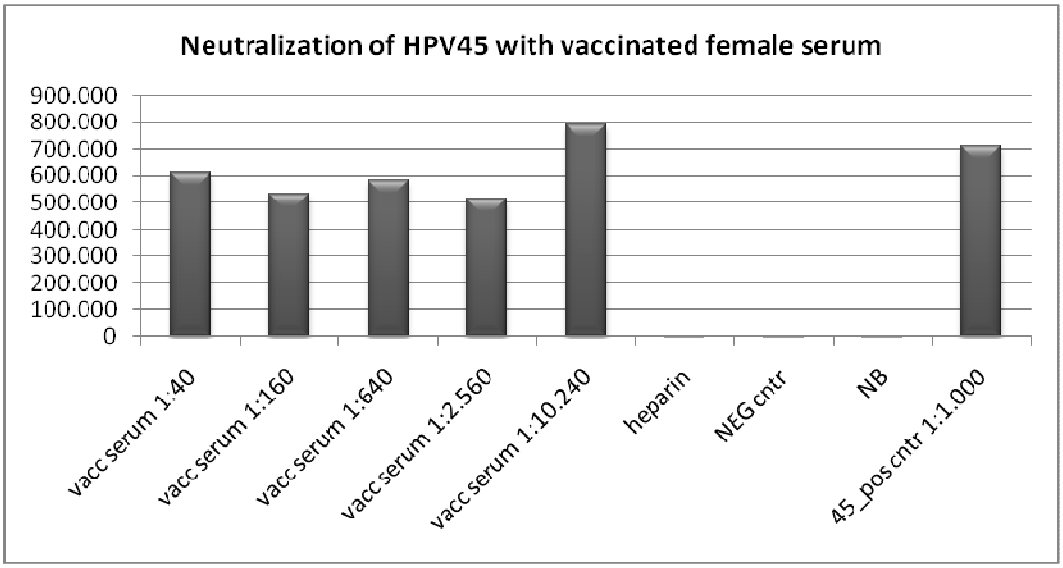


Figure 37. Neutralization of HPV45 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. No neutralizing effect on HPV45 VLPs was observed.

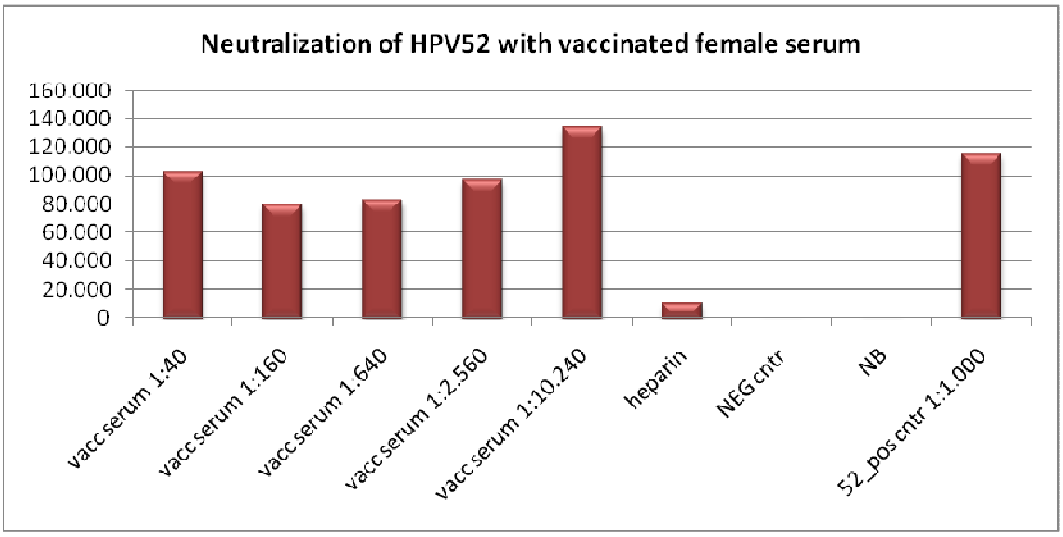


Figure 38. Neutralization of HPV52 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. No neutralizing effect on HPV52 VLPs was observed.

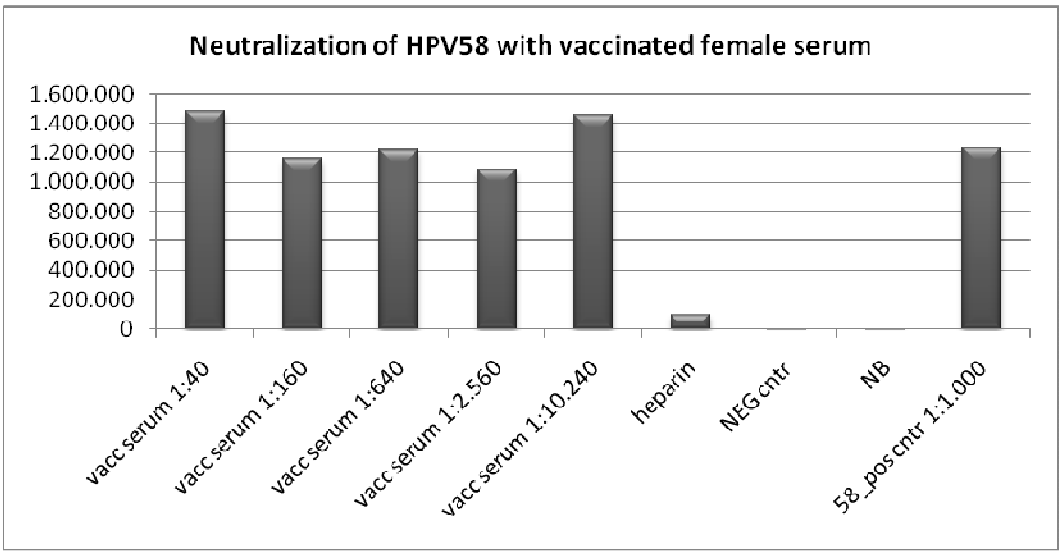


Figure 39. Neutralization of HPV58 VLPs with a vaccinated female serum, collected one year after the completion of the vaccination cycle. No neutralizing effect on HPV58 VLPs was observed.

Same neutralization assays were repeated by using a serum sample from a vaccinated male. There was no neutralizing effect against HPV31, 45, and 52, but a mild effect towards HPV58 (neutralizing antibody titer corresponding to 1:40) (figures 40, 41, 42, 43).

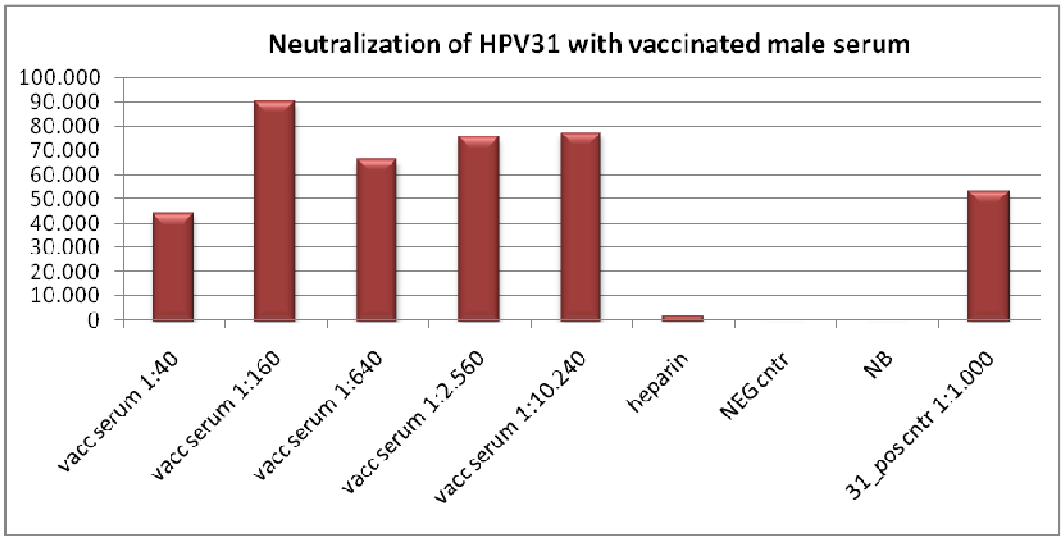


Figure 40. Neutralization of HPV31 VLPs with a vaccinated male serum. No neutralizing effect on HPV31 VLPs was observed.

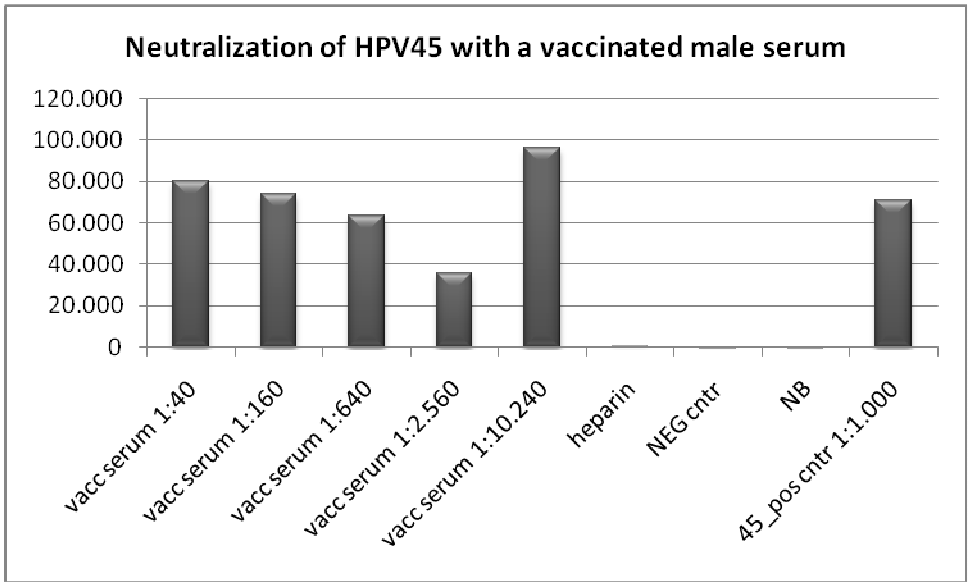


Figure 41. Neutralization of HPV45 VLPs with a vaccinated male serum. No neutralizing effect on HPV45 VLPs was observed.

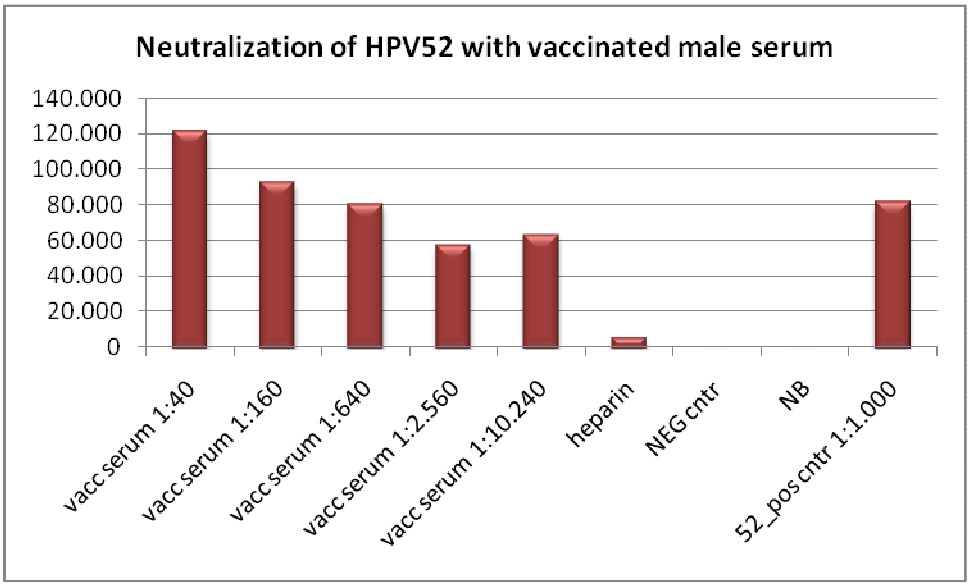


Figure 42. Neutralization of HPV52 VLPs with a vaccinated male serum. No neutralizing effect on HPV52 VLPs was observed.

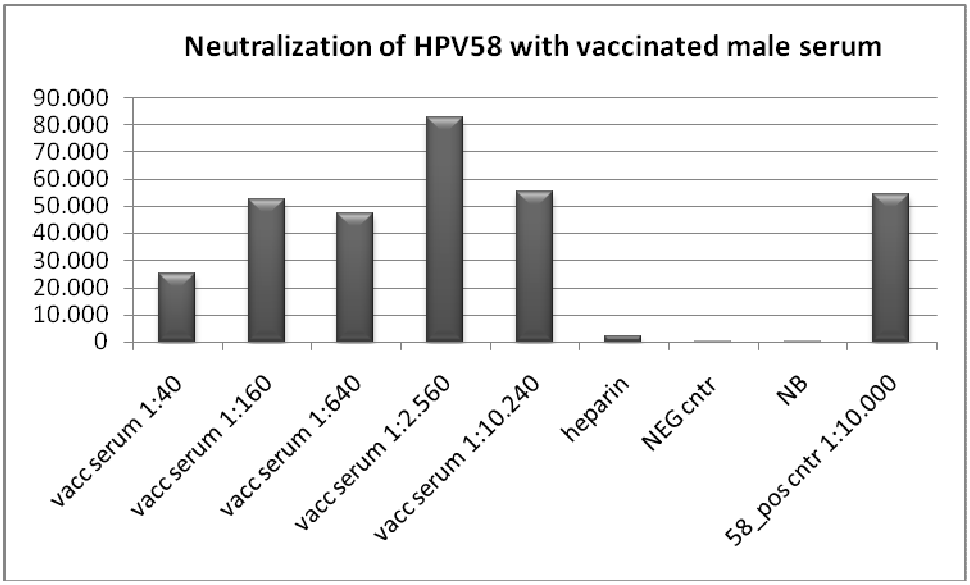


Figure 43. Neutralization of HPV58 VLPs with a vaccinated male serum. Neutralizing titer is equal to 1:40.

4.3. Clinical study to evaluate neutralizing antibodies induced by prophylactic HPV vaccines: summary of results

An observational cross-sectional study aiming to evaluate humoral immune responses against HPV in adolescents and healthy adults vaccinated with the two anti-HPV vaccines, GARDASIL® or CERVARIX™, was designed and started on

the 24th October 2011, after approval by ethics committee of Padova University Hospital (prot. 2413P). Almost 100% of subjects presented HPV type-specific (i.e. HPV16 and 18) neutralizing antibodies after the completion of the quadrivalent vaccine, with slight decline of antibody detection rate over time. Lower percentages of subjects presented neutralizing antibodies against HPV 6 and 11. Regarding non vaccinal high risk HPV types, HPV31 specific cross-neutralizing antibodies were observed in 50% of subjects vaccinated with the quadrivalent vaccine. The presence of neutralizing antibodies against four HPV vaccinal types was also evaluated in two vaccinated males. Finally, considering a group of females vaccinated with the bivalent vaccine, all subjects presented higher titers of neutralizing antibodies against HPV16 and HPV18 than subjects vaccinated with the quadrivalent vaccine. HPV31 cross-neutralizing antibodies were observed in 100% of subjects, while HPV45 and HPV58 cross-neutralizing antibodies were observed in small percentages of subjects. None presented cross-NAbs against HPV52.

4.3.1. Clinical study protocol to compare GARDASIL® or CERVARIX™ immunogenicity in vaccinated subjects

An observational cross-sectional study aiming to evaluate antibody responses against HPV in adolescents and healthy adults vaccinated with the two anti-HPV vaccines, GARDASIL® or CERVARIX™, was designed and started on the 24th October, 2011, after approval by ethics committee of Padova University Hospital (prot. 2413P).

Several partners were involved:

- Microbiology and Virology Unit, Padova University Hospital;
- Department of Histology, Microbiology and Medical Biotechnologies, Padova University;
- Hygiene and Public Health Unit, ULSS16 Padova;
- Hygiene and Preventive Medicine Unit, IRCCS Burlo Garofolo, Trieste;
- Prevention Department ASS4 "Medio Friuli", Udine;
- Microbiology and Virology Unit, Sant'Orsola Malpighi, Bologna University Hospital.

The duration of the study was 24-months and enrollment of 200 subjects aged from 12 years (the population of girls for whom free and active HPV vaccination is offered) to 45 years (the population of women for whom HPV vaccination is indicated) for both vaccines was planned. This study aimed to implement and standardize immunoassays in order to detect the immune response induced by HPV vaccine and to assess and compare the immunogenicity and cross-protection of HPV vaccines offered in Italy, CERVARIX™ and GARDASIL®. The objective of the

project was to promote the activation of immunological surveillance of the population who received prophylactic HPV vaccine.
The following primary and secondary goals were defined:
1) Standardization, validation and clinical definition of reference intervals of the following immunological tests: ELISA and PBNA for the determination of total and neutralizing antibodies to HPV, B-cell ELISPOT assay for the measurement of memory B-cells, T-cell ELISPOT assay for the measurement of memory T-cells induced by vaccination, in order to implement these assays in monitoring protocols of HPV vaccination;
2) Determination of the effectiveness of HPV vaccination (rates of serum conversion and development of cell-mediated immune response) in the vaccinated population;
3) Collection of data useful for the evaluation of immunological correlates of protection;
4) Comparison of the immunogenicity and the degree of cross-reaction of GARDASIL® and CERVARIX™ vaccines.
Criteria for inclusion in the study were: female sex, completion of vaccination no more than 6 months before, absence of concomitant chronic inflammatory diseases and cancer, absence of acute infections.
Criteria for exclusion from the study were: non-completion of vaccination, the presence of neoplastic diseases and chronic inflammatory diseases, pregnancy, male sex, fever and other symptoms of active infection.
Data analysis aimed:

1) to evaluate the distribution of type-specific total and neutralizing antibody titer against HPV16 and HPV18 in subjects who completed the third prophylactic vaccine dose, grouped by age and type of vaccine received;
2) to detect the positivity rate (the number of women with neutralizing antibody titer higher than 1:40), the percentage of subjects vaccinated with HPV L1 VLPs which were detectable in B-cells and memory T-cells by specific ELISPOT assay ("responders"), the induction of cross-neutralizing antibodies in vaccinated individuals (positivity rate and neutralizing antibody titer);
3) to compare the immunogenicity and cross-reactivity of the two vaccines CERVARIX™ and GARDASIL®.

4.3.2. PBNA in patients with natural infection

As control, the presence of HPV type specific neutralizing antibodies was evaluated in the serum of subjects (8 males and 3 females, age range: 24- 47 years old) who tested positive for HPV16 or HPV6 in the urethral or cervical swabs (table 2).

Patient (n.)	HPV infection	NAbs titer
1	HPV16	1:2,560
2	HPV6	1:160
3	HPV16	< 1:40
4	HPV16	< 1:40
5	HPV16	< 1:40
6	HPV16	< 1:40
7	HPV16	< 1:40
8	HPV16	< 1:40
9	HPV16	< 1:40
10	HPV16	< 1:40
11	HPV6	< 1:40

Table 2. HPV type specific neutralizing antibody titers in patients with HPV16 or HPV6 natural infection.

In 9 cases of natural infection caused by HPV16, only one subject presented HPV16 neutralizing antibody titer equal to 1:2,560. In 2 subjects with natural infection caused by HPV6, only one presented neutralizing antibody titer equal to 1:160.

4.3.3. PBNA in Gardasil® vaccinated subjects

Neutralization assays were performed on serum samples selected from a group of 100 subjects (all females; age range: 12-46 years old; mean age: 15,66 years old) collected in Veneto Region, where the quadrivalent Gardasil® vaccine was offered. The study group included 81 subjects investigated within 1-6 month since the completion of the three doses of vaccine and 7, 7, and 5 subjects investigated at 2, 3, and 4 years after vaccination, respectively. At 1-6 months after the completion of the vaccination cycle, 100% vaccines had neutralizing antibodies (NAbs) against HPV16, 98,8% had NAbs against HPV18, while 91% had NAbs against HPV6 and 50% had NAbs against HPV11. The NAbs titer ranged widely from 1:40 to over 1:10,240 and

was lower for NAbs against HPV6 and HPV11 than for NAbs against HPV16 and HPV18. A progressive reduction of NAbs titer was observed with time from vaccination and, at 4 years from vaccination, 80% of subjects had NAbs against HPV16, HPV18 and HPV6, and 60% against HPV11 (table 3).

Time post vaccination	HPV 16-NAbs pos	HPV 18-NAbs pos	HPV 6-NAbs pos	HPV 11-NAbs pos
1-6 months	81/81 (100%)	80/81 (98,8%)	20/22 (91%)	6/12 (50%)
24 months	7/7 (100%)	7/7 (100%)	7/7 (100%)	5/7 (72%)
36 months	7/7 (100%)	5/7 (72%)	6/7 (85%)	5/7 (72%)
48 months	4/5 (80%)	4/5 (80%)	4/5 (80%)	3/5 (60%)

Table 3. Percentages of subjects vaccinated with Gardasil® who presented HPV type-specific NAbs at different time points after the completion of vaccination.

For the analysis of the results, geometric mean of neutralizing titer (GMT) obtained by neutralization assays was calculated. Subjects were divided into groups according to the time elapsed between the completion of the vaccination and the neutralization assay: 1st year (Figure 44), 2nd year (Figure 45), 3rd year (Figure 46) and 4th year (Figure 47). The situation is summarized in figure 48 and table 4.

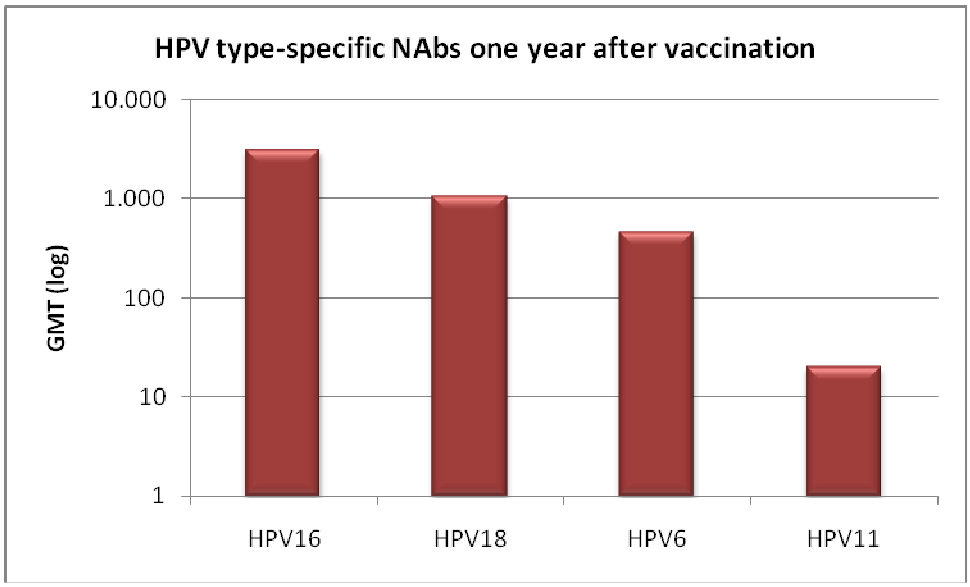


Figure 44. GMT of different HPV type-specific NAbs at one year after the completion of vaccination (logarithmic scale).

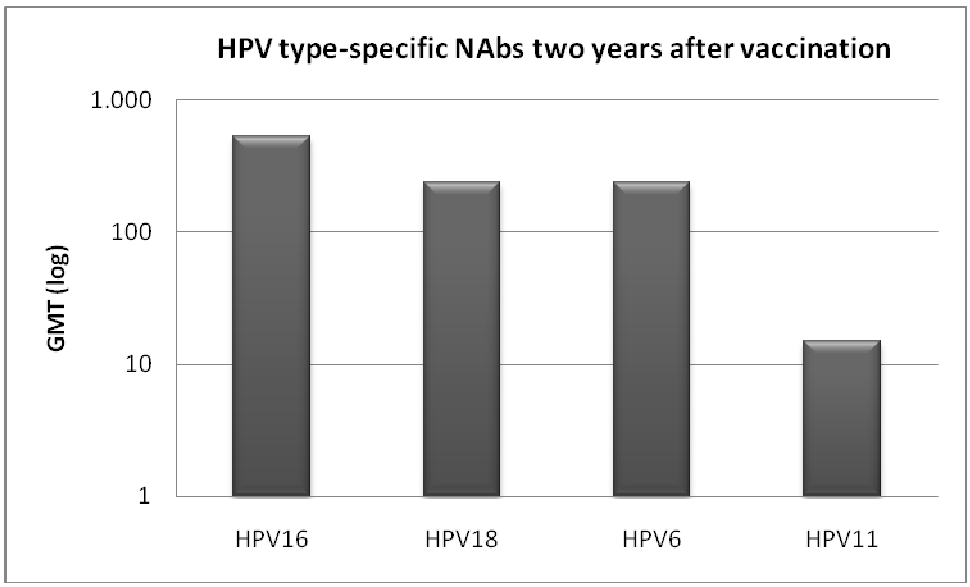


Figure 45. GMT of different HPV type-specific NAbs at two years after the completion of vaccination (logarithmic scale).

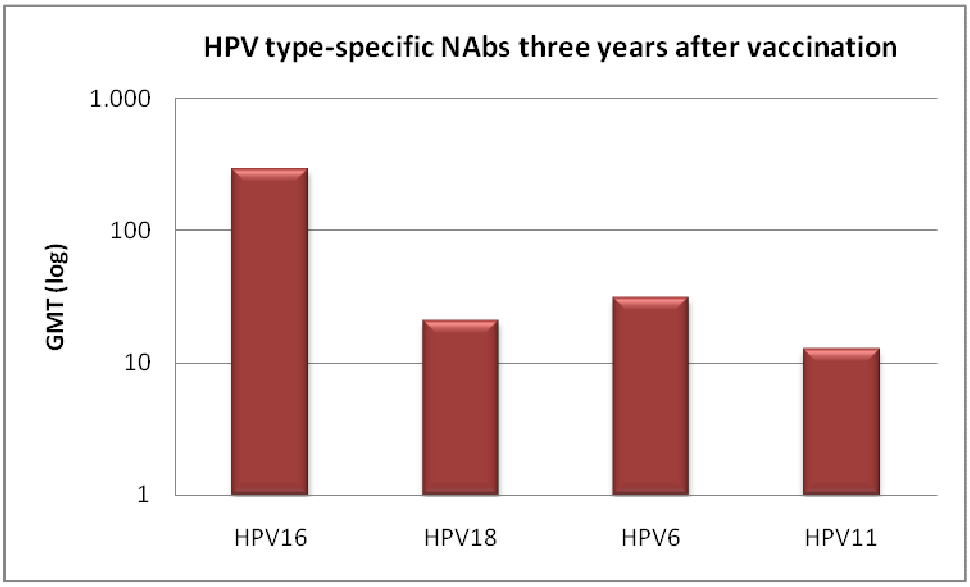


Figure 46. GMT of different HPV type-specific NAbs at three years after the completion of vaccination (logarithmic scale).

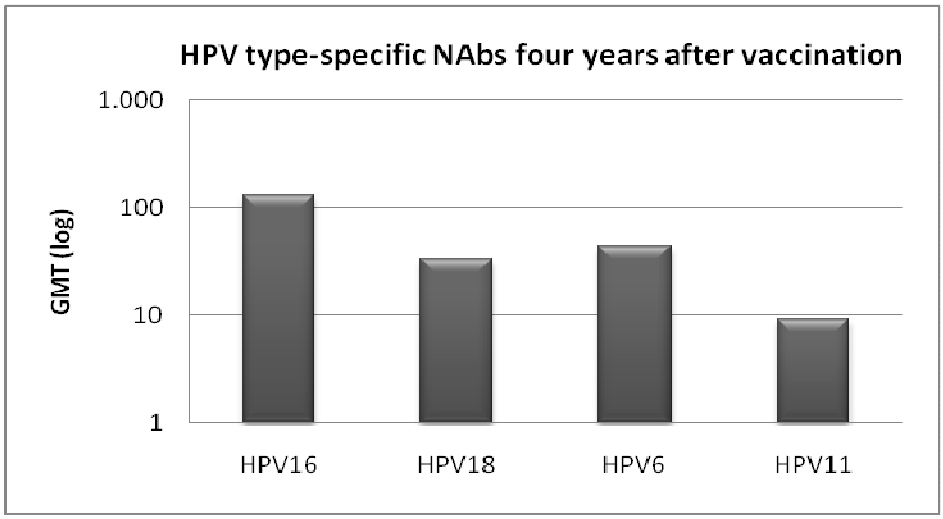


Figure 47. GMT of different HPV type-specific NAbs at four years after the completion of vaccination (logarithmic scale).

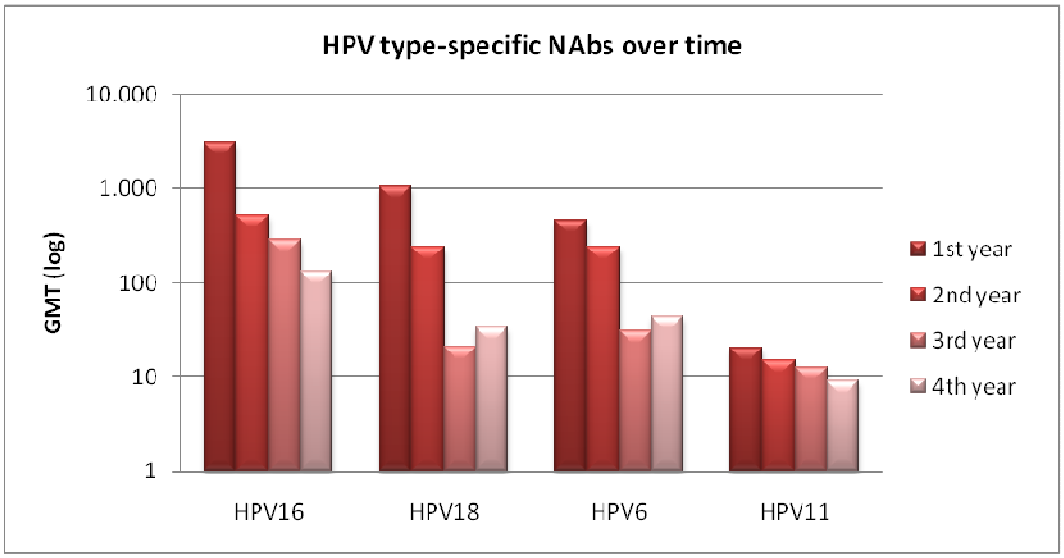


Figure 48. Histogram summarizing the GMT of HPV type-specific NAbs over the years after the completion of the vaccination (logarithmic scale).

Time post vaccination	HPV16 (GMT; 95% CI)	HPV18 (GMT; 95% CI)	HPV6 (GMT; 95% CI)	HPV11 (GMT; 95% CI)
1st year	2886 (±881)	1004 (±733)	457 (±1750)	20 (±649)
2nd year	525 (±134)	238 (±204)	238 (±134)	15 (±173)
3rd year	290 (±2788)	21 (±172)	31 (±2788)	13 (±202)
4th year	133 (±273)	33 (±65)	44 (±68)	9 (±19)

Table 4. GMTs calculated on the basis of HPV type-specific neutralizing titer, with confidence interval (CI) of 95%. For the calculation of the GMTs and 95% CI, neutralizing antibody titer <1:40 was arbitrarily considered equal to a value of 1.

Neutralizing antibody titers corresponding to four vaccinal HPV genotypes were also analyzed one by one. In figures 49, 50, 51 and 52 the progress of the titers as a function the time elapsed since the completion of the vaccination.

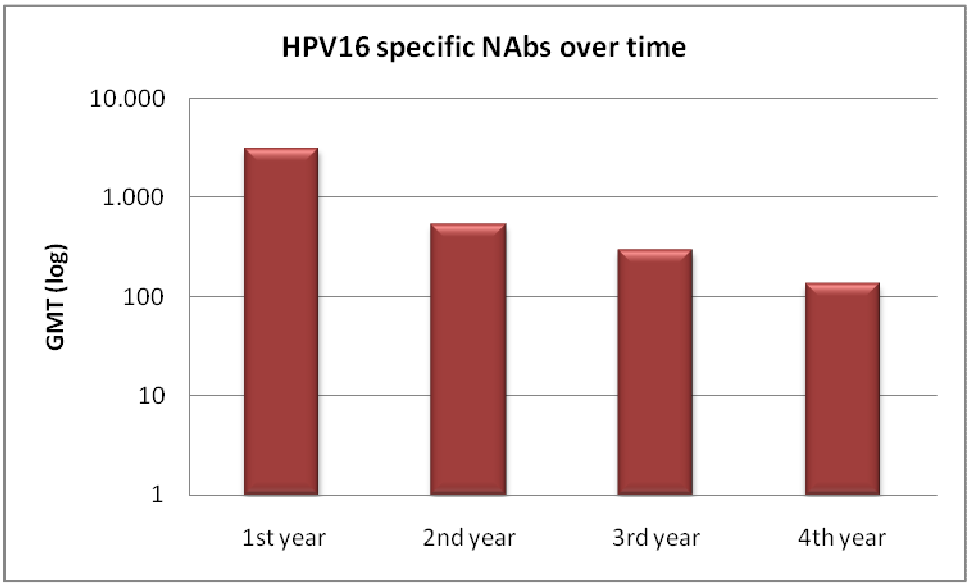


Figure 49. GMT of neutralizing antibodies specific for HPV16 over the years (logarithmic scale).

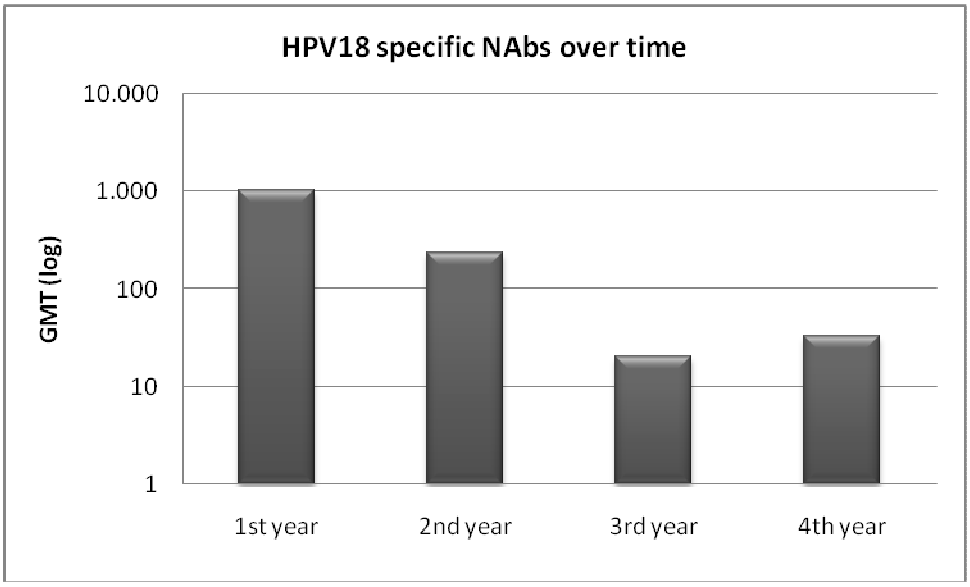


Figure 50. GMT of neutralizing antibodies specific for HPV18 over the years (logarithmic scale).

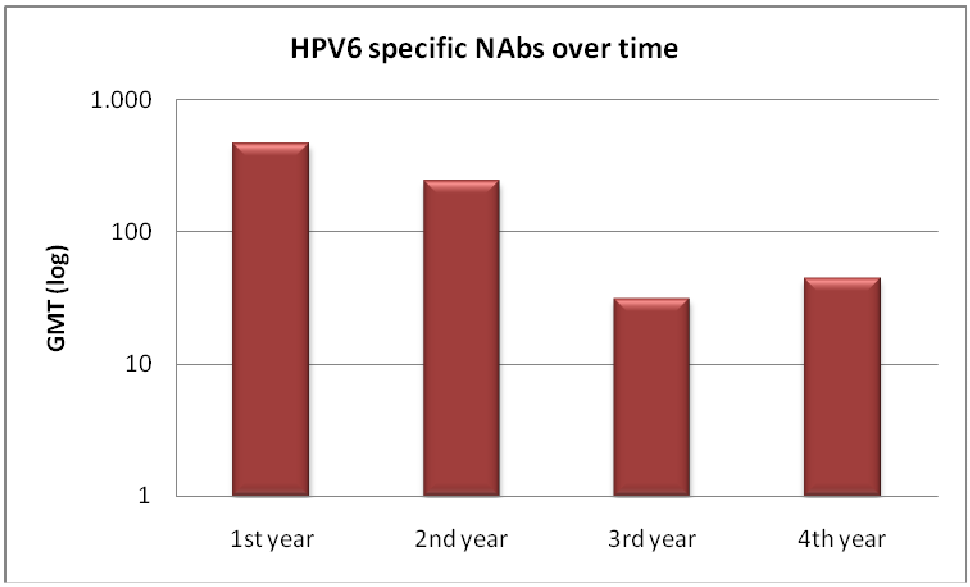


Figure 51. GMT of neutralizing antibodies specific for HPV6 over the years (logarithmic scale).

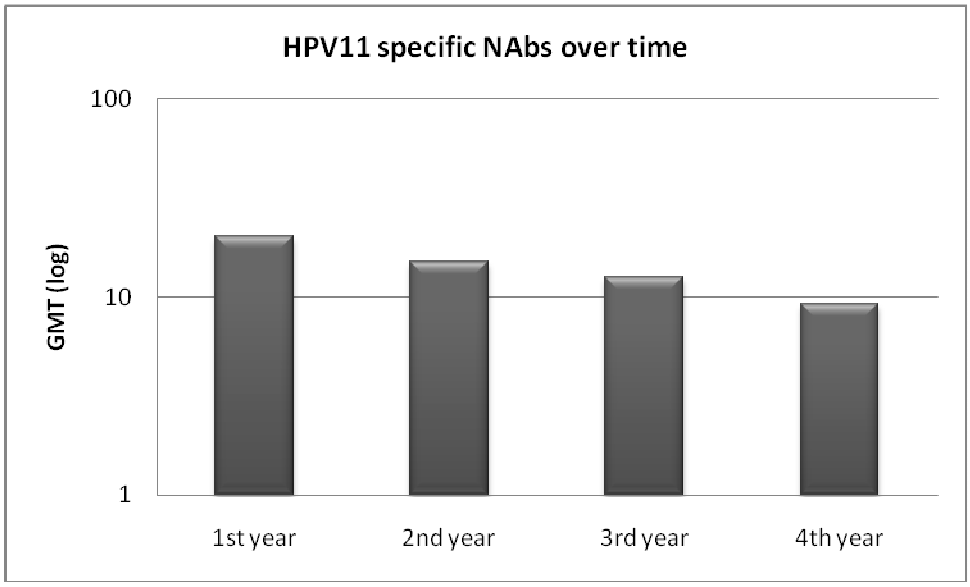


Figure 52. GMT of neutralizing antibodies specific for HPV11 over the years (logarithmic scale).

Low level of cross-NAbs against HPV31 (1:40) was detected in 50% of the 6 subjects evaluated at 1-6 months after vaccination, while no cross-NAbs were detected against HPV45, HPV52 and HPV58. Results are summarized in table 5.

Time post vaccination	HPV 31-NAbs pos	HPV 45-NAbs pos	HPV 52-NAbs pos	HPV 58-NAbs pos
1-6 months	3/6 (50%)	0/6 (0%)	0/6 (0%)	0/6 (0%)

Table 5. Percentages of subjects vaccinated with Gardasil® who presented, or not, specific cross-NAbs against HPV31, 45, 52 and 58.

High-titer neutralizing antibody response after vaccination was also observed in two males (age range: 34-41 years old; mean age: 37,5 years old) for all four vaccinal HPV genotypes. Results are presented in figure 53.

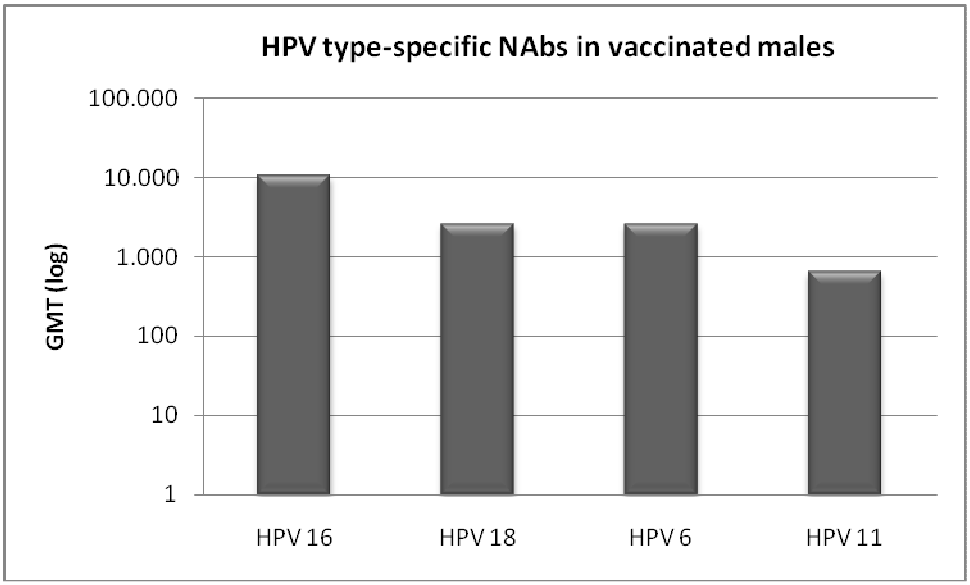


Figure 53. Histogram summarizing the GMTs of neutralizing antibody specific for HPV16, 18, 6 and 11 in vaccinated males.

4.3.4. PBNA in Cervarix™ vaccinated subjects

Neutralization assays were performed on serum samples selected from a group of 6 subjects (all females; age range: 11-20 years old; mean age: 13,99 years old) collected within 1-6 month since the completion of the three doses of vaccine in Emilia-Romagna Region, where the bivalent Cervarix™ vaccine was offered. At 1-6 months after the completion of the vaccination cycle, 100% vaccines had neutralizing antibodies against HPV16 and HPV18. Titers were higher than titers observed in Gardasil® vaccinated subjects, ranging from 1:10,240 to over 1:20,480. 100% of subjects presented also NAbs against HPV31, whereas 16.6% presented cross-NAbs

against HPV45 and HPV58. None presented cross-NAbs against HPV52. Results are summarized in table 6 and figure 54.

Time post vaccination	HPV 16-NAbs pos	HPV 18-NAbs pos	HPV 31-NAbs pos	HPV 45-NAbs pos	HPV 52-NAbs pos	HPV 58-NAbs pos
1-6 months	6/6 (100%)	6/6 (100%)	6/6 (100%)	1/6 (16.6%)	0/6 (0%)	1/6 (16.6%)

Table 6. Percentage of subjects vaccinated with Cervarix™ who presented specific NAbs against HPV16, HPV18, and specific cross-NAbs against HPV31, HPV45, HPV52 and HPV58.

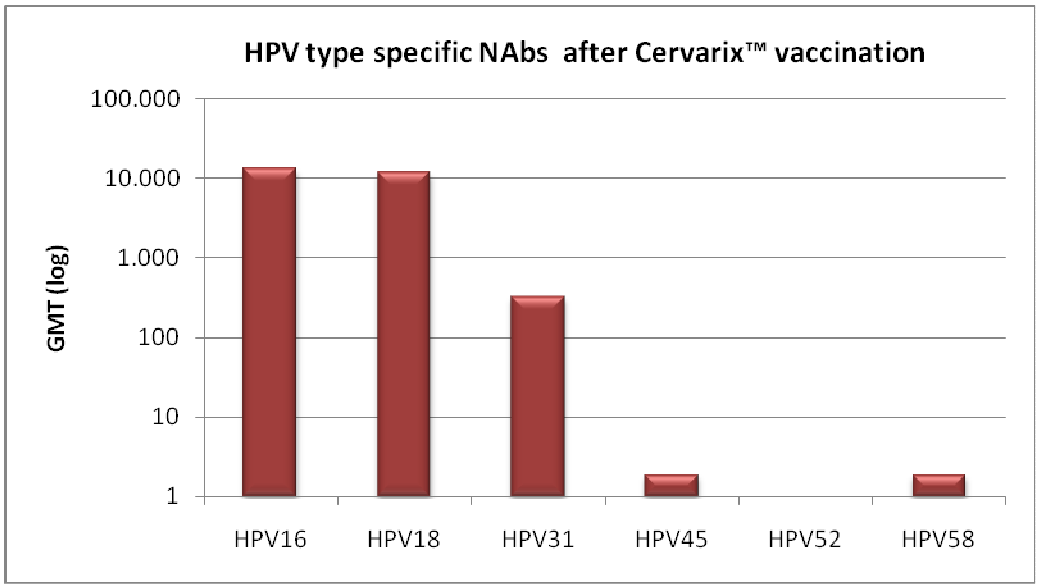


Figure 54. Histogram summarizing the GMTs of neutralizing antibody for different HPV types in Cervarix™ vaccinated females, 1-6 months after the completion of the vaccination.

5. DISCUSSION AND CONCLUSIONS

This study investigated the induction of HPV neutralizing and cross-neutralizing antibodies in subjects receiving the prophylactic bivalent and quadrivalent HPV vaccines. A range of efficacy trials with bivalent and quadrivalent HPV vaccines conducted globally demonstrated their high efficacy and immunogenicity (Villa *et al.*, 2006a; Joura *et al.*, 2008; Schwarz and Leo, 2008; Future I/II Study Group *et al.*, 2010; Roteli-Martins *et al.*, 2012; Khatun *et al.*, 2012). Nevertheless, immunological correlates of protection and quantification of antibody levels required to confer protection against HPV have not been defined yet (Stanley *et al.*, 2006; Schwarz and Leo, 2008).

This PhD research study showed high immunogenicity induced by both vaccines in adolescent girls and demonstrated a slight decrease in GMTs of NAbs over time in subject vaccinated with the quadrivalent vaccine, for whom long-term data were available. Seroconversion was observed in approximately 100% of subjects within one year after vaccination, combined with the presence of cross-neutralizing antibodies against other high risk non-vaccinal HPV types., i.e. HPV31, 45 and 58.

It's well known that vaccine-mediated protection against HPV is based on neutralizing antibodies induction, in particular against L1 capsid protein (Day *et al.*, 2007). This protein is the main component of two vaccinal preparations (Gardasil®, Cervarix™) that are available on the market since 2006 with the purpose to prevent the onset of cervical cancer (Joura *et al.*, 2008). These vaccines are made of *virus-like particles* that are able to stimulate the immune system, promoting the appearance of high antibody titers and specific memory immune responses (Paavonen *et al.*, 2007). Both have be proven to be effective and safe to prevent HPV16 and 18 related CIN2+ or greater lesions and to provide different levels of cross-protection against serotypes 31, 33, 45, 52 and 58, not included in the vaccine (Smith *et al.*, 2007; Einstein *et al.*, 2011a; Einstein *et al.* 2011b; Kemp *et al.*, 2011; Kemp *et al.*, 2012; Malagón *et al.*, 2012). Their long-term efficacy, however, has not yet been completely proven and several gaps in our understanding of the vaccines' performance remain.

Vaccine immunogenicity data derive mainly from studies performed by manufacturer companies and standardized assays are not commercially available. Pre-clinical data suggest that neutralizing antibodies constitute the primary mechanism of protection against infection with HPV, thus indicating that measuring the antibody response elicited by HPV vaccination should be performed using neutralization assays or, at a minimum, assays that correlate with neutralization activity. The use of neutralization assays as primary immune readouts for large clinical trials is challenging because HPV is unable to grow on monolayer cell culture. Even if the recent introduction of PBNA makes the use of neutralization assays possible in the context of clinical trials,

PBNA is laborious and difficult to perform, requires considerable expertise and experience to standardize, and the detection reagents are very expensive. However, the PBNA may be the best available assay to test functional anti-HPV antibodies and, as it is independent of vaccine material, is an unbiased method for measuring responses to different vaccine VLPs. For all these reasons, PBNA should therefore be considered as a benchmark assay for detecting neutralizing antibodies (Dessy *et al.*, 2008). WHO guidelines for HPV vaccines have also indicated that "neutralization assays are considered 'the gold standard' for assessing the protective potential of antibodies induced by the HPV vaccines"(WHO, 2007; WHO, 2008).

In this context, we developed and set up two different assays in order to evaluate the presence of whole and type-specific antibody response induced by prophylactic HPV L1 VLP vaccines. The first method is an ELISA which does not differentiate between type-specific conformational antibodies and type-common antibodies, which tend to be specific for epitopes presented by denatured L1 capsid protein and are usually not neutralizing. The second method is the PBNA, which can discriminate between conformational and non conformational epitopes and can evaluate the presence of HPV type-specific neutralizing antibodies. Both the direct ELISA and the PBNA were used for the assessment and registration of GSK's cervical cancer vaccine, CervarixTM, worldwide (Dessy *et al.*, 2008; Kemp *et al*, 2012).

In this PhD research study, different batches of VLPs containing structural proteins (L1 and L2) of vaccinal and non vaccinal high risk HPV types, i.e. HPV6, 11, 16, 18, 31, 45, 52, 58 were produced with high transducing property (10^9 t.u./ml). First set up experiments have been made by employing single HPV-type specific VLPs in neutralization assays with sera from a vaccinated female and from a vaccinated male one year after vaccination with Gardasil®, in order to make assays reliable and reproducible. High HPV type-specific neutralizing antibody titers were observed in PBNA, ranging from 1:1,280 against HPV11 to 1:2,560 against HPV18 and HPV6, reaching the titer of 1:10,240 against HPV16. Low NAb titers against HPV31 (1:160) and HPV58 (1:40) were observed, respectively. Results obtained after different HPV-type specific PBNA were confirmed with ELISA experiments, thus indicating the good correlation between these two methods, as reported (Dessy *et al.*, 2008). The measurement obtained with ELISA assay represents a substantial proportion of the total immune response to HPV VLP vaccination, as reported by Brown and collaborators (Brown *et al.*, 2011). The non-restrictive inclusion of VLP-binding antibodies with heterogeneous characteristics as the measured outcome of ELISA necessarily reduces the overall assay specificity for a particular subset of the immune response, such as neutralizing antibodies. Therefore, using the ELISA measurement to represent the HPV protective antibody response is likely an overestimate of the level of protective antibodies in vaccinated women (Brown *et al.*, 2011). This is the

reason why we decided to perform only PBNA to assess the antibody response induced by vaccination in a cross-sectional clinical study on vaccinated subjects, enrolled at our clinical study protocol.

Patients with HPV natural infection established by molecular test in different anogenital swabs were evaluated for the presence of HPV type-specific neutralizing antibodies in their sera, in order to establish a threshold of humoral immunity induced by the virus during natural infection. In two cases, satisfactory NAb titers were observed against HPV6 (1:160) and HPV16 (1:2,560), while other patients had no detectable neutralizing antibody response. These results confirm studies reporting that the immune antibody response induced by natural infection is very weak and not all the individuals are able to counteract HPV infection by mounting an effective humoral response (Stanley *et al.*, 2006; Stanley *et al.*, 2012).

In vaccinated subjects, we demonstrated that both the quadrivalent and the bivalent vaccines induced high levels HPV type-specific NAbs against vaccine HPV types 16 and 18. One year after completion of Gardasil® vaccination, 100% vaccinees had NAbs against HPV16, 98.8% had NAbs against HPV18, while 91% had NAbs against HPV6 and 50% had NAbs against HPV11. NAbs titers ranged widely from 1:40 to over 1:10,240 and were lower against HPV6 and HPV11 than against HPV16 and HPV18. One year after vaccination, GMTs ranged from 2886 to 20 and were higher for HPV16 and HPV18 than for HPV 6 and HPV11. These values were similar to those reported seven months after the completion of quadrivalent HPV vaccination by Joura and collaborators (Joura *et al.*, 2008). However, lower GMTs were observed in our subjects, in particular for HPV11 as compared with data reported by the same research group seven months after the completion of the vaccination cycle (Joura *et al.*, 2008). This was probably due to the small number of subjects which have been analyzed and to the assay employed for the analysis. Most studies, in fact, reported the use of competitive Luminex-based immunoassay (cLIA), instead of the gold standard method, PBNA (Villa *et al.*, 2006b; Frazer *et al.*, 2007; Olsson *et al.*, 2007; Joura *et al.*, 2008). Among Cervarix™ vaccinated subjects, although the sample size was smaller, NAbs titers against HPV16 and HPV18 reached values over to 1:20,480. Similar data were reported by Einstein and collaborators, who observed higher GMTs of anti-HPV16 and 18 serum neutralizing antibodies measured in women of 18–26 years old who received the bivalent vaccine than in women who received the quadrivalent vaccine (Einstein *et al.*, 2011a). In agreement with these findings, a large trial with the bivalent vaccine called PATRICIA (The PApilloma TRIal against Cancer In young Adults) demonstrated also a high efficacy against CIN2+ lesions associated with HPV16 and HPV18 infections, against CIN3+ and adenocarcinoma in situ (AIS) irrespective of HPV DNA in the lesion (Lehtinen *et al.*, 2012; Roteli-Martins *et al.*, 2012; Szarewski *et al.*,

2012). In our series of subjects vaccinated with the quadrivalent vaccine, a progressive reduction of NAbs titers over time was observed and, at 4 years after vaccination, 80% of subjects had NAbs against HPV16, HPV18 and HPV6, and 60% against HPV11. Likewise, Einstein and collaborators observed higher titers of NAbs at month 7 and a *plateau* phase until 24 months for both vaccines. GMTs of anti-HPV16 and 18 NAbs measured by PBNA in serum remained, however, significantly higher up to month 24 in women aged 18–26 years old who received the bivalent vaccine than in women who received the quadrivalent vaccine. (Einstein *et al.*, 2011a). Follow-up studies on Gardasil® vaccinated subjects reported the decline of neutralizing antibody titers 48 months after vaccination in 15-26 years old women (Joura *et al.*, 2008), accompanied by the persistence of antibodies up to 60 months following initiation of the primary vaccination series in 16-23 years old women enrolled in a double-blind, placebo-controlled study (Olsson *et al.*, 2007). According Villa and collaborators, quadrivalent vaccine-induced anti-HPV GMTs remained at or above those following natural infection, through 5 years (Villa *et al.*, 2006a). High GMTs of anti-HPV16 and anti-HPV18 total IgG antibodies in women aged 15-25 years vaccinated with Cervarix™ were demonstrated during a 5.5 years follow up by using ELISA and PBNA (Schwarz and Leo, 2008). More recently, sustained immunogenicity and efficacy was reported up to 8.4 years follow-up by Roteli-Martins and collaborators for the bivalent vaccine (Roteli-Martins *et al.*, 2012). It would be interesting to evaluate and compare the efficacy induced by both vaccines also in our study. Monitoring of the kinetic profile of NAbs over time for subjects vaccinated with Cervarix™ is ongoing in our study.

Regarding results obtained on cross-neutralization, low level cross-NAbs against HPV31 (1:40) was detected in 50% (3/6) of subjects at 1-6 months after quadrivalent vaccination, while no cross-NAbs were detected against HPV45, HPV52 and HPV58. On the contrary, all subjects (6/6) vaccinated with the bivalent vaccine had cross-NAbs against HPV31 and 16.6% (1/6) of them showed cross-NAbs against HPV45 and HPV58. None of them presented cross-NAbs against HPV52. Likewise, Malagon and collaborators reported higher cross-protective efficacy against HPV31 and 45 for the bivalent vaccine as compared with the quadrivalent vaccine, in particular 77% vs 46% and 79% vs 8%, respectively (Malagon *et al.*, 2012). The immune responses induced by two vaccines against HPV31 and HPV45 were also compared in an observer-blind study up to month 24, in women who were HPV DNA-negative and seronegative prior to vaccination for the HPV type analyzed. GMT measured by PBNA and ELISA were found to be similar between vaccines for HPV31 and 45. However, according Einstein and collaborators, there was a trend for higher seropositivity with the bivalent vaccine (13.0–16.7%) as compared with the quadrivalent vaccine (0.0–5.0%) for HPV45 with PBNA, but not with ELISA

(Einstein *et al.*, 2011b). Kinetics findings of the antibody response to vaccine cross-related types HPV31 and HPV45 were confirmed also by Kemp and collaborators during a 36 months follow up on Cervarix™ (Kemp *et al.*, 2012). Interestingly, Kemp and collaborators observed significant correlations between HPV16 and HPV31 neutralizing titers and between HPV18 and HPV45 neutralizing titers, despite 100-fold lower (Kemp *et al.*, 2011). Data from the end-of-study analysis of PATRICIA showed that the bivalent vaccine provides also cross-protective efficacy against 6-month persistent infection and CIN2+ associated with HPV33, HPV31, HPV45, and HPV51. Consistent vaccine efficacy for all endpoints across all cohorts was seen only for HPV33 (Wheeler *et al.*, 2012). So far, although our study is close to better evaluate NAbs titers and cross-NAbs induced by the bivalent vaccine in larger sampling, data collected are encouraging and allowed us to make a picture of the NAbs response in young females vaccinated against HPV in our Region.

In conclusion, this study showed that both the quadrivalent and bivalent HPV vaccines are immunogenic and induce high titers of NAbs against vaccine HPV types, in particular against HPV16 and 18, accompanied by the induction of cross-NAbs, especially against HPV31.

6. ABBREVIATIONS

AIS: adenocarcinoma *in situ*
BCA: bicinchoninic acid
CI: confidence interval
CIN: cervical intraepithelial neoplasia
CIS: carcinoma *in situ*
cLIA: competitive Luminex immunoassay
CMI: cell-mediated immunity
cRIA: competitive radioimmunoassay
CTL: cytotoxic T lymphocytes
DMEM: Dulbecco's Modified Eagle Medium
DNA: deoxyribonucleic acid
DPBS: Dulbecco's Phosfate-Buffered Saline
EGFP: enhanced green fluorescence protein
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunosorbent spot
EV: epidermodysplasia verruciformis
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
GMTs: geometric mean antibody titers
HPV: Human papillomavirus
HRP: horseradish peroxidase
HSPGs: heparan sulphate proteoglycans
IgA: immunoglobulin A
IgG: immunoglobulin G
IL: interleukin
INF: interferon
kb: kilobases
kDa: kiloDalton
LB: Luria-Bertani
MAb: monoclonal antibody
NAbs: neutralizing antibodies
NCI-CCR: National Cancer Institute - Center for Cancer Research
NMSC: non-melanoma skin cancers
ori: origin of replication
PBNA: pseudovirion-based neutralization assay
PBS: phosphate buffered saline
PCR: polymerase chain reaction

RLU: *relative light unit*
RRP: recurrent respiratory papillomatosis
SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SEAP: secreted embrionic alkaline phosphatase protein
SIL: squamous intraepithelial lesions
SV40: Simian vacuolating virus 40
TB: Terrific Broth
TBE: Tris-borate-EDTA buffer
TBS: Tris-buffered saline
TLR9: Toll-like receptor 9
TNF: tumor necrosis factor
VaIN: Vaginal Intraepithelial Neoplasia
VIN: Vulvar Intraepithelial Neoplasia
VLPs: *virus-like particles*
WHO: World Health Organization

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8. PUBLICATIONS AND ABSTRACTS

List of scientific publications and abstracts produced during the Biomedicine PhD School.

PUBLICATIONS

- Luisa Barzon, Monia Pacenti, Elisa Franchin, Laura Squarzon, Enrico Lavezzo, Stefano Toppo, Thomas Martello, Margherita Cattai, Riccardo Cusinato, Giorgio Palù. "Novel West Nile virus lineage 1a full genome sequences from human cases of infection in north-eastern Italy, 2011" *Clin Microbiol Infect.* 2012 Aug 31. doi: 10.1111/1469-0691.12001.

- Luisa Barzon, Monia Pacenti, Elisa Franchin, Thomas Martello, Enrico Lavezzo, Laura Squarzon, Stefano Toppo, F Fiorin, G Marchiori, GP Scotton, Francesca Russo, Margherita Cattai, Riccardo Cusinato, Giorgio Palù. "Clinical and virological findings in the ongoing outbreak of West Nile virus Livenza strain in northern Italy, July to September 2012". *Euro Surveill.* 2012 Sept 6; 17(36): pii:20260.

- Luisa Barzon, Monia Pacenti, Elisa Franchin, Thomas Martello, Enrico Lavezzo, Laura Squarzon, F Fiorin, G Marchiori, Francesca Russo, Margherita Cattai, Riccardo Cusinato, Giorgio Palù. "New endemic West Nile virus Lineage 1a in Northern Italy, July 2012". *Euro Surveill.* 2012 Aug 2;17(31). pii: 20231.

- Luisa Barzon, Monia Pacenti, Riccardo Cusinato, Margherita Cattai, Elisa Franchin, Silvana Pagni, Thomas Martello, Stefania Bressan, Laura Squarzon, Anna Maria Cattelan, Giampietro Pellizzer, Piergiorgio Scotton, Anna Beltrame, Federico Gobbi, Zeno Bisoffi, Francesca Russo, Giorgio Palù. "Human cases of West Nile virus infection in north-eastern Italy, 15 june to 15 november 2010". *Euro Surveill*, 2011 Aug 18; 16(13) pii: 19949.

- Luisa Barzon, Valentina Militello, Enrico Lavezzo, Elisa Franchin, Elektra Peta, Laura Squarzon, Marta Trevisan, Silvana Pagni, Federico Dal Bello, Stefano Toppo, and Giorgio Palù. "Human Papillomavirus Genotyping by 454 Next Generation Sequencing Technology". *J Clin Virol.* 2011 Oct 52(2):93-7.

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ABSTRACTS

- Laura Squarzon, Monia Pacenti, Lorena Gottardello, Giorgio Palù, Luisa Barzon. Independent study on the immunogenicity of the prophylactic human papillomavirus vaccines: preliminary results on the quadrivalent vaccine. (Abstract book of the 11th National Congress of the Italian Society for Virology. Selected Oral Communication C10, pag.19).

- Thomas Martello, Monia Pacenti, Laura Squarzon, Elisa Franchin, Samuele Asnicar, Enrico Lavezzo, Alessandro Sinigaglia, Stefano Toppo, Margherita Cattai, Riccardo Cusinato, Sebastian Ulbert, Silke Corbach-Soehle, Luisa Barzon, Giorgio Palù. West Nile virus in northern Italy:surveillance activity and pathogenicity studies. (Abstract book of the 11th National Congress of the Italian Society for Virology. Selected Oral Communication C23, pag. 26).

- Luisa Barzon, Monia Pacenti, Laura Squarzon, Riccardo Cusinato, Thomas Martello, Elisa Franchin, Margherita Cattai, Giorgio Palù. Surveillance of West Nile virus in Veneto Region, Italy, 2011. (Abstract book of 22nd European Congress of Clinical Microbiology and Infectious Diseases. Selected Oral Communication n. O 465, pag. 71).

- Laura Squarzon. West Nile surveillance in Veneto Region. (Workshop: Climate change and Health Effects - Impact, preparedness, guidelines and good examples. Selected Oral Communication).

- Laura Squarzon. miRNAs in viral-host interactions. (First Padua-Ulm Retreat Topics in Molecular Medicine. Selected Oral Communication).

- Laura Squarzon. Immunological and functional studies on human papillomavirus and West Nile virus prophylactic vaccines. (Abstract book del Convegno di presentazione dell'attività di ricerca dei dottorandi del XXV ciclo della macroarea 2 - Scienze della vita, II anno di corso, Palazzo del Bo, Padova, Italy, Selected Oral Presentation, pag. 77).

- Luisa Barzon, Laura Squarzon, Monia Pacenti, Riccardo Cusinato, Margherita Cattai, Elisa Franchin, Silvana Pagni, Thomas Martello, Stefania Bressan, Francesca Russo, Giorgio Palù. Enhanced Surveillance of West Nile Disease, Dengue, and Chikungunya in Veneto Region, 2010. (Abstract book of the 10th National Congress of the Italian Society for Virology, Orvieto, Italy, Poster).

- Valentina Militello, Enrico Lavezzo, Elisa Franchin, Elektra Peta, Laura Squarzon, Marta Trevisan, Silvana Pagni, Federico Dal Bello, Stefano Toppo, Luisa Barzon, and Giorgio Palù. Human Papillomavirus Genotyping by 454 Next Generation Sequencing Technology. (Abstract book of the 10th National Congress of the Italian Society for Virology, Orvieto, Italy, Poster).

- Luisa Barzon, Elisa Franchin, Laura Squarzon, Enrico Lavezzo, Stefano Toppo, Thomas Martello, Stefania Bressan, Silvana Pagni, Margherita Cattai, Anna Piazza, Monia Pacenti, Riccardo Cusinato, Giorgio Palù. Epidemiology and molecular characterization of West Nile virus infection in north-eastern Italy. (Abstract book of the 4th European Congress of Virology, Cernobbio, Italy, Selected Oral Presentation, pag. 30).

- Luisa Barzon, Elisa Franchin, Laura Squarzon, Enrico Lavezzo, Stefano Toppo, Thomas Martello, Stefania Bressan, Silvana Pagni, Margherita Cattai, Anna Piazza, Monia Pacenti, Riccardo Cusinato, Giorgio Palù. Epidemiology and molecular characterization of West Nile virus infection in north-eastern Italy (Abstract book of the 20th European Congress of Clinical Microbiology and Infectious Diseases, Selected Oral Presentation n. O 551).

- Luisa Barzon, Valentina Militello, Elektra Peta, Enrico Lavezzo, Stefano Toppo, Marta Trevisan, Laura Squarzon, Elisa Franchin, Giorgio Palù. Deep sequencing for accurate and high-throughput HPV genotyping in clinical samples. (Abstract book of the 20th European Congress of Clinical Microbiology and Infectious Diseases, Selected Oral Presentation n. O 106).

- Luisa Barzon, Elisa Franchin, Laura Squarzon, Enrico Lavezzo, Stefano Toppo, Thomas Martello, Stefania Bressan, Silvana Pagni, Margherita Cattai, Anna Piazza, Monia Pacenti, Riccardo Cusinato, Giorgio Palù. Epidemiology and molecular characterization of West Nile virus infection in north-eastern Italy. (Abstract book of the 4th European Congress of Virology, Cernobbio, Italy, Poster n. 025).

- Enrico Lavezzo, Valentina Militello, Elektra Peta, Marta Trevisan, Laura Squarzon, Elisa Franchin, Stefano Toppo, Luisa Barzon, Giorgio Palù. Deep sequencing for accurate and high-throughput HPV genotyping in clinical samples. (Abstract book of the 4th European Congress of Virology, Cernobbio, Italy, Poster n. 763, pag. 213).